

## Characterization of Dendritic Cells and Macrophages Generated by Directed Differentiation from Induced Pluripotent Stem Cells

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**Key Words.** Induced pluripotent stem cells • Dendritic cells • Macrophages • MHC • Embryonic stem cells • Cell therapy

### ABSTRACT

Methods have been established to generate dendritic cells (DCs) from mouse and human embryonic stem (ES) cells. We designated them as ES-DCs and mouse models have demonstrated the induction of anti-cancer immunity and prevention of autoimmune disease by in vivo administration of genetically engineered ES-DCs. For the future clinical application of ES-DCs, the histoincompatibility between patients to be treated and available human ES cells and the ethical concerns associated with human ES cells may be serious obstacles. However, recently developed induced pluripotent stem (iPS) cell technology is expected to resolve these issues. This report describes the generation and characterization of DCs derived from mouse iPS cells. The iPS cell-derived DCs (iPS-DCs) pos-

sessed the characteristics of DCs including the capacity of T-cell-stimulation, antigen-processing and presentation and cytokine production. DNA microarray analyses revealed the upregulation of genes related to antigen-presenting functions during differentiation into iPS-DCs and similarity in gene expression profile in iPS-DCs and bone marrow cell-derived DCs. Genetically modified iPS-DCs expressing antigenic protein primed T-cells specific to the antigen in vivo and elicited efficient antigen-specific anti-tumor immunity. In addition, macrophages were generated from iPS cells (iPS-MP). iPS-MP were comparable with bone marrow cell-derived macrophages in the cell surface phenotype, functions, and gene expression profiles. *STEM CELLS* 2009;27:1021–1031

Disclosure of potential conflicts of interest is found at the end of this article.

### INTRODUCTION

Dendritic cells (DCs) are the most potent antigen-presenting cells (APC) which are known to play major roles in the priming of naive T-cells and also in the maintenance of immunological self-tolerance, by promoting T-cells with regulatory functions or by inducing anergy of T-cells. Several groups have previously established methods to generate APC or DCs from mouse [1, 2] and human [3–5] embryonic stem (ES) cells (ES-DCs). Genetic engineering of ES-DCs can readily be done by the introduction of transgenes into undifferentiated ES cells and subsequent differentiation of the ES cell clones into ES-DCs. By genetic engineering, we can generate ES-DCs capable of modulating immune response in an antigen-specific manner. Mouse systems have demonstrated the induction of anti-cancer immunity [6–10] and the prevention of autoimmune disease [11, 12] by in vivo administration of genetically engineered ES-DCs.

Considering the future clinical application of ES-DCs technology, however, the unavailability of human ES cells genetically identical to the patients to be treated is a problem. In addition, ethical concerns related to the use of human ES cells are anticipated to be serious obstacles which will hinder the realization of the medical use of human ES-DCs.

It was recently revealed that ES cell-like pluripotent stem cells, designated as induced pluripotent stem (iPS) cells, can be generated by the simultaneous introduction of several genes for reprogramming factors, such as Oct3/4, Sox2, Klf4, and c-Myc, into somatic cells [13–20]. The issue of histoincompatibility between patients to be treated and ES cells may be overcome by the generation of iPS cells from somatic cells of the patients such as fibroblasts. The major ethical issues related to human ES cells would be avoided by aid of iPS cell technology, because the use of human embryos is not necessary for the generation of iPS cells.

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Differentiation of iPS cells into various cells belonging to the three germ layers has been demonstrated by the analysis of teratomas generated from mouse and human iPS cells. In addition, the pluripotency of iPS cells is obvious by the contribution of iPS cell-derived cells to various organs of the chimeric mice developed from iPS cell-introduced blastocysts [14]. As for the in vitro generation of cells of mesodermal lineage from iPS cells, differentiation into cardiac myocytes and endothelial cells from mouse iPS cells has been recently reported [21–23]. However, it remains to be elucidated whether fully differentiated and functional hematopoietic cells can be generated from iPS cells by directed differentiation in vitro. In the present study, we generated DCs and macrophages from mouse iPS cells (iPS-DCs and iPS-MP), and characterized them by morphological, functional, and gene-expression analyses.

## MATERIALS AND METHODS

### Cell Lines, Cytokines, Chemicals, and Peptides

The mouse embryonic fibroblast-derived iPS cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 15% ES cell-qualified fetal calf serum (FCS; Gibco-Invitrogen, Carlsbad, CA; <http://www.invitrogen.com>), 1,000 U/ml leukemia inhibitory factor, 50 U/ml penicillin, 50 mg/ml streptomycin, non-essential amino acids, and 50  $\mu$ M 2-mercaptoethanol (2-ME) on feeder cell layers of mitomycin C-treated mouse primary embryonic fibroblasts (PEF). Mouse bone marrow stromal cells, OP9 [24], were maintained in DMEM supplemented with 20% FCS and seeded onto gelatin-coated dishes before used as feeder cells. The T-cell hybridomas, RF33.70 [25], recognizing ovalbumin (OVA)<sub>257–264</sub> in the context of K<sup>b</sup> and DO11.10 [26], recognizing OVA<sub>323–339</sub> in the context of I-A<sup>d</sup>, were maintained in RPMI-1640 medium supplemented with 10% FCS. MO4 [27], a C57BL/6-derived B16 melanoma cell line expressing OVA, was maintained in RPMI-1640 medium supplemented with 10% horse serum. Recombinant mouse interleukin (IL)-4, tumor necrosis factor (TNF)- $\alpha$ , granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) were purchased from Peprotec (London, U.K.; <http://www.peprotech.com>). Lipopolysaccharide (LPS) from *Escherichia coli* and OVA protein was purchased from Sigma Chemical (St. Louis, MO; <http://www.sigma-chem.com.au>), agonistic anti-CD40 monoclonal antibody (mAb, clone HM-40-3) was from PharMingen (San Jose, CA; <http://www.bdbiosciences.com>) and OK432 was purchased from Chugai Pharmaceutical (Tokyo, Japan; [http://www.chugai-pharm.co.jp/hc/chugai\\_top\\_en.jsp](http://www.chugai-pharm.co.jp/hc/chugai_top_en.jsp)). OVA<sub>257–264</sub> peptide, SIINFEKL, was synthesized on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan; <http://www.shimadzu.com>) and purified by HPLC.

### Differentiation Culture

The procedure for induction of differentiation of iPS cells into DCs is composed of three steps (supporting information Fig. 1). Step 1: iPS cells were suspended in  $\alpha$ -MEM supplemented with 20% FCS and seeded ( $1 \times 10^5$  cells per dish) onto OP9 cell layers in 100-mm dishes. On day 6 or 7, the cells were treated with PBS/0.25% trypsin/1 mM ethylenediaminetetraacetic acid (EDTA; trypsin/EDTA) for 10 minutes, recovered with medium containing FCS, and subjected to step 2 culture or stocked frozen for future use. Step 2: Cells harvested from step 1 culture were suspended in  $\alpha$ -MEM supplemented with 20% FCS, GM-CSF (1,000 U/ml), and 2-ME (50  $\mu$ M) and plated onto freshly prepared OP9 cell layers. Cells recovered from one dish of step 1 culture were seeded onto 8–10 dishes. Thereafter, at 6 days after the passage, floating cells were recovered by pipetting and then were subjected to step 3 culture or stocked frozen. Step 3: The cells were transferred to bacteriological Petri dishes ( $5 \times 10^5$

cells/100-mm dish) without feeder cells and cultured in RPMI-1640 medium supplemented with 10% FCS, GM-CSF (1,000 U/ml) and 2-ME. To induce complete maturation of iPS-DCs, cells cultured for 10–14 days in Petri dishes were transferred to new dishes and cultured in RPMI-1640/10% FCS supplemented with GM-CSF (1,000 U/ml), IL-4 (10 ng/ml), TNF- $\alpha$  (5 ng/ml), and anti-CD40 mAb (10  $\mu$ g/ml). For the generation of macrophages, the cells recovered from step 2 were cultured in bacteriological Petri dishes or tissue culture-coated dishes ( $1-2 \times 10^6$  cells/100-mm dish) in RPMI-1640 medium supplemented with 10% FCS, 5% horse serum, M-CSF (100 ng/ml), and 2-ME.

### Generation of DCs and Macrophages From Bone Marrow Cells

Femoral and tibial bone marrow cells were obtained from DBA/2 mice. To generate DCs (BM-DCs), cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 1,000 U/ml GM-CSF, and 50  $\mu$ M 2-ME for 7 days in Petri dishes. To generate macrophages (BM-MP), cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 5% horse serum, 100 ng/ml M-CSF, and 50  $\mu$ M 2-ME for 7–10 days in Petri dishes. For the analysis, macrophages were harvested using trypsin/EDTA.

### Microscopic Analysis

Unfixed cells in the culture plates were stained with phycoerythrin (PE)-conjugated anti-Flk1 monoclonal antibody (1.25  $\mu$ g/ml) in DMEM supplemented with 10% FCS for 1 hour and washed three times. Cytospin specimens were stained with May-Grünwald-Giemsa and mounted in Entellan *neu* (Merck, Darmstadt, Germany; <http://www.merck.com>). Bright-field, phase-contrast, and fluorescence microscopic analysis were done on an inverted microscope (IX70, Olympus, Tokyo, Japan; <http://www.olympus-global.com/en/global>) and microscopic images were captured by using a digital camera unit DP70 (Olympus).

### Flow Cytometric Analysis

The staining of cells and analysis on a flow cytometer (FACScan, Becton Dickinson, San Jose, CA; <http://www.bd.com>) was done as described previously [2]. The following monoclonal antibodies (mAb) conjugated with fluorescence isothiocyanate (FITC) or PE were used for staining: anti-mouse Flk-1 (clone Avas12a1, rat IgG2a, eBioscience, San Diego, CA; <http://www.ebioscience.com>), anti-mouse CD45 (clone 30-F11, rat IgG2b, eBioscience), anti-mouse CD11b (clone M1/70, IgG2b, Pharmingen), anti-mouse CD11c (clone N148, hamster IgG, Chemicon, Temecula, CA; <http://www.chemicon.com>), anti-mouse CD80 (clone RMMP-1, rat IgG2a, Caltag), anti-mouse CD86 (clone RMMP-2, rat IgG2a, Caltag), anti-F4/80 (A3-1, rat IgG2b, Serotec Ltd., Oxford, U.K.; <http://www.serotec.com>), mouse IgG2a control (clone G155-178, Pharmingen), mouse IgG2a control (clone G155-178, Pharmingen), rat IgG2a control (clone LO-DNP-16, Caltag), rat IgG2a control (clone LODNP-57, Beckman-Coulter, Fullerton, CA; <http://www.beckmancoulter.com>), and hamster IgG control (clone 530-6, Caltag). Intracellular staining with FITC-conjugated Fab fragment of anti-influenza virus hemagglutinin (HA) antibody (clone 3F10, rat IgG<sub>1</sub>, Roche Diagnostics, Basel, Switzerland; <http://www.roche-applied-science.com>) was done using IntraPrep (Immunotech, Marseille, France; [http://www.beckmancoulter.com/products/pr\\_immunology.asp](http://www.beckmancoulter.com/products/pr_immunology.asp)). Two-color staining with PE-conjugated tetramer of H-2K<sup>b</sup>-OVA<sub>257–264</sub>-complex (MBL, Nagoya, Japan; <http://www.mbl.co.jp/e/index.html>) in combination with FITC-conjugated anti-CD8 (clone KT15, Beckman-Coulter) was done according to the manufacturer's instructions.

### Mixed Lymphocyte Reaction

Splenic T-cells were isolated from female C57BL/6 mice by using a pan-T-cell isolation kit (Miltenyi Biotec, Belgisch-Bladbach, Germany; <http://www.miltenyibiotec.com>) and then they were used as responders. Graded numbers of stimulator cells were X-ray irradiated (35 Gy) and cocultured with responders ( $1.5 \times 10^5$ ) in wells of 96-well round-bottomed culture plates for

4 days. [ $^3\text{H}$ ]-methyl-thymidine (247.9 Gbq/mmol) was added (0.037 Mbq per well) during the last 16 hours of the culture. At the end of the culture, the cells were harvested onto glass fiber filters (Wallac, Turku, Finland; <http://www.perkinelmer.com>) and the incorporation of [ $^3\text{H}$ ]-thymidine was measured by scintillation counting.

### Antigen Presentation Assay

iPS-DCs were seeded into 96-well flat-bottomed culture plates ( $1 \times 10^4$  cells per well) with indicated concentrations of OVA protein, IL-4 (10 ng/ml), and anti-CD40 mAb (10  $\mu\text{g}/\text{ml}$ ) and cultured overnight. Subsequently, DO11.10 hybridoma cells were added to the wells ( $5 \times 10^4$  cells per well) and the culture was continued for further 24 hours. At the end of the culture, the concentration of IL-2 in the culture supernatant was measured by ELISA (eBioscience). For the assay with OVA-transfectant iPS-DCs, the indicated numbers of iPS-DCs were cocultured with hybridoma cells, DO11.10 or RF33.70, in the absence of exogenously added antigen for 24 hours and production of IL-2 was also measured by ELISA.

### Analysis of the Activation of NKT Cells by iPS-DCs Loaded with $\alpha$ -Galactosylceramide

Splenic T-cells of (C57BL/6  $\times$  BALB/c) F1 (CBF1) mice were isolated using nylon-wool columns. Mature iPS-DCs were cultured in the presence of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer; 100 ng/ml) or vehicle (0.00025% Polysorbate-20) alone for 18 hours, washed, and cocultured with splenic T-cells ( $1.6 \times 10^5$  DCs +  $4 \times 10^6$  T-cells per well in 24-well culture plates). After 24 hours, the cells were recovered and analyzed on their cytotoxic activity by a 4-hour  $^{51}\text{Cr}$ -release assay using YAC-1 cells ( $5 \times 10^3$  cells per well) as targets in 96-well round-bottomed culture plates. In the analysis of the stimulation of NKT cells in vivo, iPS-DCs loaded with either  $\alpha$ -GalCer or vehicle alone were intraperitoneally injected into H-2-matched CBF1 mice ( $1.2 \times 10^6$  cells per mouse). After 24 hours, the mice were sacrificed and the cytotoxic activity of whole spleen cells pooled from three mice for each group were analyzed using YAC-1 cells as targets, as described earlier.

### Quantitation of Cytokine Production by iPS-DCs

iPS-DCs were cultured in 48-well culture plates ( $1.5 \times 10^5$  cells/200  $\mu\text{l}$  per well) in the presence or absence of IL-4 (10 ng/ml), anti-CD40 mAb (10  $\mu\text{g}/\text{ml}$ ), TNF- $\alpha$  (10 ng/ml), LPS (1  $\mu\text{g}/\text{ml}$ ), and OPK432 (5 or 25  $\mu\text{g}/\text{ml}$ ). After 3 days of culture, culture supernatant was collected and concentration of IL-12p70 and TNF- $\alpha$  was determined by using ELISA kits (eBioscience).

### cDNA Microarray Analysis

iPS cell-derived cells in culture step 2, iPS-DCs, iPS-MP, BM-DCs, and BM-MP were recovered from the culture and subjected to cDNA microarray analysis, without purification of specific cell fractions. Total cellular RNA was extracted by using an RNeasy kit (Qiagen, Hilden, Germany, <http://www1.qiagen.com>). Integrity of RNA samples was verified by using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, <http://www.agilent.com>) and the purity and concentration was checked based on A260/280 (A260/280: 1.98~2.04). For each cell sample, cDNA was synthesized by using MMLV-RT using 300 ng of RNA as template and the cDNA was used as template for synthesis of cyanine-3 (Cy-3)-labeled cRNA by using T7 RNA polymerase. A total of 1.65  $\mu\text{g}$  each of Cy-3-labeled cRNA was hybridized to a microarray slide (Whole Mouse Genome Oligo Microarray 4  $\times$  44K; Agilent Technologies; <http://www.home.agilent.com>) for 17 hours and washed, according to the manufacturer's instructions (Protocol: One-Color Microarray-based Gene Expression Analysis, version 5.7). The slides were scanned on Agilent DNA Microarray Scanner and data were processed and normalized using the GeneSpring GX9.0 software program.

### Plasmid Construction

A cDNA fragment coding for a truncated form of ovalbumin, OVA<sub>241-340</sub>, was prepared by PCR amplification using full length cDNA for OVA as a template with PCR primers 5'-*cctcgagcccgccgccaccatggctagcatgttggtctgttgctgat*-3' (Xho I and Nhe I sites are indicated by underline) and 5'-*cttaagcgtagctcggacgtcgtatgggtactctctgcctgcttcattgattc*-3'. The design of these primers results in the cloning of OVA downstream of the Kozak sequence and the addition of the HA epitope (MYPYDVPDYA) to the carboxyl terminus of OVA fragment. The cDNA fragment for OVA<sub>241-340</sub>-HA was cloned into pCAG-INEO, a mammalian expression vector driven by a CAG promoter and containing the internal ribosomal entry site (IRES)-neomycin resistance gene cassette to generate pCAG-OVA-INEO. A cDNA fragment for amino terminal portion of invariant chain (Ii<sub>1-80</sub>) was prepared by PCR amplification using full-length cDNA for human Ii as a template with PCR primers, 5'-*acctcgagcccgccgccaccatggatgacagcgcgacattatctc*-3' and 5'-*aagctagcaagcttcacgacgaggttccag*-3', and inserted into the Xho I-Nhe I site of pCAG-OVA-INEO to generate the Ii-OVA<sub>241-340</sub> expression vector, pCAG-Ii-OVA-INEO.

### Transfection of iPS Cells by Electroporation

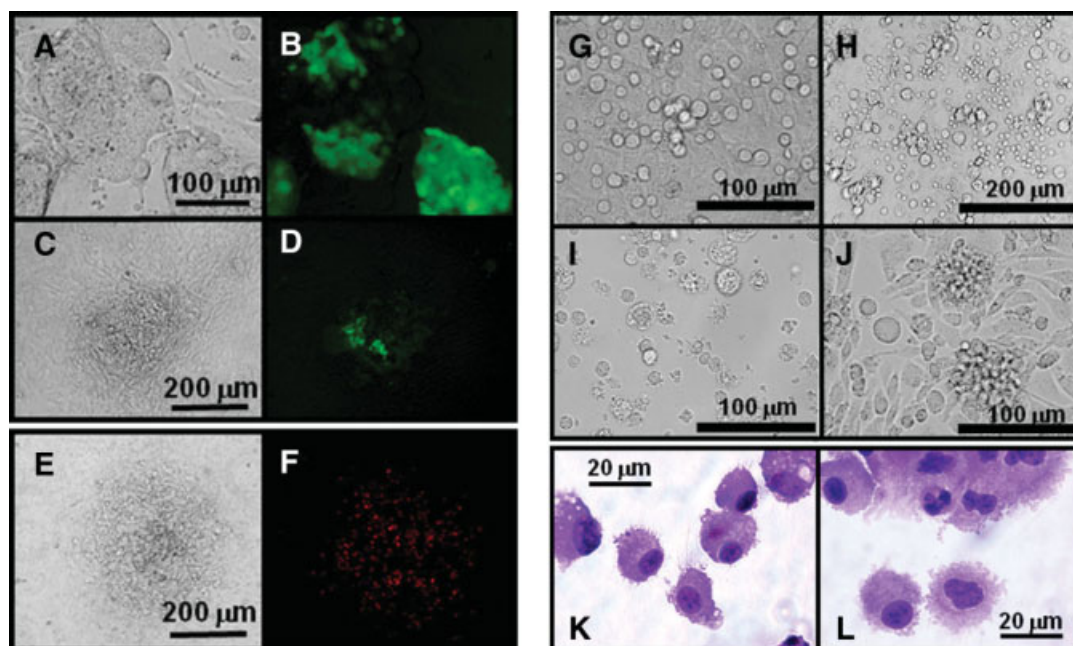
iPS cells maintained on layers of PEF were harvested by using trypsin/EDTA and suspended in DMEM ( $3 \times 10^7$  cells per milliliter) and  $1.2 \times 10^7$  cells were electroporated in a 4-mm-gap cuvette under 225 V and 600  $\mu\text{F}$  with 30  $\mu\text{g}$  of plasmid DNA. After electroporation, cells were cultured on neomycin-resistant PEF feeder layers in 100-mm culture dishes in the presence of G418 (500  $\mu\text{g}/\text{ml}$ ) for 9–10 days. Subsequently, drug-resistant colonies were picked up and transferred into 24-well culture plates. iPS cell transfectant clones with high levels of expression of the transgene were selected based on the resistance to high-dose (3 mg/ml) of G418. Thereafter, the expression of the transgene after differentiation of the transfectants was examined by intracellular staining with anti-HA mAb and flow cytometric analysis.

### Analysis of In Vivo Priming of Antigen-Specific T-Cells by iPS-DCs

Transfectant or nontransfectant iPS-DCs were stimulated with IL-4, TNF- $\alpha$ , and anti-CD40 mAb and injected intraperitoneally into C57BL/6 mice ( $1.5 \times 10^5$  cells per mouse). Eight days after the injection, spleen cells were isolated from the injected mice and pooled for each group of three mice. After hemolysis, spleen cells were cultured in 24-well culture plates ( $3 \times 10^6$  cells/2 ml per well) in RPMI-1640 supplemented with horse serum (10%), recombinant human IL-2 (100 U/ml), and OVA<sub>257-264</sub> peptide (0.001  $\mu\text{M}$ ). After 5 days, the cells were harvested and OVA-specific cytotoxic T lymphocyte (CTL) activity was analyzed by 5 hour- $^{51}\text{Cr}$ -release assay using OVA-peptide-pulsed or unpulsed EL-4 cells as targets. The percentage of specific lysis was calculated as:  $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{maximal release} - \text{spontaneous release})$ . Spontaneous release and maximal release were determined in the presence of medium or 1% Triton X-100, respectively. The experiments using mice were done according to the plan approved by animal research committee of Kumamoto University. The frequency CD8 $^+$  T-cells specific to OVA<sub>257-264</sub> was analyzed using the PE-labeled tetramer of K $^b$ -OVA<sub>257-264</sub>-complex as described earlier.

### Tumor Challenge Experiments

OVA-transfectant or nontransfectant iPS-DCs were injected intraperitoneally into C57BL/6 mice ( $1.0 \times 10^5$  cells per mouse) on day-10, and MO4 cells ( $2 \times$  or  $3 \times 10^5$  cells per mouse) were inoculated subcutaneously into the shaved right flank region on day 0. The tumor size was measured on days 11, 15, and 18 and the tumor volume was calculated as follows: tumor volume ( $\text{mm}^3$ ) = (length  $\times$  width  $\times$  height).



**Figure 1.** Morphological changes from iPS cells to iPS-DC. Phase-contrast images (A, C) and fluorescence images showing expression of green fluorescence protein (B, D) of undifferentiated Nanog-iPS cell colonies on primary embryonic fibroblasts feeder layer (A, B) and a differentiating colony on OP9 feeder layer at day 3 of the step 1 culture (C, D). A phase-contrast image (E) and a fluorescence image showing expression of Flk-1 (F) of a colony at day 6 of the step 1 culture. Phase-contrast images of iPS cell-derived hematopoietic cells at day 3 (G) and day 6 (H) in step 2 and day 15 (I, J) in step 3 are shown. (K, L): Cytopsin specimens of iPS-DC recovered from step 3 culture were stained with May-Grünwald-Giemsa. Cells in (I–L) had been stimulated for 2–4 days with IL-4, TNF- $\alpha$ , and anti-CD40 mAb.

### Chemotaxis Assay

A multiwell chemotaxis assay was done by using 24-well Transwell permeable support chamber (pore size 5  $\mu$ m; Corning, NY; <http://www.corning.com/index.aspx>). Cell suspensions ( $1 \times 10^6$  cells per ml) in serum-free culture medium (AIMV, Gibco-Invitrogen) were added to the upper compartments (0.1 ml per well) and the indicated concentration of C5a in AIMV medium was added to the lower compartments (0.6 ml per well). Assay plates were incubated at 37°C for 90 minutes and then the cells on the upper surface of the microporous membranes were removed by using swabs. Subsequently, the membranes were fixed with methanol for 5 minutes and stained with May-Giemsa solution (Muto Chemicals, Tokyo, Japan). The stained cells on the lower surface of the membranes were counted and the data were indicated as the number of cells per 1 mm<sup>2</sup>.

### Phagocytosis Assay

The cell suspensions in RPMI-1640/10% FCS/2-ME were added to 48-well culture plates ( $2 \times 10^5$  cells/200  $\mu$ L per well) and incubated at 37°C for 2 hours to allow the cells to adhere to the plates. FITC-labeled Zymosan A particles (Molecular Probes Inc., Eugene, OR, <http://probes.invitrogen.com>) were added to the wells ( $4.8 \times 10^6$  particles/200  $\mu$ L per well) and, after incubation for the indicated period, cells were rinsed with PBS and harvested by using trypsin/EDTA. The cells were treated with trypan blue to quench FITC of the cell-surface attached particles, washed and then analyzed on FACSscan flow cytometer.

### Measurement of Nitric Oxide Production

The cells suspended in phenol-red-free DMEM/5% FCS were seeded into 96-well plates ( $1 \times 10^5$  cells/0.2 ml per well) in the presence or absence of IFN- $\gamma$  (200 U/ml) and LPS (100 ng/ml). After 24 hours of incubation, concentration of NO<sub>2</sub> + NO<sub>3</sub> in the culture supernatant was determined based on Griess method by using a nitric oxide assay kit (Dojindo, Kumamoto, Japan; <http://www.dojindo.com>).

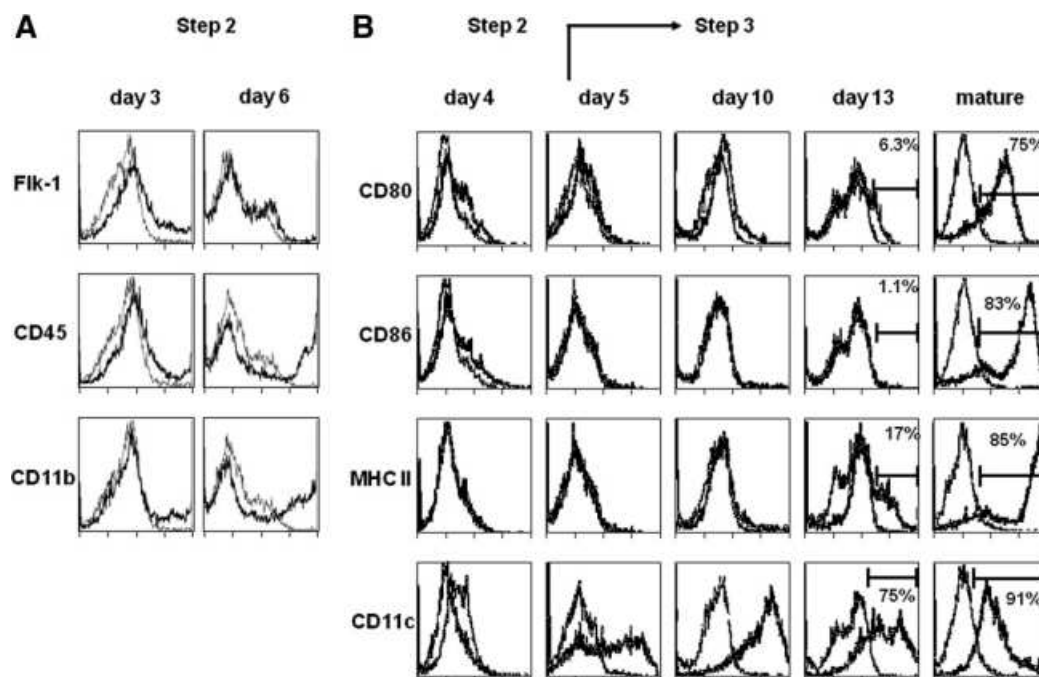
## RESULTS

### Generation of DCs From iPS Cells

The present study mainly examined iPS-MEF-Ng-38C-2 (38C-2), one of the previously established mouse iPS cell clones [14], for the capacity to differentiate into functional DCs. 38C-2 was developed by introduction of the four genes (Oct3/4, Sox2, Klf4, and c-Myc) for reprogramming factors into embryonic fibroblasts and subsequent selection based on the expression of the Nanog gene. The procedure to induce the differentiation of iPS cells into DCs, composed of three steps as shown in supporting information Figure 1, was basically the same as that developed previously using mouse ES cells [2].

Undifferentiated iPS cells were maintained on the feeder layers of PEF. They were similar to ES cells in morphology (Fig. 1A) and growth properties. Nanog-selected iPS cells carry a transgene of the Nanog genome inserted with a cDNA coding for green fluorescence protein (GFP) in the 5'-untranslated region. The expression of GFP was observed in approximately half of the undifferentiated Nanog-iPS cells (Fig. 1B), as reported previously [14]. To initiate the differentiation, iPS cells were transferred onto OP9 feeder layers (step 1). After 3 days, mesodermally differentiated flat colonies appeared. The expression of GFP was scarcely observed in the differentiated colonies (Fig. 1C, 1D), thus indicating that the promoter of Nanog gene was turned off along with the mesodermal differentiation of iPS cells. On day 6, most of the colonies exhibited a differentiated morphology. They were completely negative for the expression of GFP and positive for cell surface expression of Flk-1/VEGFR2 (Fig. 1E, 1F).

On day 6 or 7 of step 1, cells were harvested by using trypsin/EDTA and dissociated into single cells. Subsequently,



**Figure 2.** Surface phenotypes of iPS-derived cells in differentiation culture. (A): The cells at days 3 and 6 in step 2 culture were examined for the expression of Flk-1, CD45, and CD11b. (B): The cells in step 2 and step 3 culture were examined for the expression of CD80, CD86, MHC class II, and CD11c. The staining patterns of specific antibodies (thick lines) and isotype-matched controls (thin lines) are shown. The numbers in the panels of day13 and mature DC indicate percentages of cells positive for CD80, CD86, or MHC class II.

the cells were transferred onto freshly prepared OP9 feeder layers and cultured in the presence of GM-CSF, to start step 2. On the next day, homogenous small cells, resembling primitive hematopoietic progenitor cells, appeared (Fig. 1G). The iPS cell-derived round cells, expressing Flk-1 and CD45 (Fig. 2A), gradually increased and became morphologically heterogeneous (Fig. 1H). The addition of exogenous GM-CSF was essential for the propagation of the cells, thus indicating that the cells proliferated in response to GM-CSF. At day 6 in culture step 2, more than half of the floating cells highly expressed CD11b (Fig. 2A), thus suggesting their commitment to myeloid cell lineage. Step 2 culture was continued for 6–7 days.

At the end of step 2, floating or loosely adherent cells were recovered by pipetting and transferred them into bacteriological Petri dishes without feeder cells (step 3). After 5–7 days, most of floating cells showed irregular shape with some protrusions (supporting information Fig. 2). In addition, some of the transferred cells adhered to the dish surfaces like macrophages. Based on the morphology, the floating cells with protrusions were named iPS-DCs (iPS cell-derived dendritic cells). iPS-DCs expressed CD11c, but did not express CD80, CD86, and MHC class II until day 10 of the step 3 culture (Fig. 2B). At day 13, some of them expressed CD80 and MHC class II, thus suggesting spontaneous partial maturation. After day 10 of the step 3 culture,  $1\text{--}2 \times 10^6$  iPS-DCs were recovered from one Petri dish. The number of cells increased about 400–600 times from the initiation of the differentiation until differentiation into iPS-DCs.

To induce full maturation of iPS-DCs, we transferred the floating cells into new Petri dishes, and added IL-4, TNF- $\alpha$ , and anti-CD40 mAb to the cells simultaneously. In 2 or 3 days, most of the cells exhibited morphology of typical mature DCs, with many long protrusions or veil-like protrusions and some of the cells formed clusters (Fig. 1I–1L). Flow cytometric analysis demonstrated that high levels of

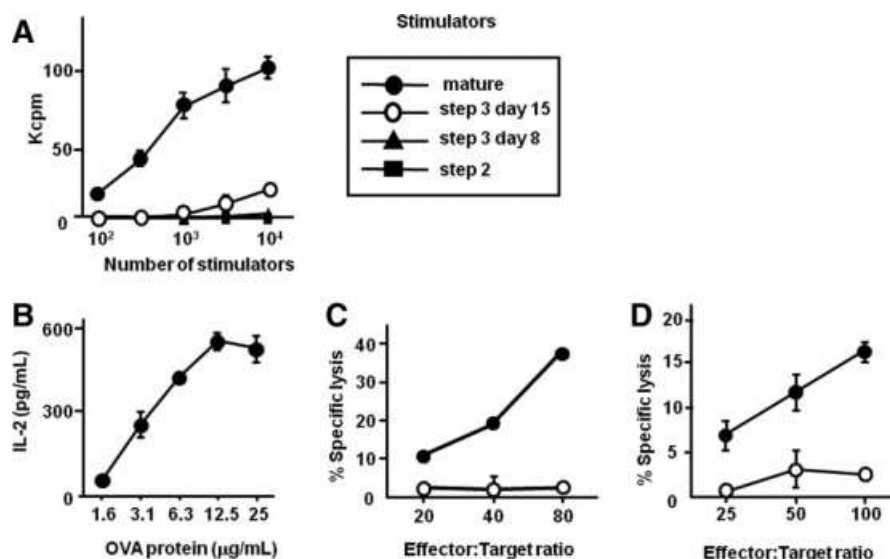
cell-surface expression of CD80, CD86, and MHC class II in the mature iPS-DCs (Fig. 2B).

One of the Fbx15-selected iPS cell clones, iPS-MEF-FB-20A-10<sup>13</sup> (20A-10), was also subjected to the differentiation culture. The 20A-10 iPS cells differentiated and proliferated well in the culture steps 1 and step 2, resulting in appearance of a large number of myeloid lineage cells at the end of step 2 (supporting information Fig. 3A). In the step 3 culture with GM-CSF intended for generation of DCs, 20A-10-derived myeloid cells further grew, exhibited some protrusions, and expressed CD11c. However, they were refractory to maturation. Even when stimulated with the simultaneous addition of IL-4, TNF- $\alpha$  and anti-CD40 mAb, they expressed very low level of cell-surface MHC class II and did not express CD80 and CD86 (supporting information Fig. 3B).

### Functions and Gene-Expression of iPS-DCs

Nanog-selected iPS cells including the clone 38C-2 were derived from PEF with a mixed genetic background composed of 75% DBA (H-2<sup>d</sup>), 12.5% C57BL/6 (H-2<sup>b</sup>), and 12.5% 129S4 (H-2<sup>b</sup>) [14], and thus their H-2 haplotype could be b/b, b/d, or d/d. Before the analyses of the immunological functions of iPS-DCs, we determined the H-2 haplotype of 38C-2 iPS cells by flow cytometric and PCR-based analyses and that was found to be d/b (supporting information Fig. 4).

To examine the capacity of iPS-DCs to stimulate T-cells, allogeneic mixed lymphocyte reaction (MLR) assay was conducted using iPS cell-derived cells of several different differentiation stages as stimulators. We used splenic T-cells isolated from naive C57BL/6 mice (H-2<sup>b</sup>) as allogeneic responder T-cells. As shown in Figure 3A, the floating cells harvested from step 2 culture (pre-iPS-DCs) or at day 8 of step 3 (immature iPS-DCs) exhibited either no or a very low level of activity to stimulate naive T-cells. Partially matured iPS-DCs harvested on day 15 of the step 3 culture induced a small but definite proliferative response of T-cells. In contrast,



**Figure 3.** Antigen-processing and presenting function of iPS-DC in vitro. (A): Pre-iPS-DC harvested from step 2 culture, immature iPS-DC (day 8 of step 3), partially matured iPS-DC (day 15 of step 3) and fully matured iPS-DC were cocultured with T-cells from C57BL/6 mice in wells of 96-well round-bottomed plates and cultured for 4 days. Proliferation of the T-cells was measured based on the incorporation of [<sup>3</sup>H]-thymidine. (B): iPS-DC were cultured in 96-well flat-bottomed culture plates in the presence of indicated concentrations of ovalbumin protein, IL-4, and anti-CD40 mAb for overnight. Subsequently, T-cell-hybridoma cells (DO11.10) were added and further cultured for 24 hours. The concentration of IL-2 produced by the hybridoma in the culture supernatant was measured by ELISA. (C): Mature iPS-DC were loaded with either  $\alpha$ -GalCer (closed symbols) or vehicle alone (open symbols) for 18 hours, washed and cocultured with splenic T-cells of H-2-matched (C57BL/6  $\times$  BALB/c) F1 (CBF1) mice. After 24 hours of culture, cytotoxic activity of the cultured cells against YAC-1 cells were analyzed by 4-hour Cr-release assay. (D): iPS-DC were loaded with either  $\alpha$ -GalCer (closed symbols) or vehicle alone (open symbols) as in (C) and intraperitoneally injected into CBF1 mice ( $1.2 \times 10^6$  cells per mouse). After 24 hours, spleen cells isolated from the three mice for each group were pooled and their cytotoxic activity against YAC-1 cells were analyzed by 4-hour Cr-release assay. Abbreviation: Kcpm, kilo counts per minute.

iPS-DCs treated with maturation stimuli for 2 days exhibited a very high magnitude of T-cell-stimulating activity (Fig. 3A), consistent with their high levels of cell-surface expression of MHC class II and costimulatory molecules (Fig. 2B).

To examine the antigen-processing and presentation function of iPS-DCs, the presentation of OVA antigen to a T-cell hybridoma, DO11.10, recognizing OVA<sub>323–339</sub> in the context of I-A<sup>d</sup>, was analyzed. iPS-DCs harvested at day 10-day of culture step 3 were cultured in the presence of OVA protein, IL-4 and anti-CD40 mAb in 96-well culture plates, to allow them to capture the antigenic protein and to mature. After 18 hours, DO11.10 hybridoma cells were added and then were further cultured. In this assay, IL-2 in the culture supernatant produced by DO 11.10 indicated the presentation of OVA-derived epitope on the I-A<sup>d</sup> of iPS-DCs. The results shown in Figure 3B indicate that DO11.10 produced IL-2 depending on the concentration of OVA protein loaded to iPS-DCs, thus demonstrating processing of the antigenic protein and presentation of the I-A<sup>d</sup>-restricted epitope by iPS-DCs.

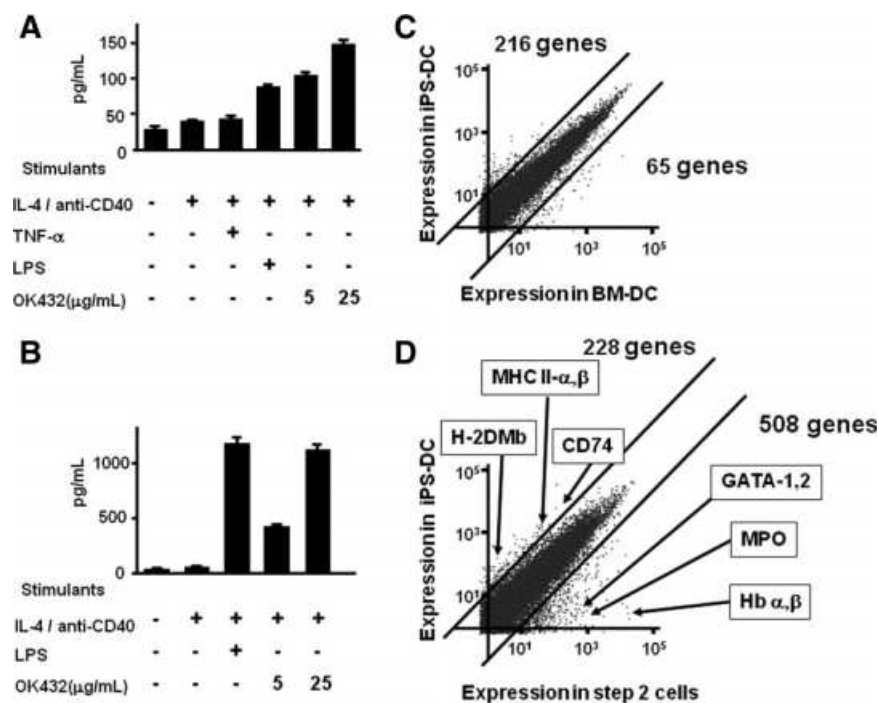
NKT cells are a group of T-cells expressing invariant T-cell receptors and recognize lipid ligands, for example  $\alpha$ -GalCer, in the context of CD1d, a nonclassical MHC class I. On stimulation with  $\alpha$ -GalCer, NKT cells rapidly produce large amount of cytokines, resulting in activation of conventional T-cells and NK cells.  $\alpha$ -GalCer-loaded DCs are known to be efficient in activation of NKT cells [7, 28–30]. We also analyzed the capacity to present  $\alpha$ -GalCer and activate NKT cells. Mature iPS-DCs were loaded with  $\alpha$ -GalCer or vehicle alone and cocultured with H-2-matched splenic T-cells from (C57BL/6  $\times$  BALB/c) F1 (CBF1) mice (H-2<sup>b/d</sup>) for 24 hours. As shown in Figure 3C, the cells cocultured with  $\alpha$ -GalCer-loaded iPS-DCs but not those cultured with vehicle-loaded iPS-DCs exhibited activity to kill YAC-1 cells, indicating

activation of NKT cells by  $\alpha$ -GalCer-loaded iPS-DCs. To examine the in vivo stimulation of NKT cells, iPS-DCs loaded with either  $\alpha$ -GalCer or vehicle alone were intraperitoneally injected into CBF1 mice. Spleen cells isolated from the mice injected with  $\alpha$ -GalCer-loaded iPS-DCs showed higher cytotoxic activity against YAC-1 cells than those from mice injected with vehicle-loaded iPS-DCs (Fig. 3D), thus demonstrating in vivo activation of NKT cells by  $\alpha$ -GalCer-loaded iPS-DCs.

To know how long iPS-DCs could survive in vivo, we injected carboxyfluorescein succinimidyl ester (CFSE)-labeled iPS-DCs ( $2 \times 10^6$  per mouse) into H-2-matched CBF1 mice at the tail-base and analyzed the frequency of CFSE-positive cells in the draining para-aortic lymph nodes after 3, 5, 8, 14, and 21 days (supporting information Fig. 5). As a result, we observed that injected iPS-DCs survived more than 14 days in the draining lymph nodes.

Next, the production of IL-12 and TNF- $\alpha$  by iPS-DCs upon treatment with stimulatory ligands was analyzed (Fig. 4A, 4B). The addition of IL-4 and anti-CD40 mAb exhibited little effect to enhance the production of IL-12p70. The addition of either LPS or OK432 but not TNF- $\alpha$  significantly enhanced the production of IL-12. The addition of LPS and OK432 exhibited dramatic effect to enhance the production of TNF- $\alpha$ . Collectively, the production of IL-12 and TNF- $\alpha$  by iPS-DCs was significantly enhanced on stimulation with bacteria-derived stimulatory ligands, similarly as the case of physiological DCs.

The global gene-expression profile of iPS-DCs was examined by using DNA microarrays. As shown in Figure 4C, the gene expression profile of iPS-DCs was similar to that of BM-DCs. We analyzed the change of gene expression profile during the step 3 culture, by comparing the data of cells recovered at the end of step 2 and those of iPS-DCs (Fig.



**Figure 4.** Cytokine production and gene-expression profiles of iPS-DC. (A, B): iPS-DC were cultured in the presence or absence of IL-4 (10 ng/ml), anti-CD40 mAb (10  $\mu$ g/ml), TNF- $\alpha$  (10 ng/ml), LPS (1  $\mu$ g/ml), and OK432 (5 or 25  $\mu$ g/ml) as indicated. After 3 days of culture, culture supernatant was collected and concentration of IL-12p70 (A) and TNF- $\alpha$  (B) was determined by ELISA. (C): A scattered plot comparing global gene-expression profiles of BM-DC and iPS-DC. The numbers of more than 10-fold differentially expressed genes among the total of 22,506 genes (array spots) analyzed are shown. The results of flow cytometric analysis of iPS-DC and BM-DC are shown in Figure 1A (step 3, day 13) and supporting information Figure 6, respectively. (D): A scattered plot comparing gene-expression profiles of step 2 culture cells (pre-iPS-DC) and iPS-DC. The levels of expression of genes for H-2DM $\beta$  (H-2DMb), MHC class II  $\alpha$  and  $\beta$ , MHC class II-associated invariant chain (Ii/CD74), GATA-1, GATA-2, MPO, and hemoglobin (Hb)  $\alpha$  and  $\beta$  are indicated. Among the total of 23,481 genes (array spots) analyzed, the number of genes with more than 10-fold increase or decrease in the level of expression is shown. The results of flow cytometric analysis of step 2 cells are shown in Figure 1A (step 2, day 6). Abbreviations: LPS, lipopolysaccharide; MPO, myeloperoxidase; TNF, tumor necrosis factor.

4D). The results showed that 228 out of 23,481 analyzed genes were upregulated more than 10 times (listed in supporting information Table 1) and such dramatically upregulated genes included those directly associated with antigen-presenting functions (Fig. 4D), such as MHC class II, CD74, and H2-DM. On the other hand, a number of genes were expressed highly in the step 2 cells and at very levels in iPS-DCs, for example hemoglobin  $\alpha$  and  $\beta$  chains, myeloperoxidase (MPO), GATA-1, and GATA-2 genes (Fig. 4D). These down-modulated genes were probably expressed by erythroid and myeloid precursor cells included in the cells of step 2 which disappeared along with differentiation into iPS-DCs in the step 3 culture.

### Genetic Modification of iPS-DCs

Genetic modification is a valuable means for modifying the function of DCs for medical application. For example, by means of the forced expression of antigenic proteins and immunostimulatory molecules, we can generate DCs vaccines potentially inducing immune response to specific antigens [6]. In the present study, we generated an expression vector from which a model antigen, OVA protein, was expressed as a fusion protein with MHC class II-associated invariant chain (Ii). Ii-fused OVA protein (Ii-OVA) produced in the APC was expected to be transported to endosomes and digested and thus epitopes derived from this protein could be presented in the context of MHC class II, in addition to MHC class I.

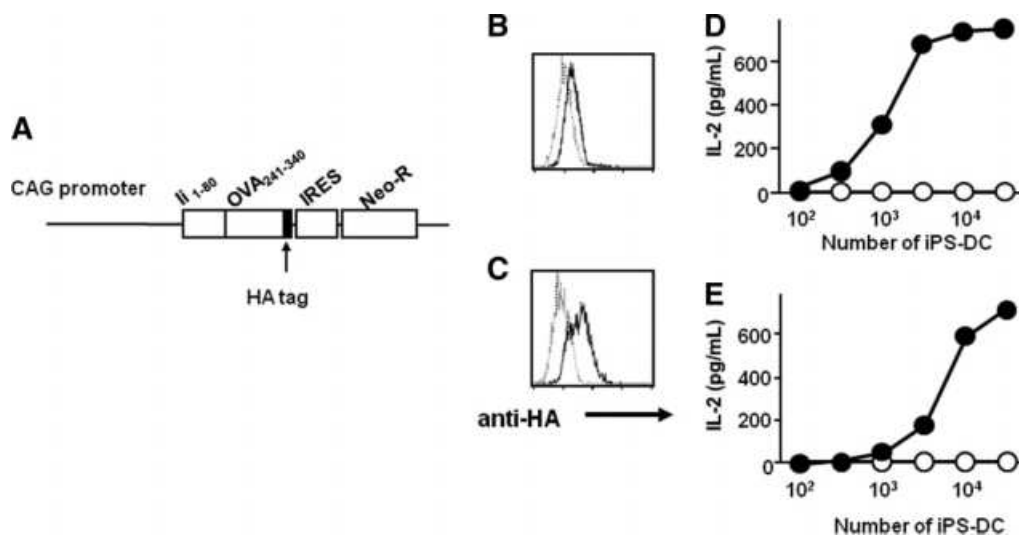
As shown in Figure 5A, the expression vector for Ii-OVA was created by ligating the cDNA fragment encoding for aminoterminal portion of Ii (p1-81), including an endosome tar-

geting signal sequence (di-leucine motif), with that encoding for a truncated form of OVA protein (OVA<sub>241-340</sub>) attached with an HA-tag. 38C-2 iPS cells were introduced with the vector and selected by culture in the presence of G418 and then 48 transfectant iPS cell clones were isolated. The expression of the transgene after differentiation to iPS-DCs was detected by flow cytometric analysis following intracellular staining with anti-HA mAb. The results of flow cytometric analysis of the parental 38C-2 iPS cell-derived DCs (nontransfectant iPS-DCs) and the Ii-OVA-transfectant iPS-DCs (iPS-DCs/OVA) are shown in Figure 5B and 5C, respectively.

iPS-DCs/OVA were examined for the presentation of OVA-derived epitopes in the context of MHC class I and MHC class II molecules. iPS-DCs/OVA or nontransfectant iPS-DCs were cocultured with T-cell hybridomas, RF33.70 (recognizing OVA<sub>257-264</sub> in the context of H-2K<sup>b</sup>) and DO11.10 (recognizing OVA<sub>323-339</sub> in the context of I-A<sup>d</sup>). As shown in Figure 5D and 5E, iPS-DCs/OVA stimulated both of the T-cell hybridomas to produce IL-2, whereas nontransfectant iPS-DCs did not. Collectively, we verified the expression by iPS-DCs of transgene introduced before differentiation and confirmed that iPS-DCs possess a physiological intracellular antigen presentation machinery.

### Priming of Antigen-Specific Cytotoxic T-Cells In Vivo by Genetically Modified iPS-DCs

To analyze the capacity of iPS-DCs/OVA to prime OVA-specific T-cells in vivo, we injected iPS-DCs/OVA or nontransfectant iPS-DCs intraperitoneally into C57BL/6 mice. In this experiment, injected iPS-DCs were derived from 38C-2 iPS



**Figure 5.** Genetic modification of iPS-DC to express antigenic protein. (A): The structure of pCAG-IiOV, an expression vector for Ii-OVA chimeric protein attached with HA-Tag. (B, C): Nontransfected iPS-DC (B) or Ii-OVA transfected iPS-DC (C) were stained intracellularly with FITC-conjugated anti-HA mAb (thick lines) or left unstained (thin dotted lines) and analyzed by flow cytometry. (D, E): Nontransfected iPS-DC (open symbols) or Ii-OVA transfected iPS-DC (closed symbols) were cocultured with T-cell hybridomas, RF33.70 (D, recognizing OVA<sub>257-264</sub> in the context of K<sup>b</sup>) or DO11.10 (E, recognizing OVA<sub>323-339</sub> in the context of I-A<sup>d</sup>). Abbreviations: HA, hemagglutinin; IRES, internal ribosomal entry site.

cells (H-2<sup>b/d</sup>) and the recipient mice were of C57BL/6 strain (H-2<sup>b/b</sup>). Therefore, iPS-DCs were semiallogeneic to the recipient mice sharing H-2<sup>b</sup>-haplotype with the recipient. This experimental design was based on our previous observation that ES-DCs could prime antigen-specific CTL restricted to the MHC molecule of the shared allele in such semiallogeneic recipient mice [8, 10]. Eight days after the injection, splenocytes were isolated from the mice and cultured in the presence of OVA<sub>257-264</sub> peptide, the major H-2K<sup>b</sup>-restricted epitope derived from OVA protein. After 5 days, cultured cells were recovered and examined for the frequency and cytotoxic activity of H-2K<sup>b</sup>-OVA<sub>257-264</sub>-specific CD8<sup>+</sup> T-cells (Fig. 5A).

The results showed that 1.56% of the CD8<sup>+</sup> T-cells in the cultured spleen cells isolated from the mice injected with iPS-DCs/OVA were positively stained with tetramer of K<sup>b</sup>-OVA<sub>257-264</sub>-complex (Fig. 5B). On the other hand, these were 0.22% in the cells from the mice injected with nontransfected iPS-DCs (Fig. 5C). The results shown in Figure 5D indicated that cytotoxic T-cells specific to the OVA epitope was primed in vivo by iPS-DCs/OVA but not by nontransfected iPS-DCs. These results demonstrate that iPS-DCs genetically engineered to express an antigenic protein primed cytotoxic T-cells specific to the antigen upon in vivo administration.

We examined whether antigen-specific anti-tumor immunity was induced by iPS-DCs expressing antigenic protein or not. Ten days after the intraperitoneal injection of iPS-DCs/OVA or nontransfected iPS-DCs, the recipient C57BL/6 mice were challenged by subcutaneous injection of MO4, an OVA expressing melanoma cell line derived from a C57BL/6 mouse. As shown in Figure 6E, the growth of tumor in the mice was almost completely inhibited by pretreatment with iPS-DCs/OVA but not by nontransfected ES-DCs. Therefore, it was demonstrated that in vivo transfer of genetically modified iPS-DCs expressing model tumor antigen efficiently induced antigen-specific anti-tumor immunity.

### Generation of Macrophages From iPS Cells

As described earlier, in the step 3 of iPS-DCs differentiation culture, some adherent cells were observed and they showed

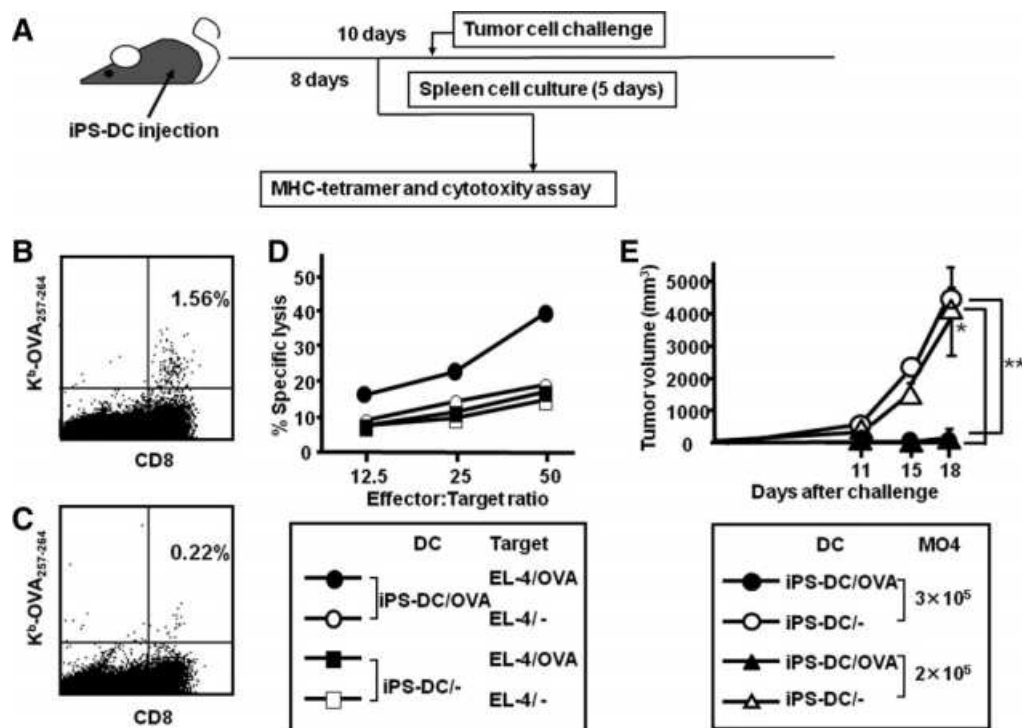
macrophage-like morphology. To promote the differentiation into macrophages, M-CSF was added instead of GM-CSF in the step 3 culture. As a result, a large number of macrophage-like adherent cells appeared from 38C-2 (Nanog-selected) iPS cells (Fig. 7A, 7B). These were designated as iPS-MP (iPS cell-derived macrophages). The iPS-MP were harvested by treatment with trypsin/EDTA and then were characterized.

A flow cytometric analysis shown in Figure 7C revealed that iPS-MP expressed F4/80 and CD11b, as macrophages generated from bone marrow cells (BM-MP) did. The level of expression of CD14 in iPS-MP was very low. iPS-MP showed C5a-induced chemotaxis (Fig. 7D). Their phagocytic capacity was assessed by using FITC-labeled zymosan particles. As shown in Figure 7E, iPS-MP ingested zymosan particles in a similar rate as BM-MP did. Stimulation with LPS and IFN- $\gamma$  synergistically induced production of nitric oxide by iPS-MP (Fig. 7F).

Consistent with the results of the functional analysis, gene expression profile of iPS-MP revealed by cDNA microarray analysis was relatively similar to that of BM-MP (Fig. 7G). In the analysis of change of gene expression from precursor cells (recovered from step 2 culture) to iPS-MP (Fig. 7H), we found that the expression of some genes associated with function of macrophages was drastically increased. For example, expression of CD14, Msr (macrophage scavenger receptor) two, and CD36 were upregulated more than 20 times during the iPS-MP differentiation. The 157 genes with more than a 10 time-increase in the level of expression during the differentiation from culture step 2 to iPS-MP were listed in the supporting information Table 2.

Collectively, culture of iPS cell-derived myeloid lineage cells harvested from the culture step 2 in the presence of exogenous M-CSF resulted in the generation of macrophages (iPS-MP). The characteristics of iPS-MP as macrophages were confirmed by their morphology, cell surface phenotypes, functions and also gene-expression profile.

An Fbx15-selected iPS cell clone 20A-10 also showed morphology and cell surface markers of macrophages, when subjected to the differentiation culture for iPS-MP (supporting information Fig. 3C, 3D). Therefore, in the present study,



**Figure 6.** Activation of T-cells in vivo by iPS-DC. (A): A schematic depiction of the experiment to analyze the priming of antigen-specific T-cells by iPS-DC in vivo. iPS-DC (iPS-DC/OVA) or nontransfectant iPS-DC were injected intra-peritoneally into C57BL/6 mice ( $1.5 \times 10^5$  cells per mouse). Eight days after the injection, spleen cells were isolated from the mice and cultured in the presence of OVA<sub>257–264</sub> peptide. After 5 days, cultured cells from iPS-DC/OVA-injected (B) or nontransfectant iPS-DC-injected (C) were stained with tetramer of H-2K<sup>b</sup>-OVA<sub>257–264</sub> in combination with anti-CD8 mAb. (D): OVA-specific CTL activity was analyzed by <sup>51</sup>Cr-release assay using OVA-peptide-pulsed (closed symbols) or unpulsed (open symbols) EL-4 cells as targets. (E): Ten days after the injection of iPS-DC/OVA or non-transfectant iPS-DC into C57BL/6 mice ( $1 \times 10^5$  cells per mouse), the mice were subcutaneously injected with MO4, an OVA expressing melanoma cell line, (two  $\times$  or  $3 \times 10^5$  cells per mouse, 5–6 mice per group), at the shaved right flank. After that, the tumor size was measured and the tumor volume was calculated as: tumor volume (mm<sup>3</sup>) = (length  $\times$  width  $\times$  height). \*One mouse out of five mice pre-treated with non-transfectant iPS-DC and challenged with  $2 \times 10^5$  MO4 cells died between days 15 and 18. \*\*The results of iPS-DC/OVA-treated and non-transfectant iPS-DC-treated mouse groups were different with statistical significance for both  $2 \times 10^5$  and  $3 \times 10^5$  MO4 cell-injected groups ( $p < .001$  by Student's *t* test). Abbreviations: DC, dendritic cells; OVA, ovalbumin.

20A-10 iPS cells differentiated into macrophages but not into DCs.

## DISCUSSION

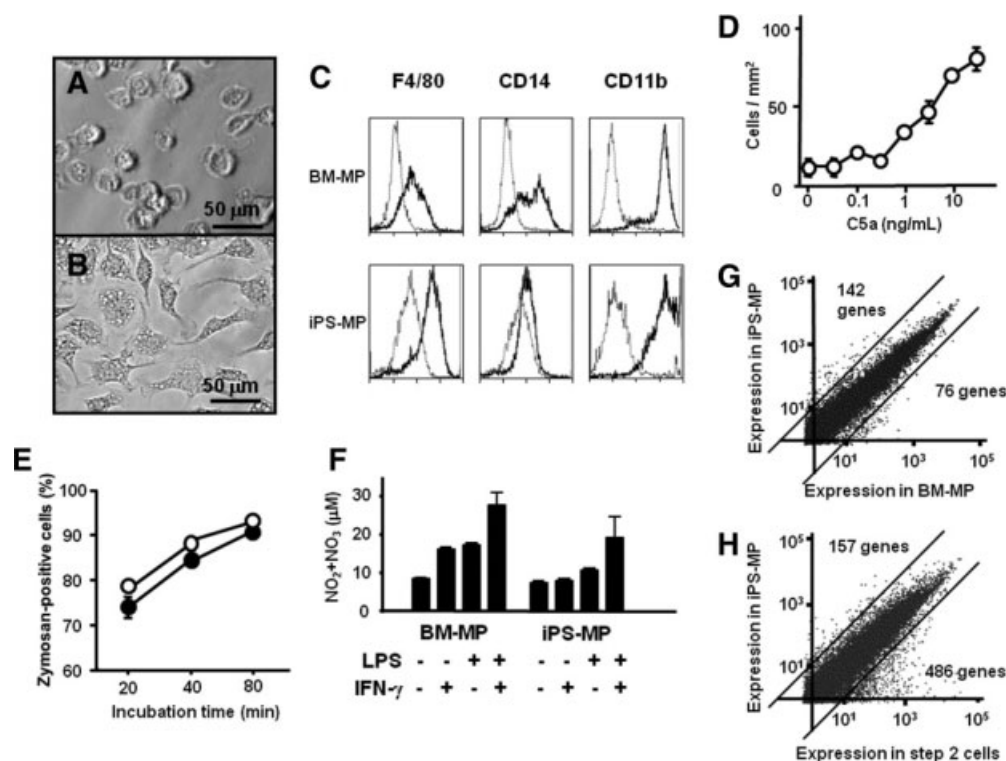
The present study demonstrated that Nanog-selected iPS cells (38C-2) were capable of differentiating into functional DCs and macrophages. The morphological changes observed during the differentiation of the iPS cells into DCs were very similar to that observed in the differentiation culture for ES-DCs [2]. However, there was some delay (1–2 days in step 1 and 2 and 2–4 days in step 3) in the kinetics of differentiation process of iPS cells, as in comparison with most of mouse ES cell lines. On the other hand, the yield of differentiated cells (up to 600 times the cell number from undifferentiated iPS cells to differentiated iPS-DCs) was higher than that in the cases of most of ES cell lines (usually up to 400 times). In contrast to 38C-2 iPS cells, we could generate macrophages but not DCs from 20A-10 (Fbx15-selected) iPS cells.

The difference in the potential of differentiation among ES cells and the two clones of iPS cells might be due to the expression of transgenes for reprogramming factors in iPS cell-derived cells during differentiation. To assess this possibility, we analyzed the expression of the transgene-derived reprogramming factors in iPS cells before and after differentiation by reverse

transcription polymerase chain reaction (RT-PCR) (supporting information Fig. 7). The expression of all four transgene-derived reprogramming factors was detected during the culture steps 2 and 3 in 38C-2 (Nanog-selected) iPS cells, although the expression of Klf4 was scarce. As for Fbx15-selected 20A-10 iPS cells, three transgene-derived reprogramming factors other than Oct3/4 were detected during differentiation. Collectively, most of the transgene-derived reprogramming factors were expressed during differentiation in the both of iPS cell clones, and they possibly exerted some influence on the differentiation process. However, the reason for the difference in the differentiation potential between the two iPS cell clones was not clarified by this RT-PCR experiment.

As a means to induce T-cell-mediated anti-cancer immunity, vaccination with DCs loaded with tumor antigen-derived peptides or tumor cell lysates are being clinically tested. For this purpose, human DCs are generated from monocytes obtained from peripheral blood of the patients. However, because monocytes cannot be propagated in vitro, apheresis, a procedure sometimes invasive for the patients, is necessary to obtain sufficient number of monocytes as the source of DCs. iPS cells, like ES cells, possess practically unlimited propagation capacity and may be an ideal source for DCs to be used in such immunotherapy.

Embryoid body-mediated differentiation to generate macrophages from mouse ES cells has been reported by several groups [31–34]. In the present study, we demonstrated



**Figure 7.** Characterization of iPS cell-derived macrophages. Phase-contrast images of iPS-MP in a bacterial Petri dish (A) and in a tissue culture-coated dish (B) are shown. (C): The expression of F4/80, CD14, and CD11b on BM-MP and iPS-MP was analyzed by flow cytometry. (D): C5a-induced chemotaxis of iPS-MP was analyzed using 24-well culture plates with permeable membrane insets. (E): BM-MP (open circles) or iPS-MP (closed circles) in 48-well culture plates were added with FITC-labeled Zymosan A particles. After incubation for the indicated period, cells were harvested by using trypsin/EDTA, treated with trypan blue, washed and then analyzed on a FACScan. (F): Production of nitric oxide by BM-MP and iPS-MP stimulated with IFN- $\gamma$  and/or LPS was analyzed. The cells were seeded onto 24-well culture plates ( $1 \times 10^5$  cells/0.2 ml per well) in the presence or absence of the stimulants, as indicated. After 24 hours of incubation, culture supernatant was collected and concentration of  $\text{NO}_2 + \text{NO}_3$  was determined by the Griess method. (G): A scattered plot comparing of the gene-expression profiles of BM-MP and iPS-MP is shown. The numbers of more than 10-fold differentially expressed genes among the total of 21,896 genes (array spots) analyzed are shown. The results of flow cytometric analysis of BM-MP and iPS-MP are shown in (C). (H): A scattered plot comparing the gene-expression profiles of step 2 culture cells and iPS-MP is shown. Among the total of 22,552 genes (array spots) analyzed, the number of genes with more than 10-fold increase or decrease in the level of expression is shown. The results of flow cytometric analysis of step 2 cells are shown in Figure 1A (step 2, day 6). Abbreviation: LPS, lipopolysaccharide.

efficient generation of functional macrophages from mouse iPS cells. Although we had not performed the embryoid body-mediated methods to generate macrophages, the reported ES cell-derived macrophages seemed to be similar to iPS-MP. Physiologically, macrophages play important roles in the defense mechanism against various infectious organisms and also in maintenance of homeostasis by ingestion and digestion of dead cells occurring in the body. The technology to generate large number of functional macrophages from iPS cells may be applicable to the development of novel macrophage-based medical technology.

## SUMMARY

DCs and macrophages were generated by directed in vitro differentiation of mouse iPS cells. The iPS cell-derived DCs (iPS-DCs) possessed the characteristics of DCs including the capacity of T-cell-stimulation, antigen-processing, and presentation and cytokine production. There was some delay in the kinetics of differentiation process of iPS cells, as in comparison with most of mouse ES cell lines. On the other hand, the yield of differentiated cells was higher than that in the cases of most of mouse ES cell lines. Genetically modified iPS-DCs

expressing antigenic protein primed T-cells specific to the antigen in vivo. Macrophages generated from iPS cells (iPS-MP) were comparable with bone marrow cell-derived macrophages in the cell surface phenotype, functions, and gene expression profiles.

## ACKNOWLEDGMENTS

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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