



Contents lists available at ScienceDirect

Allergy International

journal homepage: <http://www.elsevier.com/locate/alit>

Original article

Mast cells derived from human induced pluripotent stem cells are useful for allergen tests

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ARTICLE INFO

Article history:

Received 25 April 2017

Received in revised form

3 August 2017

Accepted 9 August 2017

Available online xxx

Keywords:

Allergen-specific IgE

Allergy test

Human mast cells

Induced pluripotent stem cells

Luciferase

Abbreviations:

iPSCs, induced pluripotent stem cells;
 MCs, mast cells; NF- κ B, nuclear factor-kappa B; iPSC-MCs, mast cells differentiated from induced pluripotent stem cells;
 iMAT, induced mast cell activation test;
 Fc ϵ RI, high-affinity IgE receptor;
 HRT, histamine release test; BAT, basophil activation test; EXiLE, IgE crosslinking-induced luciferase expression; BAT-LR, BAT low responders; cedar, cedar pollen antigen; mite, mite antigen; HD, house dust antigen; ragweed, ragweed pollen antigen;
 bFGF, fibroblast growth factor-2;
 BMP4, bone morphogenetic protein 4;
 SCF, stem cell factor/c-kit ligand;
 TPO, thrombopoietin; FL, Fms-like tyrosine kinase 3 ligand; PMA, phorbol 12-myristate 13-acetate; PBS, phosphate-buffered saline; PE, phycoerythrin; iPSC-NB7AMCs, MCs differentiated from transfectant clone NB7A of iPSCs

ABSTRACT

Background: Several methods have been developed to detect allergen-specific IgE in sera. The passive IgE sensitization assay using human IgE receptor-expressing rat cell line RBL-2H3 is a powerful tool to detect biologically active allergen-specific IgE in serum samples. However, one disadvantage is that RBL-2H3 cells are vulnerable to high concentrations of human sera. Only a few human cultured cell lines are easily applicable to the passive IgE sensitization assay. However, the use of human induced pluripotent stem cells (iPSCs) to generate human mast cells (MCs) has not yet been reported.

Methods: The nuclear factor-kappa B (NF- κ B)-responsive luciferase reporter gene was stably introduced into a human iPSC line 201B7, and the transfectants were induced to differentiate into MCs (iPSC-MCs). The iPSC-MCs were sensitized overnight with sera from subjects who were allergic to cedar pollen, ragweed pollen, mites, or house dust, and then stimulated with an extract of corresponding allergens. Activation of iPSC-MCs was evaluated by β -hexosaminidase release, histamine release, or luciferase intensity.

Results: iPSCs-MCs stably expressed high-affinity IgE receptor and functionally responded to various allergens when sensitized with human sera from relevant allergic subjects. This passive IgE sensitization system, which we termed the induced mast cell activation test (iMAT), worked well even with undiluted human sera.

Conclusions: iMAT may serve as a novel determining system for IgE/allergens in the clinical and research settings.

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Introduction

Allergen-specific IgE levels are commonly used to diagnose allergy *in vitro*.^{1–3} ImmunoCAP (IgE CAP test) is the most popular system for measuring IgE.⁴ However, it often yields clinically

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Peer review under responsibility of Japanese Society of Allergy.

<http://dx.doi.org/10.1016/j.alit.2017.08.008>

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irrelevant results for various reasons, such as cross-reactivity and monovalency of antigen determinants.^{5–7} IgE antibodies are considered biologically inactive because they cannot cross-link the high-affinity IgE receptor (FcεRI) via an allergen on the surface of mast cells (MCs) and basophils. To detect biologically active and clinically relevant IgE-allergen interactions, a histamine release test (HRT) using peripheral blood basophils was developed.^{8,9} Recently, a simpler basophil activation test (BAT) was developed that uses flow cytometry and CD63 or CD203c as a basophil activation marker.^{10–12} These basophil-based tests have been widely used for allergies to foods, environmental factors, and drug hypersensitivities. However, they both require fresh blood, and basophils from approximately 10% of blood donors are non/low-responders and unresponsive in HRT/BAT assays.^{13–17}

To overcome these problems, a passive IgE sensitization assay was developed with cultured cell lines expressing human FcεRI. Human FcεRI genes were transfected into a rat basophilic leukemia line RBL-2H3.^{18–22} Recently, an advanced passive IgE sensitization assay named IgE crosslinking–induced luciferase expression (EXiLE) was reported.^{23,24} EXiLE uses modified RBL-2H3 cells in which a luciferase reporter gene was introduced with human FcεRI genes to sense FcεRI cross-linking signals with high sensitivity. These passive IgE sensitization assays detect biologically active IgE in serum samples that can be stored for a long time. However, because of their rat origin, RBL-2H3-derived cell lines are sensitive to human serum, and so, human serum samples must be diluted more than 10 fold in the assay.^{21,23} In certain cases, levels of components that affect the IgE-mediated activation of basophils are increased in the serum.^{25,26} To reflect the influence of such serum factors, undiluted serum or serum at a high concentration is desirable.²⁷ Thus, human MC and/or basophil cell lines are more suitable for these assays.

Human MCs develop from hematopoietic stem cells, express FcεRI, and are crucial initiator cells in inflammatory reactions, including allergic diseases.²⁸ Although several human MC lines, such as HMC-1, LAD2 and LUVA,²⁹ have been established, their availability in the passive IgE sensitization assay is unclear.

In this study, we generated MCs from human induced pluripotent stem cells (iPSCs). The resulting iPSC-MCs stably expressed FcεRI and functionally responded to allergens when sensitized with allergic subject sera. We named this system the “induced mast cells activation test” or iMAT. iMAT worked well with undiluted human sera and was applicable to various serum samples, including those from BAT low-responder subjects.

Methods

Blood specimens and antigens

The whole blood and serum samples were obtained from volunteers at BML, Inc. All of the volunteers gave written informed consent and subject anonymity was preserved using methods approved by the internal Ethics Committee (09-009) in accordance with the Declaration of Helsinki.

Volunteers included two subjects without cedar pollen allergy (H1, H2), six with cedar pollen allergy (A1–A6), and two BAT low responders with cedar pollen allergy (BAT-LR1, BAT-LR2). Table 1 provides the age and sex profiles of the subjects. For all sera, total IgE levels and specific IgE levels to cedar pollen antigen (cedar), mite antigen (mite), house dust antigen (HD) and ragweed pollen antigen (ragweed) were measured using the CAP-FEIA system (Phadia, Uppsala, Sweden) (Table 1). All sera were stored at –80 °C until use. All antigens were purchased from Torii Pharmaceutical (Tokyo, Japan).

Table 1
Profiles of the subjects.

Subject	Age, sex	Total IgE (IU/mL)	IgE CAP test, U _A /mL (Class)				BAT (%)
			Cedar	Mite	HD	Ragweed	
H1	61, M	3339	0.29 (0)	8.45 (3)	6.88(3)	3.67 (3)	93.2
H2	45, M	38	0.10 (0)	0.10 (0)	0.10 (0)	0.10 (0)	
A1	48, M	451	22.10 (4)	83.20 (5)	72.00 (5)	0.10 (0)	
A2	46, M	1521	6.40 (3)	21.80 (4)	20.30 (4)	0.15 (0)	92.4
A3	45, M	200	30.70 (4)	2.82 (2)	5.34 (3)	0.10 (0)	
A4	39, M	102	3.51 (3)	12.30 (3)	9.52 (3)	0.10 (0)	
A5	42, M	23	2.11 (2)	0.10 (0)	0.10 (0)	0.10 (0)	94.3
A6	44, M	48	13.80 (3)	0.10 (0)	0.10 (0)	0.10 (0)	
BAT-LR1	54, M	33	2.47 (2)	0.10 (0)	0.10 (0)	0.58 (1)	
BAT-LR2	54, F	92	11.80 (3)	0.10 (0)	0.10 (0)	0.10 (0)	3.3

H, subjects without cedar pollen allergy; A, cedar pollen-allergic subjects; BAT-LR, basophil activation test low responders with cedar pollen allergy; cedar, cedar pollen antigen; mite, mite antigen; HD, house dust antigen; ragweed, ragweed pollen antigen; BAT, basophil activation test.

iPSC culture

A human iPSC line 201B7 was purchased from iPSC Academia Japan, Inc. and cultured as described.³⁰ Briefly, 201B7 was cultured on mitomycin C-treated mouse embryonic fibroblast feeder cells (Kitayama Labs, Nagano, Japan) in KnockOut Dulbecco's Modified Eagle Medium (Invitrogen, Rockville, MD), supplemented with 20% knockout serum replacement (Invitrogen), 1 mM L-glutamine (Invitrogen), 0.1 mM non-essential amino acids (Sigma, St. Louis, MO), 0.1 mM α-mercaptoethanol (Sigma) and 4 ng/mL recombinant human fibroblast growth factor-2 (bFGF, Repro Cell, Kanagawa, Japan). Next, for feeder cell-free cultures, iPSCs were maintained in tissue culture dishes coated with Growth Factor-Reduced Matrigel (BD, Franklin Lakes, NJ) in mTeSR1 serum-free medium (Stemcell Technologies, Vancouver, BC, Canada). The medium was replaced everyday. Passage was performed according to the manufacturer's protocol for mTeSR1 serum-free medium.

Differentiation of iPSCs to mast cells

For hematopoietic differentiation of iPSCs, we used a serum- and feeder-free culture method (Supplementary Fig. 1.2) involving a monocytic lineage differentiation system.^{31,32} Under feeder cell-free conditions, iPSCs were cultured at a density of about 30 colonies per 100-mm dish. When individual colonies reached approximately 1 mm in diameter (day 0), bone morphogenetic protein 4 (BMP4, Life Technologies, 80 ng/mL) was added to the mTeSR1 culture medium for 4 days (from day 0 to day 4). Culture medium was replaced to StemPro-34 serum-free medium (Life Technologies), supplemented with 1% nonessential amino acids (Sigma), 2 mM L-glutamine (Invitrogen), 100 U/mL penicillin (Sigma), 100 μg/mL streptomycin (Sigma) (referred to as MC basal medium), and a cytokine cocktail of 80 ng/mL vascular endothelial growth factor 165 (Peprotech, Rocky Hill, NJ), 25 ng/mL bFGF, and 100 ng/mL stem cell factor/c-kit ligand (SCF, Life Technologies) for 2 days (days 4–6). At this point, most cells were positive for hematopoietic/blood cell marker CD45, and only 0.5% were positive for CD34.

The protocol for hematopoietic progenitor expansion and initial myeloid differentiation was modified from Yanagimachi *et al.*³² Cells were cultured in MC basal medium, supplemented with 100 ng/mL SCF, 10 ng/mL interleukin (IL)-3 (Life Technologies), 10 ng/mL thrombopoietin (TPO, R & D Systems, Minneapolis, MN, USA), 100 ng/mL IL-6 (Life Technologies), and 10 ng/mL Fms-like tyrosine kinase 3 ligand (FL, Life Technologies). The medium was

replaced every 3–4 days, and non-adherent cells were collected after 3 weeks (from day 27 onward).

To generate MCs, we used a cytokine cocktail as described.^{33,34} Briefly, hematopoietic progenitor cells were cultured in MC basal medium, supplemented with 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL TPO, 100 ng/mL IL-6, and 10 ng/mL FL. The medium was replaced every 3–4 days, and non-adherent cells were collected from 2 weeks onward. The collected nonadherent cells were cultured in MC basal medium, supplemented with 100 ng/mL SCF, 10 ng/mL TPO, 100 ng/mL IL-6, and 10 ng/mL FL, but without IL-3 on PrimeSurface 24F Plate (Sumitomo Bakelite, Tokyo, Japan). The medium was replaced every 3–4 days, and cells were cultured for more than 50 days.

Transfection

The pHTS-NF- κ B plasmid containing a luciferase reporter and neomycin-resistance genes was purchased from Biomyx Technology (San Diego, CA). Serum- and feeder-free cultured iPSCs were detached with 1 mg/mL dispase (Stemcell Technologies), according to the manufacturer's protocol. Detached iPSCs were dispersed with TrypLE Select (Life Technologies). The pHTS-NF- κ B plasmid was introduced into the dispersed iPSCs by X-treme GENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). Transfectants were cultured on a monolayer of mitomycin C-exposed G418-resistant mouse primary cultured embryonic fibroblasts (Kitayama Labs) in iPSC growth medium. Clones were obtained by limiting dilution method after 100 μ g/mL G418 (Geneticin, Life Technologies) selection and screened for responsiveness to 10 nM phorbol 12-myristate 13-acetate (PMA, Wako Pure Chemical Industries, Osaka, Japan) or 10 μ M ionomycin (Wako) in a luciferase assay.

Degranulation assay

Degranulation of MCs was determined in β -hexosaminidase and histamine release assays. MCs derived from iPSCs were sensitized with a threefold dilution of sera in StemPro-34 medium overnight and stimulated with a specific antigen (e.g., cedar, mite, HD, ragweed; Torii Pharmaceuticals) diluted with StemPro-34 medium for 30 min at 37 °C. In parallel, iPSC-MCs were stimulated with an anti-human Fc ϵ R1 α monoclonal antibody CRA1 (Bio Legend, San Diego, CA) for 30 min. Supernatants were collected and residual cells were then frozen in liquid nitrogen and soon returned to a 37 °C water bath to thaw. The freeze-thaw procedure was repeated twice.

The activities of β -hexosaminidase in the supernatants and cell lysates were determined using a fluorometric assay with 4-methylumbelliferonyl-N-acetyl-D-glucosaminide-6-sulfate (Glycosynth Limited, Cheshire, UK) as a substrate (0.1 mM in 100 mM citrate, pH 4.5). After a 30-min incubation at 37 °C, the reaction was stopped with 0.25 M glycine buffer. The plate was read on EnSpire plate reader (Perkin Elmer, Waltham, MA) using 365-nm excitation and 445-nm emission filters. Histamine levels were measured by an enzyme-linked immunosorbent assay kit (Medical and Biological Laboratories, Nagoya, Japan) according to the manufacturer's protocol.

Luciferase assay

MCs derived from luciferase reporter gene-transfected iPSCs were suspended in fresh MC basal medium and inoculated onto a Prime Surface 96U plate (Sumitomo Bakelite) at a density of 1×10^4 cells/50 μ L/well. The cells were sensitized with various concentrations of sera overnight (18 h) at 37 °C, 5% CO₂ and without

washing, a specific antigen diluted with StemPro-34 medium (50 μ L/well) was added to the cell-serum mixture for 3 h at 37 °C, 5% CO₂. After the antigen stimulation, 100 μ L of luciferase substrate solution containing cell lysis reagent (ONE-Glo, Promega Corp., Tokyo, Japan) was added to the cells, and chemiluminescence was measured using EnSpire plate reader. For confirmation of IgE dependency iPSC-MCs were sensitized overnight with a threefold dilution of serum A6 that had been incubated earlier for 6 h with or without an anti-IgE antibody omalizumab (Novartis Pharma AG, Basel, Switzerland), and subsequently stimulated with an antigen as described above.

Measurements were done in duplicate or triplicate, and the average was used for analysis.

Flow cytometry

Cells were stained for 30 min in 0.3% BSA-containing phosphate-buffered saline (PBS) with phycoerythrin (PE)-labeled monoclonal antibodies to human CD34 (Affymetrix eBioscience, Santa Clara, CA; clone 4H11), CD45 (Affymetrix eBioscience; clone HI30), CD117 (c-kit) (Affymetrix eBioscience; clone YB5.B8), CRA1, HLA-DR (BD; clone L243), and CD203c (Beckman coulter, Marseille France; clone 97A6). Stained cells were washed with BSA-containing PBS and analyzed using a FACSVerse or FACSCalibur cytometry system (BD) with BD FACSuite software (BD).

Measurement of CD203c expression on basophil activation test

A commercial kit (Allergenicity Kit, Beckman Coulter, Brea, CA) was used to measure basophil CD203c expression levels, following the manufacturer's instructions. Briefly, heparinized whole blood was incubated with 0.5 JAU/mL of cedar pollen for 20 min at 37 °C in a 5% CO₂ incubator. Anti-IgE antibody (4 μ g/mL) and PBS (vehicle) were used as positive and negative controls, respectively. After incubation, PC7-conjugated anti-CD3, FITC-conjugated anti-CRTH2, and PE-conjugated anti-CD203c antibodies were added to the reaction mixtures. Red blood cells were lysed, and leukocytes were analyzed on a flow cytometer. Basophils were identified by characteristic forward and side scatter, expression of CRTH2 and absence of CD3. Percentages of activated basophils were determined using a threshold that was defined by the fluorescence intensity for CD203c over the top 3 $\pm$ 0.5% of individual unstimulated cells (negative control).

Immunohistochemistry

For detection of MC markers tryptase and chymase, cells were attached to poly-L-lysine-coated glass slides (Muto Pure Chemicals, Tokyo, Japan), fixed with 4% paraformaldehyde in PBS, and blocked by blocking buffer (3% BSA, 0.2% Triton X-100 in PBS, all from Sigma). The cells were stained with 3 μ g/mL anti-tryptase (Merck Millipore, Billerica, MA; clone G3) or 3 μ g/mL anti-chymase antibody (Merck Millipore; clone B7) in blocking buffer, followed by incubation with Alexa Fluor 488-labeled goat anti-mouse IgG₁ antibodies (Invitrogen).

Statistics

Data are presented as the mean $\pm$ S.D., and the statistical significance of the differences was evaluated by Student's *t*-test or by one-way analysis of variance followed by post-hoc analysis with the Dunnett test using GraphPad Prism version 5.04. *P* values less than 0.05 were considered significant. Data were determined in at least three separate experiments.

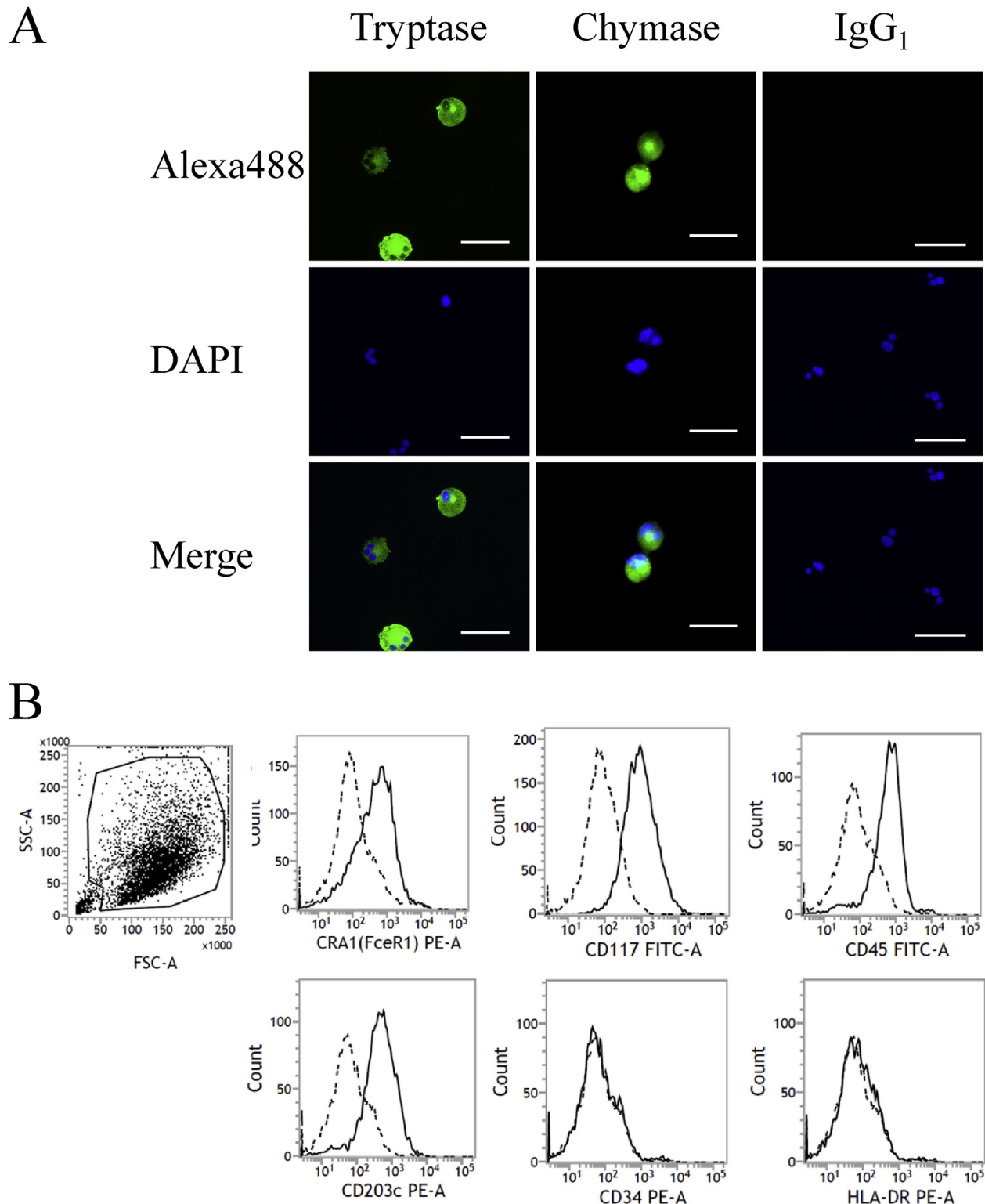


Fig. 1. MCs derived from human iPSCs showed characteristic features of human MCs. **(A)** MCs differentiated from human iPSCs were stained on day 40 with monoclonal antibodies to tryptase and chymase or isotype control mouse IgG₁ and then Alexa Fluor 488-labeled goat anti-mouse IgG₁. Nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Photographs were taken with a Zeiss Axiovert 200 M fluorescence microscope using an Axioacam HRM camera, operated by Axiovision software (Carl Zeiss). Bar = 20 μ m. **(B)** Day 40 iPSC-MCs were stained with fluorescent dye-labeled antibodies to FcεR1, CD117 (c-kit), CD45, CD203c, CD34, HLA-DR and subjected to flow cytometry. Living cells were gated on the basis of forward scatter (FSC) and side scatter (SSC), characteristics as shown in the upper left panel. Staining patterns with a specific antibody and an isotype control are depicted by solid and dashed lines, respectively, in each panel. Representative results from three experiments are shown.

Results

Generating the iPSC-MC lines

We generated MCs from human iPSCs by a serum- and feeder-free method in a chemically defined medium. The differentiated cells expressed tryptase, chymase, FcεR1, c-kit, CD45 and CD203c, but not CD34 and HLA-DR (Fig. 1), which identified them as mature MCs.^{33,34} The MC marker-positive cells reached to 80–90% of the total cells at day 40. The iPSC-MCs expanded 5–10 times during the culture. Under our culture conditions, iPSC-MC lines could be maintained for at least 6 months without losing their MC phenotype and function.

Sensitized iPSC-MCs can respond to allergens

To analyze the function of iPSC-MCs, we examined their responsiveness to FcεRI stimulation with an agonistic anti-FcεRI mAb CRA1. To evaluate iPSC-MCs degranulation, we measured release rates of both β-hexosaminidase and histamine after CRA1 treatment. iPSC-MCs responded to CRA1 in a concentration-dependent manner in both assays, though the amount of histamine released was mostly higher than that of β-hexosaminidase release (Fig. 2A, B).

Next, we examined the ability of iPSC-MCs to respond to specific allergens. iPSC-MCs were sensitized with threefold diluted serum from a cedar pollen-allergic patient, and cedar-induced degranulation was measured by a histamine release test. iPSC-MCs sensitized with serum from subject A4 degranulated in response to a

cedar allergen in a concentration-dependent manner (Fig. 2C). However, no degranulation occurred when iPSC-MCs were sensitized with serum from H1. Next, we investigated the responses of sensitized iPSC-MCs against mite, HD, and ragweed with serum A4. The sensitized iPSC-MCs responded to mite and HD, but not to ragweed, in a concentration-dependent manner (Fig. 2D). These results indicate that iPSC-MCs function similar to mature MCs.

In BAT, basophil activation rates in subjects A4 and H1 were 92.4% and 2.8%, respectively, when stimulated with cedar (Table 1). In subject A4, basophils were activated by mite (93.9%) and HD (97.0%) stimulation, but not by ragweed (4.9%) in BAT assay. These results indicate that iPSC-MCs were sensitized to an allergic serum with similar results of IgE CAP test and BAT assay.

Sensitized iPSC-NB7AMCs respond appropriately to CRA1 or specific allergens

We transfected the NF-κB-dependent luciferase reporter gene into cell line 201B7 to establish a simple assay system for cell activation. NF-κB is one of the transcription factors that is activated after FcεRI cross-linking in mast cells.^{35,36} After G418 selection, we obtained 10 clones that responded to PMA or ionomycin stimulation at various levels. The three clones with the highest responsiveness (i.e. NB7A, NB7H, and NB8H) were selected for further experiments. These clones had almost identical cell morphology and growth rates with 201B7, and were positive for pluripotent markers Oct-3/4, Tra-1-60, Nanog, SSEA-3, E-cadherin and alkaline phosphatase (data not shown). We confirmed that MC lines differentiated from these clones expressed FcεRI by day 40 (data

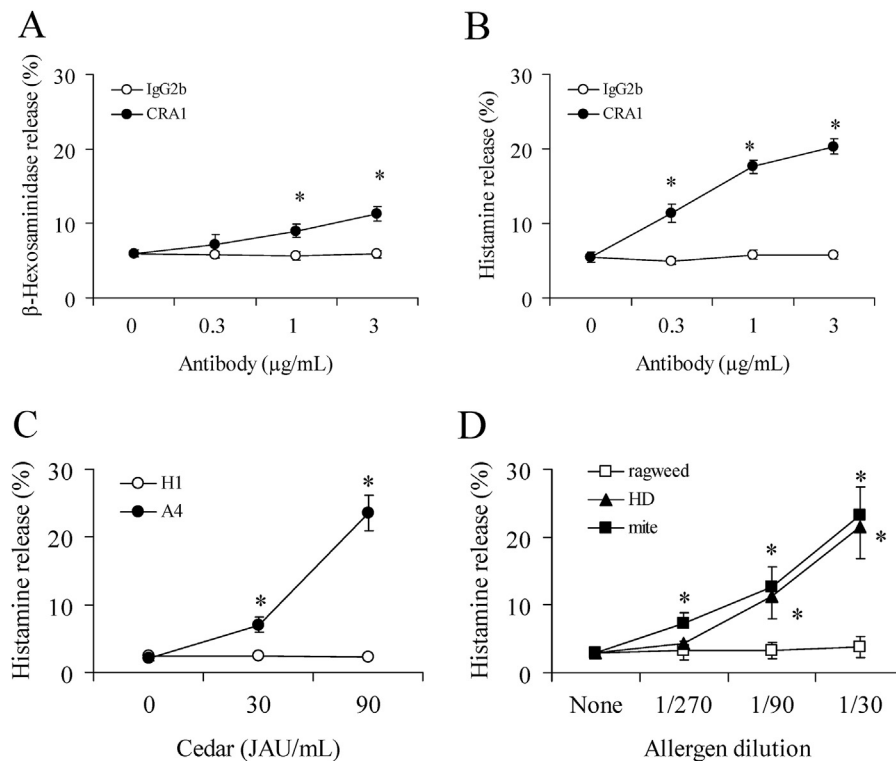


Fig. 2. Human iPSC-MCs responded to stimulation of CRA1 (anti-IgE receptor antibody) or various allergens. (A, B) iPSC-MCs were stimulated with the indicated concentrations of CRA1 (closed circles) or isotype control IgG2b (open circles) for 30 min, and β-hexosaminidase (A) and histamine (B) release rates were examined as described in the Materials and Methods. **P* < 0.05 between CRA1-treated cells and IgG2b-treated cells at the same antibody concentrations (Student's *t*-test). (C) iPSC-MCs were sensitized with serum from a cedar pollen allergy patient A4 (closed circles) or a control subject without cedar pollen allergy H1 (open circles) and stimulated with cedar pollen antigen for 30 min. Degranulation in the stimulated iPSC-MCs was assessed by histamine release (*n* = 3). **P* < 0.05 compared to vehicle controls (0 JAU/mL) (Dunnett test). (D) Histamine release in A4 serum-sensitized iPSC-MCs after stimulation with mite antigen (closed squares), house dust antigen (closed triangles) or ragweed pollen antigen (open squares) is shown. **P* < 0.05 compared to vehicle controls (None; No antigen) (Dunnett test). Data are presented as the means ± SD (*n* = 3).

not shown) and functionally responded to CRA1 stimulation in a concentration-dependent manner (Fig. 3A). In particular, MCs differentiated from clone NB7A (referred to as iPSC-NB7AMCs) showed the highest response to CRA1 stimulation (Fig. 3A). Hence, iPSC-NB7AMCs were used for further experiments.

The response of iPSC-NB7AMCs to cedar pollen was examined after the cells were sensitized with various concentration of A6 serum. Sensitized iPSC-NB7AMCs showed an increase in luminescence intensity after stimulation with cedar pollen in a serum concentration-dependent manner (Fig. 3B). Notably, the greatest response was with undiluted serum. We named this system iMAT.

Next, iPSC-NB7AMCs were sensitized for 18 h with sera from six subjects with cedar pollen allergy and two normal subjects and stimulated with cedar pollen allergen for 3 h. The iPSC-NB7AMCs sensitized with sera from subjects A1 to A6 increased

luminescence intensity after stimulation with cedar in a concentration-dependent manner, but there was no response in cells sensitized with serum from H1 or H2 (Fig. 3C). In addition, iPSC-NB7AMCs sensitized with serum A1 responded to mite and HD in a concentration-dependent manner, but not to ragweed (Fig. 3D). These results correlated well with the data from the IgE CAP test for each serum (Table 1) and those of a histamine release test with iPSC-MCs (Fig. 2D).

Sensitized iPSC-NB7AMCs respond to allergens through IgE-FcεRI interaction

To check involvement of IgE–FcεRI interaction, 3–5-week-cultured iPSC-NB7AMCs were sensitized overnight with a threefold dilution of serum A6 that had been incubated for 6 h with or

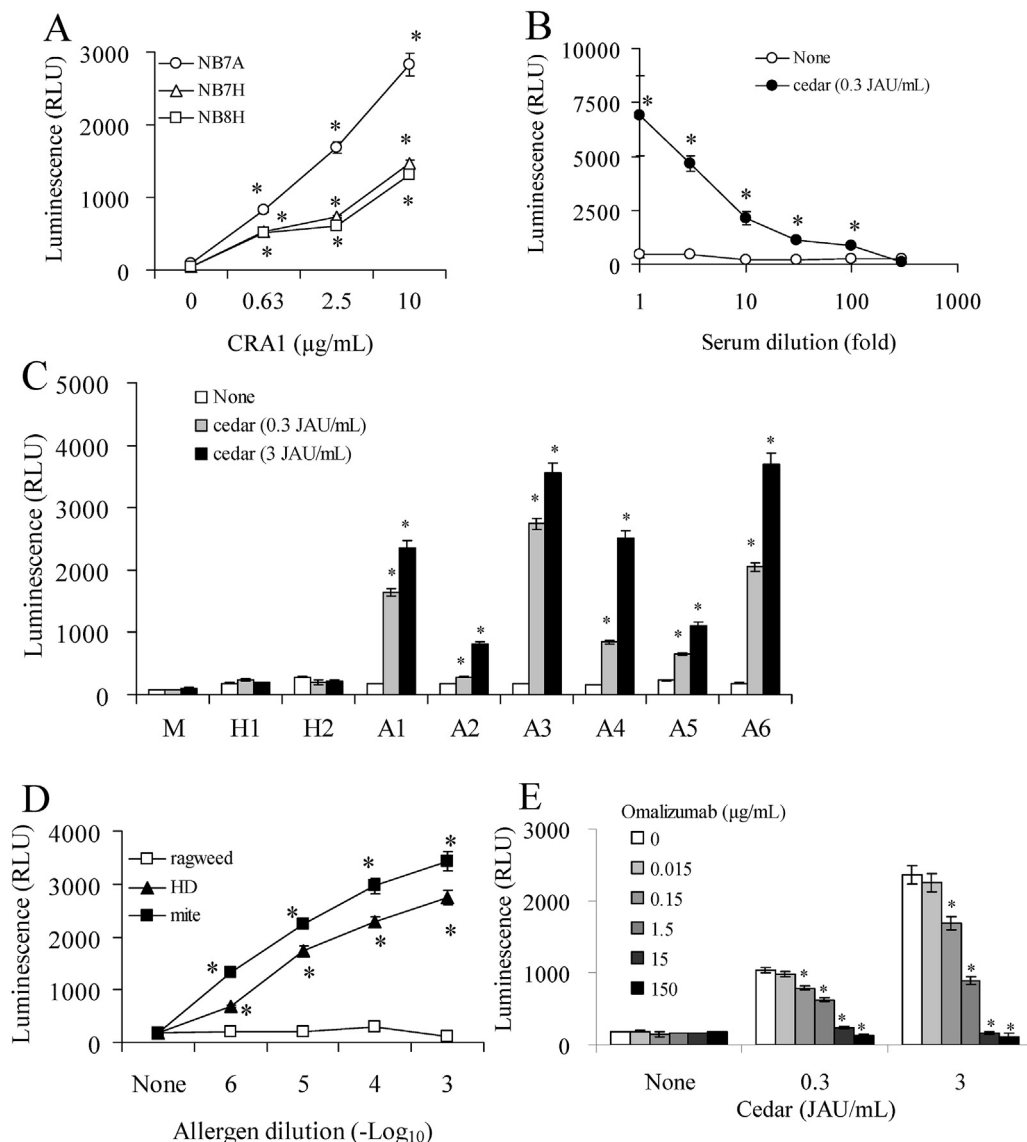


Fig. 3. iPSC-NB7AMCs responded to various antigens through IgE–FcεRI interaction. (A) Cellular luminescence of human MCs derived from three transfectants, NB7A (open circle), NB7H (open triangles) or NB8H (open squares) after stimulation with the indicated concentrations of CRA1. * $P < 0.05$ compared to vehicle controls (0 μg/mL CRA1) (Dunnett test). (B) iPSC-NB7AMCs were sensitized with various dilutions of A6 serum and stimulated with cedar pollen antigen (0.3 sJAU/mL, closed circles) or vehicle (open circles). * $P < 0.05$ between cedar (0.3 JAU/mL)-treated cells and vehicle-treated cells at the same serum dilution levels (Student's *t*-test). (C) iPSC-NB7AMCs were sensitized with eight sera (H1, H2, A1 to A6, described in Table 1) or StemPro-34 medium alone (M), and stimulated with cedar pollen antigen (0 JAU/mL; open boxes, 0.3 JAU/mL; gray boxes, 3 JAU/mL; black boxes). * $P < 0.05$ compared to vehicle controls (None) (Dunnett test). (D) iPSC-NB7AMCs were sensitized with A1 serum, and stimulated with mite antigen (closed squares), house dust antigen (closed triangles) or ragweed pollen antigen (open squares). * $P < 0.05$ compared to vehicle controls (None) (Dunnett test). (E) iPSC-NB7AMCs were sensitized with A6 serum in the presence of the indicated concentrations of omalizumab, and stimulated with cedar pollen antigen. * $P < 0.05$ compared to omalizumab-untreated controls (0 μg/mL omalizumab) (Dunnett test). Data are presented as the means $\pm$ SD ($n = 3$).

without an anti-IgE antibody omalizumab. iPSC-NB7AMCs were then added with cedar (0, 0.3 and 3 JAU/mL) and examined for luciferase activity. The increase in luminescence intensity was completely inhibited by omalizumab (Fig. 3E).

iPSC-NB7AMCs sensitized with sera from BAT low responders, respond to allergens

We next examined the response of iPSC-NB7AMCs to sera from cedar pollen allergic subjects who showed a low response in BAT (BAT-LR). Basal CD203c expression levels in basophils from H1, A4, BAT-LR1 and BAT-LR2 were around 3% (Fig. 4A). When stimulated with an anti-IgE antibody, a positive control, basophils from H1 and A4 were fully activated, but those from BAT-LR1 and BAT-LR2 were not sufficiently activated (Fig. 4A, lower central panels). The results of BAT assay with cedar in subjects H1 and A4 correlated with the results of IgE CAP test of their sera (activated basophils 2.8% and 92.4% in BAT, class 0 and class 3 in IgE CAP test, respectively), but those in BAT-LR subjects did not correlate to their IgE CAP test data (6.1% and 3.3% in BAT, class 2 and class 3 in IgE CAP test) (Fig. 4A lower right panels and Table 1).

iPSC-NB7AMCs were sensitized with sera from these subjects and stimulated with cedar. Both histamine release rates and luciferase activities were increased in the iPSC-NB7AMCs previously sensitized with sera from A4 and two BAT-LR subjects in a cedar concentration-dependent manner (Fig. 4B).

Discussion

In this study, we generated MCs from human iPSCs and confirmed that they stably express high-affinity IgE receptor and functionally respond to various allergens when previously

sensitized with human sera from relevant allergic subjects. This passive IgE sensitization system, called iMAT, worked well even in undiluted human sera and could detect allergen-specific biologically active IgE in sera from BAT low-responder subjects.

Previous studies showed that two types of MCs in human tissues.³⁷ The connective tissue type contains tryptase, chymase, MC carboxypeptidase and cathepsin G in its secretory granules and is predominantly located in skin and intestinal submucosa. The mucosal type that contains tryptase but not the other three proteases and is mainly located in the alveolar wall and small intestinal mucosa. We speculate that MCs generated in this study are classified as connective tissue type because they showed tryptase and chymase expression.

Under our culture conditions, MC lines derived from human iPSCs could be maintained for at least 6 months and retain their MC phenotype and function. For example, an iPSC-NB7AMC line (7A-1), the longest cultured line, could be regularly used as a source of MCs for iMAT assay over 8 months. Their monthly responses to 3 JAU/mL cedar pollen antigens were 230, 185, 202, 230, 185, 178, 208, and 182 luminescence units with H1 serum and 2738, 2508, 2390, 2369, 2310, 2085, 2312, and 2369 luminescence units with A4 serum in the luciferase assay. Other iPSC-NB7AMC lines (7A-2, 7A-3 and 7A-4) showed similar levels of response. iMAT requires as few as 500 cells per well in 96-well culture plate format (data not shown). Therefore, differentiated MCs in one 90-mm diameter culture dish, which are equivalent to around 10^6 MCs, are sufficient for nearly 2000 wells of passive IgE sensitization assay.

Like EXiLE, iMAT uses a luciferase-reporter gene, which simplified the assay procedure and markedly increased the allergen-detecting sensitivity, compared with a histamine release assay (Fig. 4). Furthermore, as expected from its human origin nature, iMAT could measure undiluted human sera with no sign of cell

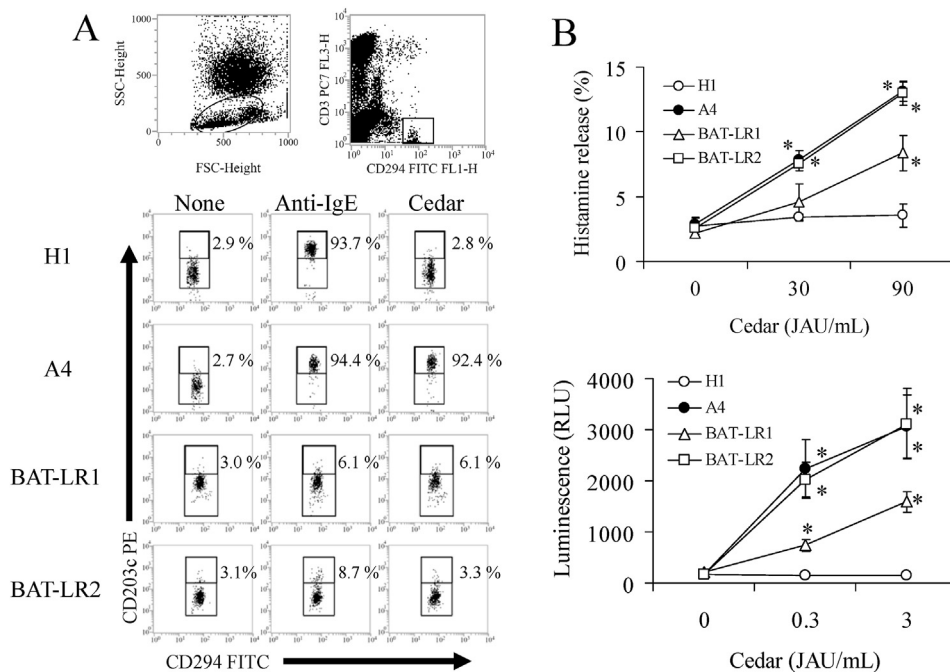


Fig. 4. iPSC-MCs sensitized with serum from BAT-LR subjects responded to specific antigens. (A) Flow cytometry of BAT using whole blood from H1, A4, BAT-LR1 and BAT-LR2. Stimulators used in this experiment were PBS (negative control), anti-IgE (positive control) or cedar pollen antigen (0.5 JAU/mL). Basophils were identified on the basis of FSC and SSC characteristics, expression of CD294 and absence of CD3 (upper two panels). On other panels, expression levels of CD203c in the gated basophils are shown. The percentages of activated basophils are indicated in each panel. Data are representative of three experiments. (B) iPSC-NB7AMCs were sensitized with serum from BAT-LR1 (open triangles), BAT-LR2 (open squares), A4 (closed circles) as positive control, or H1 (open circles) as negative control, and stimulated with cedar pollen antigen. The responsiveness of the sensitized cells were evaluated by histamine release (upper panel) and luciferase expression (lower panel) assays ($n = 3$). * $P < 0.05$ compared to vehicle controls (0 JAU/mL Cedar) (Dunnett test). Data are presented as the means $\pm$ SD.

damage (Fig. 3B). This is a key advantage over RBL-2H3 cell-based passive IgE sensitization assays. Inhibitory factors that affect IgE-mediated basophil activation are increased in sera from subjects who acquired tolerance to particular allergens through their natural history or immunotherapy.^{27,38,39} Such factors are considered clinically relevant, and the BAT assay detects the influence of such factors in whole blood. The serum factors include blocking IgG, inhibitory IgG, immune complex, and others.^{27,38,39} The inhibitory effects of the serum factors decreased dramatically with increasing dilution of autologous sera in BAT assay.²⁷ Thus, undiluted or low-dilution rate serum samples are desirable to reflect the influence of serum inhibitory factors. As shown in this study, iMAT is easily applicable to such samples and, therefore, is expected to perform similarly to the BAT assay. In addition, iMAT may be applicable to retrospective epidemiological study using a serum bank.

In this study, we showed allergen-specific biologically active IgE in sera from BAT low-responder subjects with iMAT. Basophils from some blood donors do not respond to stimulation by FcεRI cross-linking in HRT and/or BAT assays. The percentages of such non-responders are 5–25%, depending on the study.^{14–17} Although the mechanisms of the basophil unresponsiveness are not well understood, deficiency in some signal transduction molecules, such as Syk, was reported.^{40,41} Few reports have pursued allergen-specific functional IgE levels in the nonresponder subjects. Studies using iMAT might shed light on the issue of non/low responders. Apart from these causes, food supplement- or drug-induced temporary unresponsiveness of basophils was also reported. Basophil activation is reduced with daily consumption of γ-tocopherol in dust mite-allergic patients.⁴² Similarly, basophils from patients receiving long-term steroid therapy showed reduced responses to allergen stimulation.⁴³ In our preliminary data, dexamethasone did not affect the results of iMAT (data not shown). Therefore, iMAT may be useful with the donors whose basophils are temporary unresponsive in HRT or BAT assay.

We observed that receptors for anaphylatoxins C3a and C5a were slightly expressed on iPSC-MCs (data not shown). Complement-dependent activation of MCs leads to their degranulation in an IgE-independent manner.^{44–46} Also, platelet-activating factor activates MCs through its specific surface receptor in an IgE-independent manner.⁴⁶ In the future, it will be important to scrutinize the effects of these IgE-independent factors on the iMAT assay.

In conclusion, we demonstrated the effectiveness of human iPSC-derived MCs in a passive IgE sensitization system. This system, named iMAT, may serve as a novel determining system for IgE/allergens in the clinical and research settings.

Acknowledgment

We are grateful to Drs. Tatsutoshi Nakahata and Hiromitsu Nakauchi for their helpful advice.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.alit.2017.08.008>.

Conflict of interest

AI, TK, HH and KN are employees of BML, Inc. The rest of the authors have no conflict of interest.

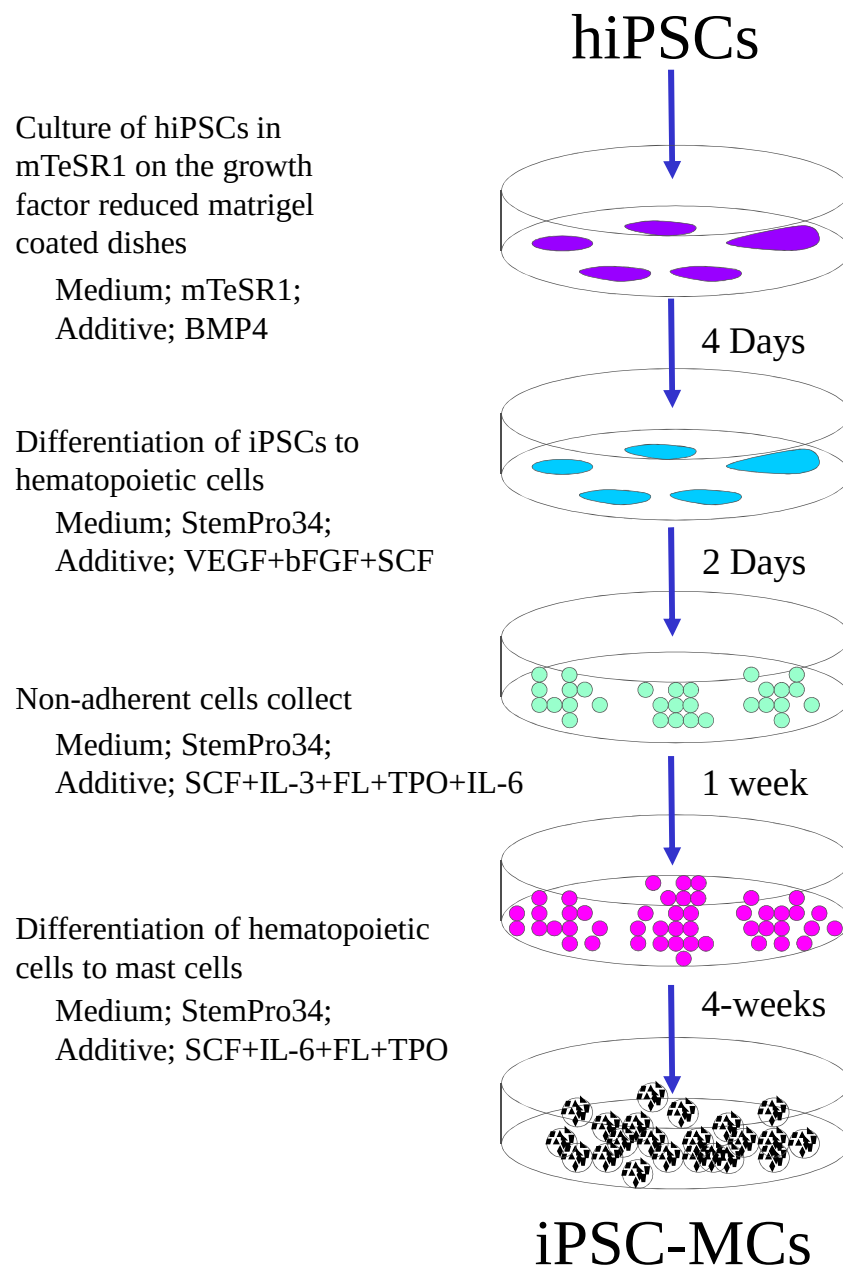
Authors' contributions

AI, YE, HH, KN, and KT designed the study and wrote the manuscript. AI and TK contributed to data collection. AI performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

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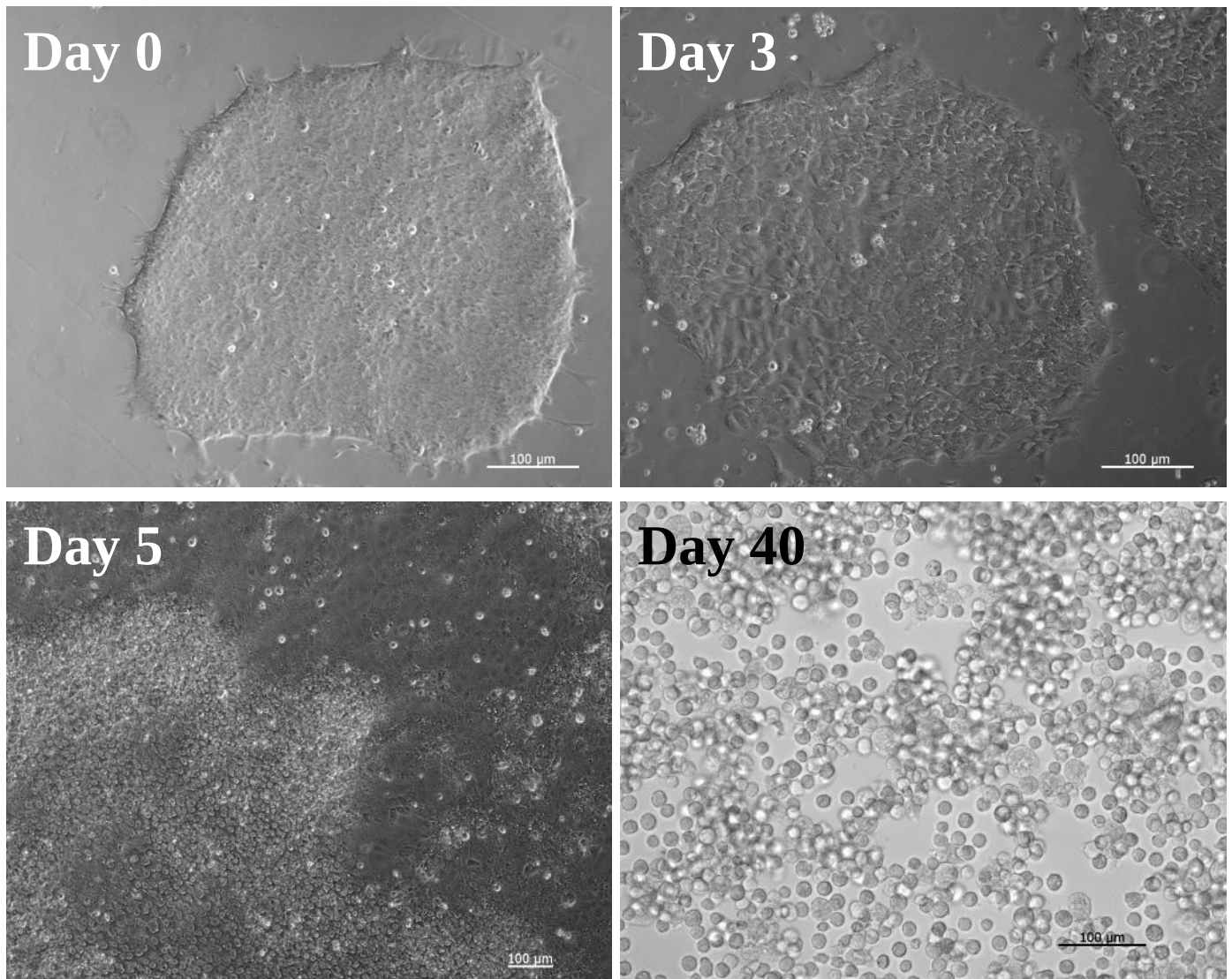
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Supplementary Fig. 1.

Illustration of cell images and the induction of MCs from human iPSCs with a serum- and feeder-free culture method.

Schematic diagrams of the induction of MCs from human iPSCs. Under feeder cell-free, iPSCs were cultured at a density of about 30 colonies per 100-mm dish. When the individual colony reached approximately 1 mm in diameter (day 0), BMP4 (80 ng/mL) was added to the mTeSR1 culture medium for 4 days (days 0-4). In turn, the culture medium was replaced with StemPro-34 serum-free medium, supplemented with 1% nonessential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (referred to as MC basal medium), and a cytokine cocktail of 80 ng/mL VEGF, 25 ng/mL bFGF and 100 ng/mL SCF for 2 days (days 4-6). The protocol for hematopoietic progenitor expansion and initial myeloid differentiation was modified from Yanagimachi et al. (32). Cells were cultured in MC basal medium supplemented with 100 ng/mL SCF, 10 ng/mL IL-3, 10 ng/mL TPO, 100 ng/mL IL-6, and 10 ng/mL FL. The medium was replaced every 3-4 days, and nonadherent cells were collected after 3 weeks (from day 27 onward).



Supplementary Fig. 2.

Phase contrast microscope photos of cells illustrated in Supplementary Fig. 1.

Day 0: Cells were cultured with mTeSR1 on the Growth Factor-Reduced Matrigel coated dish.

Day 3: Cells were cultured with mTeSR1 containing BMP4 for 3 days.

Day 5: Cells were cultured with MC basal medium containing VEGF, bFGF and SCF for 1 day.

Day 40: Cells were cultured with MC basal medium containing SCF, IL-6, FL and TPO.