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Short communication

Improved protocol for the isolation of naïve follicular dendritic cells

Kazuki Sato[a], Shin-ichiro Honda[a], Akira Shibuya[a,b], Kazuko Shibuya (MD, PhD)[a,*]

[a] Department of Immunology, Faculty of Medicine, University of Tsukuba, 1-1-1, Tennodai, Tsukuba, Ibaraki 305-8575, Japan
[b] Life Science Center of Tsukuba Advanced Research Alliance (TARA), University of Tsukuba, 1-1-1, Tennodai, Tsukuba, Ibaraki 305-8575, Japan

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ABSTRACT

Follicular dendritic cells (FDCs) in lymphoid organs play an important role in the humoral immune response. However, because the isolation of FDCs is difficult due to their very small population size and fragility under mechanical and chemical stresses, the genetic and biochemical characteristics of FDCs remain unclear. Previously, we identified FDCs as ICAM-1$^+$ cells in the CD45$^-$ non-hematopoietic cell fraction from naïve mouse spleen after cell separation by means of digestion with a combination of enzymes. In the present study, using a new combination of enzymes, we found that FDCs are highly enriched in the CD45$^-$ICAM-1$^+$CD21/35$^+$ cell fraction. CD45$^-$ICAM-1$^+$CD21/35$^+$ cells in the mouse spleen retained an antigen administered *in vivo* for more than 7 days. Moreover, CD45$^-$ICAM-1$^+$CD21/35$^+$ cells isolated from the spleen of mice administered with a cognate antigen enhanced the survival and proliferation of antigen-specific B cells *in vitro*. Our improved protocol for the isolation of naïve FDCs will be useful for the analysis of FDCs *in vitro* and *in vivo*.

1. Introduction

Follicular dendritic cells (FDCs) were identified in 1965 as antigen-retaining reticular cells in lymphoid organs (Mitchell and Abbot, 1965). FDCs are non-hematopoietic antigen-presenting cells that can be distinguished from conventional dendritic cells (Chen et al., 1978a,b; Turley et al., 2010). FDCs present antigen via CD21/35 to B cells and support the clonal expansion of B cells in germinal center and immunoglobulin class-switching, and for the B-cell-receptor gene to undergo somatic hypermutation (Victora and Nussenzweig, 2012). Thus, FDCs play an important role in the humoral immune response.

Immunohistochemical analysis has partially revealed the phenotypic characteristics of FDCs; however, because the isolation of FDCs is difficult due to their scarcity in lymphoid organs and their fragility under mechanical and chemical stresses, the molecular and functional characteristics of FDCs remain unclear. Conventionally, FDCs are isolated as FDC-M1$^+$ cells by using magnetic cell-separation technology after whole-body irradiation and enzymatic digestion (Sukumar et al., 2006). However, the molecular marker FDC-M1 is detected not only in FDCs but also in cells located in the marginal sinus and tingible body macrophages (Kranich et al., 2008; Krautler et al., 2012). In addition, whole-body irradiation induces systemic inflammation through the release of damage-associated molecular pattern molecules from damaged tissue (Schaue et al., 2015), which might cause unexpected FDC activation. Thus, FDCs isolated after whole-body irradiation are not suitable for the biological study of naïve FDCs. Although previous reports described FDCs isolated from lymph nodes of naïve mice without irradiation (Fasnacht et al., 2014; Jarjour et al., 2014; Tamburini et al., 2014), phenotypical and functional characteristics of those FDCs were unclear.

Previously, we reported a unique method to isolate FDCs from the CD45$^-$ICAM-1$^+$ cell population in the lymphoid organs of non-irradiated naïve mice by means of flow cytometry (Usui et al., 2012). In the present study, we used a new combination of enzymes for cell separation to subdivide the CD45$^-$ICAM-1$^+$ cell population into CD21/35$^+$ and CD21/35$^-$ cell populations. Here we describe this improved method for producing highly enriched preparations of FDCs.

2. Materials and methods

2.1. Mice

C57BL/6J mice were purchased from Clea Japan Inc. (Tokyo, Japan) and maintained under specific pathogen-free (SPF) conditions. Quasi-monoclonal (QM) mice, in which transgenic B cell

* Corresponding author.
E-mail addresses: s0710709@gmail.com (K. Sato), shonda@md.tsukuba.ac.jp (S.-i. Honda), ashibuya@md.tsukuba.ac.jp (A. Shibuya), kazukos@md.tsukuba.ac.jp (K. Shibuya).

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receptor specifically reacts with 4-hydroxy-3-nitrophenylacetyl (NP) antigen, are homozygous for the *Vht* gene (*Vht*/*Vht*, Jκ$^-$/Jκ$^-$, λ$^+$/λ$^+$). All experiments were performed according to the guidelines of the Animal Ethics Committee of the University of Tsukuba Animal Research Center.

2.2. Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD31 (clone, 390), R-phycoerythrin (PE)-conjugated anti-mouse ICAM-1 (3E2), anti-mouse CD45.2 (104), Alexa647-conjugated anti-mouse CD31 (390), allophycocyanin (APC)-conjugated anti-mouse CD19 (1D3), anti-mouse CD45R/B220 (RA3-6B2), Horizon V450-conjugated anti-mouse CD45.2 (104), and biotin-conjugated anti-mouse CD45 (30-F11) monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA, USA). Purified anti-mouse CD16/32 (2.4G2), and PECy7-conjugated rat IgG2a isotype control (2A3) mAbs were purchased from Tonbo Biosciences (San Diego, CA, USA). PECy7-conjugated anti-mouse CD21/35 (7G6), biotin-conjugated anti-mouse CD45R/B220 (RA3-6B2), and biotin-conjugated anti-mouse Ter119 (Ter-119) mAbs were purchased from BioLegend (San Diego, CA, USA). eFluor660-conjugated anti-mouse podoplanin (PDPN) (eBio8.1.1) mAb was purchased from eBioscience (San Diego, CA, USA). Biotin-conjugated anti-FDC-M2 (FDC-M2) mAb was purchased from ImmunoKontact (Abingdon, UK). NP-Ficoll and 2, 4, 6-trinitrophenyl (TNP)-Ficoll were purchased from Biosearch Technologies (Novato, CA, USA) and TNP-Ficoll was labeled to produce FITC-TNP-Ficoll by using a FluoReporter FITC Labeling Kit (Thermo Fisher Scientific, MA, USA), in accordance with the manufacturer's instructions.

2.3. Isolation of stromal cell subsets

Stromal cells were isolated by using a method reported previously with modifications (Usui et al., 2012). Spleen from naïve mice was minced into a homogenous paste with a scalpel on a dish plate and then treated with 2 ml of an enzyme cocktail containing 1 mg/mL collagenase D (Roche, Indianapolis, IN, USA), 100 μg/mL DNase I (Sigma, St. Louis, MO, USA) and 0.6 U/mL Dispase (Roche, Indianapolis, IN, USA) in complete Dulbecco's modified Eagle's medium (cDMEM) containing 2% fetal bovine serum (FBS). After incubation in a 24-well plate for 30 min at 37 °C in a humidified incubator, cell suspensions were passed through a 100 μm nylon cell strainer (Corning, NY, USA) and cells were resuspended in cDMEM containing 10% FBS and 5 mM ethylenediaminetetraacetic acid (EDTA). For lysis of red blood cells, cells were incubated with 1 ml of ammonium-chloride-potassium lysing buffer for one spleen for 2 min, washed once with cDMEM, and subsequently once with the washing buffer (PBS containing 2% FBS and 5 mM EDTA). Cells were resuspended in the washing buffer at 10^9/ml and incubated with 2.5 μg purified anti-mouse CD16/32 mAb for 10^8 cells for 20 min on ice. Cells were then stained with a biotin-conjugated anti-mouse mAbs cocktail containing anti-CD45 (2.5 μg), anti-B220 (1 μg), and anti-anti-Ter119 (1 μg) for 10^8 cells for 30 min on ice. Unlabeled cells were then negatively selected by magnetic separation with BD IMag SAv particles (BD Pharmingen, San Diego, CA) in accordance with the manufacturer's instructions. Finally, cells were stained with V450-conjugated anti-CD45.2, FITC or Alexa647-conjugated anti-CD31, eFlour660-conjugated anti-PDPN, PE-conjugated anti-ICAM-1, and PECy7-conjugated anti-CD21/35 mAbs for 30 min on ice, washed with the washing buffer, passed through a 100 μm-nylon cell strainer, and analyzed by flow cytometry (FACSAria, BD Biosciences). Doublet and dead cells were gated out on the basis of forward-scatter A (FSC) and FSC H properties and PI staining, respectively. CD45$^-$ICAM-1$^+$CD21/35$^+$ cells and other stromal cells were sorted by using a flow cytometry (FACSAria) (100 μm-nozzle; 20 psi). For evaluation of B cell contamination in CD45$^-$ICAM-1$^+$CD21/35$^+$ fraction, anti-B220 mAb was excluded from antibody mixture for negative selection of lineage cells. For the analyses of the FDC-M2 expression on CD45$^-$ICAM-1$^+$CD21/35$^+$ cells, the stromal cells were enriched with BD IMag PE particles (BD Pharmingen, San Diego, CA), instead of BD IMag SAv particles, after staining with PE-conjugated anti-mouse CD45.2 mAb.

2.4. Antigen retention analysis

C57BL/6J mice were immunized intravenously with 50 μg FITC-TNP-Ficoll or phosphate-buffered saline as control. Seven days later, retention of the FITC-conjugated antigen by stromal cell subsets was analyzed by means of flow cytometry.

2.5. Ex vivo antigen presentation to cognate B cells

Naïve QM and WT B cells were purified from naïve mouse spleen by using anti-B220 particles-DM (BD Pharmingen), in accordance with the manufacturer's instructions, and then labeled with 500 nM carboxyfluorescein diacetate succinimidyl ester (CFSE). To assess B-cell proliferation, CFSE-labeled B cells (5×10^3 cells) were co-cultured in cDMEM medium containing 10% FBS in 96-well round-bottom plates with purified CD45$^-$ICAM-1$^+$ cells (500 cells) or CD45$^-$ICAM-1$^+$CD21/35$^+$ cells (500 cells) cells isolated from mice given 50 μg NP-Ficoll intravenously seven days previously. Three days later, the cells were harvested and stained with APC-conjugated anti-CD19 monoclonal antibodies. The ratio of PI$^-$ cells in the CD19$^+$ gate was used as an index of B-cell survival. The proliferation of PI$^-$CD19$^+$ cells was analyzed by means of the CFSE dilution assay.

2.6. Quantitative polymerase chain reaction

Total RNA was isolated by using Isogen reagent (Nippon Gene, Tokyo, Japan). For reverse transcription, we used a high-capacity complementary DNA reverse-transcription kit (Applied Biosystems, Carlsbad, CA, USA). Real-time polymerase chain reaction (PCR) analysis of *Mfge8*, *Cxcl13*, *Fcgr2b*, *Cr2*, *Icam1*, *Vcam1*, *Fcamr*, *Stab2*, *Ccl19*, and *Pdgfrb* was performed by using an ABI 7500 sequence detector (Applied Biosystems), Power SYBR Green PCR master mix (Applied Biosystems), and the appropriate primers. The *Actb* expression level was measured as the internal control to normalize the data. The primer sequences for the target genes were as follows: *Mfge8*: forward, 5′-ATA TGG GTT TCA TGG GCT TG-3′, reverse, 5′-GAG GCT GTA AGC CAC CTT GA-3′; *Cxcl13*: forward, 5′-CAT AGA TCG GAT TCA AGT TAC GCC-3′, reverse, 5′-TCT TGG TCC AGA TCA CAA CTT CA-3′; *Fcgr2b*: forward, 5′-TGC TGT CGC AGC CAT TGT TA-3′, reverse, 5′-TGT TGG CTC CAG TCC AGA TG-3′; *Cr2*: forward, 5′-AAT TGC AAA TGG TGG TCA CA-3′, reverse, 5′-GAT CGG GGC AAT GAG TTA GA-3′; *Icam1*: forward, 5′-TTG AGA ACT GTG GCA CCG TG-3′, reverse, 5′-CAG CTC CAC ACT CTC CGG AA-3′; *Vcam1*: forward, 5′-GTG ACC TGT CTG CAA AGG AC-3′, reverse, 5′-AAA GGG ATA CAC ATT AGG GAC TG-3′; *Fcamr*: forward, 5′-CCC AGC CTG AGA ACG AGA TG-3′, reverse, 5′-AGA GAT GGG TCC TGA ACT GAG-3′; *Stab2*: forward, 5′-TCA AGA CGG AGT GCC AGT C-3′, reverse, 5′-GCA ATC TCG AAC CCC GAC A-3′; *Ccl19*: forward, 5′-TTC ACG CCA CAG GAG GAC ATC T-3′, reverse, 5′-CCA CAC TCA CAT CGA CTC TCT AGG C-3′; *Pdgfrb*: forward, 5′-TCA AGC TGC AGG TCA ATG TC-3′, reverse, 5′-CCA TTG GCA GGG TGA CTC-3′; *Actb*: forward, 5′-ACT GTC GAG TCG CGT CCA-3′, reverse, 5′-GCA GCG ATA TCG TCA TCC AT-3′. The thermal cycling conditions comprised an initial denaturation step at 95° C for 10 min, followed by 40 cycles at 95° C for 15 s and 60° C for 1 min. The mRNA level was determined relative to that in CD45$^-$ICAM-1$^+$ cells.