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Bone marrow-derived mesenchymal stem cells propagate immunosuppressive/anti-inflammatory macrophages in cell-to-cell contact-independent and -dependent manners under hypoxic culture

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ABSTRACT

Immunosuppressive/anti-inflammatory macrophage (Mφ), M2-Mφ that expressed the typical M2-Mφs marker, CD206, and anti-inflammatory cytokine, interleukin (IL)-10, is beneficial and expected tool for the cytotherapy against inflammatory diseases. Here, we demonstrated that bone marrow-derived lineage-positive (Lin+) blood cells proliferated and differentiated into M2-Mφs by cooperation with the bone marrow-derived mesenchymal stem cells (MSCs) under hypoxic condition: MSCs not only promoted proliferation of undifferentiated M2-Mφs, pre-M2-Mφs, in the Lin+ fraction *via* a proliferative effect of the MSCs-secreted macrophage colony-stimulating factor, but also promoted M2-Mφ polarization of the pre-M2-Mφs through cell-to-cell contact with the pre-M2-Mφs. Intriguingly, an inhibitor for intercellular adhesion molecule (ICAM)-1 receptor/lymphocyte function-associated antigen (LFA)-1, Rwj50271, partially suppressed expression of CD206 in the Lin+ blood cells but an inhibitor for VCAM-1 receptor/VLA-4, BIO5192, did not, suggesting that the cell-to-cell adhesion through LFA-1 on pre-M2-Mφs and ICAM-1 on MSCs was supposed to promoted the M2-Mφ polarization.

Thus, the co-culture system consisting of bone marrow-derived Lin+ blood cells and MSCs under hypoxic condition was a beneficial supplier of a number of M2-Mφs, which could be clinically applicable to inflammatory diseases.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent somatic cells able to differentiate into various cell types and support tissue regeneration and restoration *in vivo*. MSCs and tissue-resident macrophages, which supply tissue-reconstructing cells and remove cell debris from inflammation sites, respectively, are essential for injured tissue restoration and regeneration [1–3]. MSCs also exert potent immunosuppressive and anti-inflammatory effects [4–6]. MSCs modulate their anti-inflammatory effects in multiple ways in response to the different microenvironments caused by various tissue injuries [7].

MSCs have been reported to affect several immune-regulatory functions in both adaptive and innate immunity [8–10]. MSCs induce macrophage (Mφ) polarization from immune-reactive (or pro-inflammatory) M1 phenotype to immune-suppressive (or anti-inflammatory) M2 phenotype [11,12]. The latter is termed M2-Mφ, and is also known

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as MSC-educated Mφ [13]. M2-Mφs produce mediators essential in the resolution of inflammation, and thus promote wound repair [8,14,15]. M2-Mφs express a cell surface mannose receptor MR/CD206, and they release immune suppressive cytokine interleukin (IL)-10 which functions to prevent excessive inflammation [13,16–18]. Thus, M2-Mφs are expected tool for the cytotherapy against inflammatory diseases.

M2-Mφ polarization factors known to be secreted from MSC include prostaglandin (PG) E_2, PGE1α, IL-6, IL-8, TNF-α-stimulating gene (TSG) 5, and transforming growth factor (TGF)-β [13,19,20]. These soluble factors modulate the cytokine expression profile in Mφs. In regards to Mφ polarization signaling, although there is evidence for intracellular signaling involving in the M1/M2 Mφ polarization [16,21,22], the intercellular and molecular mechanisms underlying the M2-Mφ polarization by MSC remain to be fully elucidated.

The macrophage colony-stimulating factor (M-CSF)/colony-stimulating factor (CSF-1) is a key regulator of monocyte (Mo)/Mφ lineage cells: M-CSF stimulates growth or proliferation of Mo/Mφ lineage cells, especially of undifferentiated precursor cells in the bone marrow [23], and induces the differentiation to Mφs *in vitro* and *in vivo* [24]. M-CSF and granulocyte macrophage colony-stimulating factor (GM-CSF) have generally been used to generate an activated Mφ population *in vitro* [21,25,26]. Joshi et al. referred to Mφs grown with GM-CSF as M1-Mφs, and Mφs grown with M-CSF/CSF-1 as M2-Mφs [26]. However, Murray et al. argued that since there is no compelling evidence to assign M-CSF- and GM-CSF-derived Mφs as M1-Mφs and M2-Mφs, respectively, such assignment of this terminology should be abandoned [18]. Consequently, the effect of M-CSF on the M2-Mφ polarization remains obscure.

In this study, we established a novel culture system of mouse bone marrow-derived cells under hypoxic conditions, in which lineage positive (Lin+) blood cells vigorously proliferated in cooperation with linage negative (Lin–) cells/MSCs. In addition, Lin+ blood cells in contact with Lin– cells/MSCs differentiated into CD206-positive immunosuppressive M2-Mφs. Here, we investigated molecular mechanisms underlying the Lin– cells/MSCs-induced propagation of M2-Mφs. These findings will contribute to the generation of large numbers of anti-inflammatory M2-Mφs *ex vivo* for the cytotherapy against inflammatory diseases.

2. Materials and methods

2.1. Reagents

Recombinant mouse M-CSF, selective intercellular adhesion molecule (ICAM)-1 receptor inhibitor, RWJ 50271 (TOCRIS brand) and selective vascular cell adhesion molecule (VCAM)-1 receptor inhibitor, BIO 5192 (TOCRIS brand) were purchased from R & D Systems Inc. (Minneapolis, MN, USA). Brefeldin A (Solution, 1000X) was purchased from Affymetrix (Santa Clara, CA, USA). Blocking/neutralizing antibodies: anti-mouse ICAM1/CD54 hamster monoclonal antibody (Clone 1A29; NBP2-22540) was purchased from Novus Biologicals (Littleton, CO, USA), and anti-mouse VCAM-1 rat monoclonal antibody (Clone 1. BB.619; ab61993) was purchased from Abcam (Cambridge, UK).

2.2. Mice

This study was approved by the Ethics Committee for Animal Research of Iwate Medical University (approval number: 27-001). Animal breeding, care, and experiments were performed in accordance with the Guidelines for the Animal Experiments of Iwate Medical University and the Act on Welfare and Management of Animals of Japan. In this study, 2–3-week old (regardless of sex) transgenic td-Tomato- and EGFP- mice [27,28], and wild-type mice based on C57BL6/J were used.

2.3. Primary culture of bone marrow-derived cells

Bone marrow aspirates were harvested by flushing the tibias of 2 mice by syringes with 20-gauge needles, according to the standard method. Freshly isolated bone marrow was suspended in PBS containing 2% fetal bovine albumin (Sigma-Aldrich, Co., St. Louis, MO, USA), 2 mM EDTA, and 1 mg/ml each of penicillin and streptomycin, and centrifuged at 800*g* for 5 min. The obtained bone marrow cells were plated onto a type I collagen-coated 10 cm dish (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) in the MSC-expansion Media (StemXVivo®, R & D Systems) supplemented with 100 μg/ml each of penicillin and streptomycin and cultured under hypoxic conditions (5% O_2—5% CO_2). Seven days after the first plating, the medium was aspirated from the dish, and the dish was washed with PBS. Fresh medium was subsequently added to the dish, and the culture was continued again for one week with the medium changed on day 4 from the first medium change. On day 14, the bone marrow-derived cells were harvested by using 0.05% Trypsin and 5 mM EDTA and re-plated on a new dish (Passage 1/P1) at 1/4 cell concentration of the original plate. Passage 2/P2 was carried out on day 7. Bone marrow-derived cells obtained at P2 were used for the experiments described below, except for analysis of passage-dependent changes of marker expressions. In some cases, human MSC line UE7T-13 was used instead of primarily cultured Lin– cells/MSCs for our co-culture system consisting of MSCs and Lin+ blood cells derived from mice bone marrow.

2.4. Cell separation from the bone marrow-derived cells using the MACS separator system

Lin– cells/MSCs and Lin+ blood cells were prepared by the MACS separator (magnetic labeling system) with the mouse Lineage Cell Depletion Kit (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). The separated Lin– cells/MSCs and Lin+ blood cells were reconstitutively cultured as necessary. The separated Lin– cells/MSCs were cultured in the MSC-expansion medium, while the separated Lin+ blood cells were cultured in DMEM (HG) containing 10% FBS using type-IV collagen-coated dishes unless otherwise noted. Conditioned media from Lin– cells/MSCs, Lin+ blood cells, and the P2 culture of bone marrow-derived cells for mass spectrometry analysis were collected at 2 days from the time point of confluent. Cell morphology and fluorescence were observed under a fluorescence microscope, Olympus IX70 (Olympus Co., Tokyo, Japan).

2.5. Flow cytometry

Cells (1.0×10^6 cells) were suspended in PBS containing 2% FBS and 2 mM EDTA, and incubated with FITC- or PE-conjugated rat primary antibodies for 10 min at 4 °C. For the negative control experiments, rat isotype IgG (Miltenyi Biotec) for each antibody was used. Antibodies used were anti-mouse (m) CD90-FITC, anti-mSca-1-FITC, anti-mCD45-FITC, anti-mCD11b-FITC, anti-mIL-10-PE (Miltenyi Biotech), and anti-mCD206-FITC (BioLegend, San Diego, CA, USA). For IL-10 labeling, cells were fixed and permeabilized using the Inside Stain Kit (Miltenyi Biotec.). Acquisition was performed with an EPICS XL EXPO 32 ADC System (Beckman Coulter, Brea, CA, USA).

2.6. RNA isolation and qRT-PCR

Total RNAs from separated Lin– cells/MSCs, and Lin+ blood cells, or co-culture of these cells were isolated with ISOGEN II reagent (Nippon Gene, Toyama, Japan). First-strand cDNA was synthesized using the PrimeScript RT reagent Kit (Takara-Bio, Shiga, Japan), and then PCR was performed on a Thermal Cycler Dice Real Time System (Takara-Bio) using the SYBR Premix Ex Taq II (Takara-Bio). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for sample normalization. The custom oligonucleotide primers (Takara-Bio) used are listed in Supplementary material Table 1.

2.7. Primer array

Gene expression levels of a range of cytokines and cytokine receptors were measured using a PrimerArray consisting of mouse Cytokine-cytokine receptor interaction (PN001, Takara Bio) in combination with a Thermal Cycler Dice Real Time System (TaKaRa Bio) and PrimerArray Analysis Tool version 2.0 (Takara Bio), according to the manufacturer's instructions. Genes with expression levels more than 5-fold in comparison between Lin+ blood cells and Lin− cells/MSCs are listed in Table 1.

2.8. Immunofluorescence analysis

Cells were subcultured in 8-well chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) at a density of 1 or 2 × 10^4 cells/well. After culture in various conditions, cells were fixed in 4% paraformaldehyde for 10 min and permeabilized with 0.1% Tween-20 or Triton X-100 for 5 min. After background reduction with 1–3% BSA and 10% normal goat serum (KPL, Gaithersburg, MD, USA) for 1 h at room temperature, the cells were labeled with anti-mannose receptor/CD206 rabbit polyclonal antibody (1:1000; Abcam), for 2 h at room temperature, or with anti-mouse M-CSF mouse monoclonal antibody (1:100; SantaCluz, Dallas, TX, USA) or anti-rabbit M-CSF receptor polyclonal antibody (1:100; SantaCluz) overnight at 4 °C. For negative controls, normal IgG of the cognate species of animals were used instead of the primary antibodies. After being washed with PBS, the cells were incubated with Alexa Fluor® 488-conjugated goat anti-rabbit or anti-mouse IgG (1:1000; Molecular Probes, Leiden, The Netherlands) for 1 h at room temperature. The cells were mounted with DAPI Fluoromount-G (SouthernBiotech Com., Birmingham, AL, USA). The fluorescent signal was detected and photographs were taken using a DP72 digital camera equipped to the Olympus IX71 fluorescence microscope.

2.9. Mass spectrometry analysis

Aliquots of 200 μL of conditioned media were harvested and then concentrated by ultrafiltration with Microcon-10 filters (cutoff, 10 kDa; Merck). The concentrated media were treated with an equal volume of sample buffer (Laemmli 2x concentrate; Sigma-Aldrich) and separated by SDS-PAGE using a 10–20% acrylamide gradient gel. Protein bands were stained with silver stain (Ez Stain silver, ATTO, Tokyo, Japan). Digestion of proteins in the gel pieces was carried out according to a previously described method [29]. The peptides were eluted with a gradient of 5–45% acetonitrile in 0.1% formic acid by nano-liquid chromatography (LC) (Advance LC & PAL System; AMR, Inc., Tokyo, Japan). Tandem mass spectrometry (MS/MS) was analyzed with the use of a LTQ Orbitrap XL™ with ETD (Thermo Fisher Scientific, Waltham, MA USA) mass spectrometer, according to the manufacturer's instructions. Protein sequence database searches were performed with Mascot (Matrix Science, Boston, MA, USA) using the MS/MS peptide ions.

2.10. Enzyme-linked immunosorbent assay

The conditioned medium from each culture was centrifuged at 10,000 rpm for 5 min at 4 °C to exclude cell and cell debris contamination. The level of IL-10 in the conditioned medium was quantified using Quantikine immunoassay enzyme-linked immunosorbent assay kits for mouse IL-10 (R & D Systems).

2.11. Cell proliferation assay

Cell proliferation was evaluated by two methods: microscopic observation and determination of cellular metabolic activity. Metabolic activity of the cells was measured by WST assay (Cell Proliferation Reagent WST-1, Roche Life Science, Mannheim, Germany) [30] or alamarBlue assay (alamarBlue Reagent, AbDSerotec, Oxon, UK) [31] according to the manufacturer's instruction. Absorbance of the reduced form of the indicator was measured by using an ELISA plate reader (Tosoh Corp., Tokyo, Japan).

2.12. Reconstitutive co-culture of MSCs and blood cells

For cell-to-cell-contact co-culture, 3.7 × 10^5 cells of Lin+ blood cells and 3.7 × 10^4 cells of Lin− cells/MSCs separated from the P2 culture of bone marrow-derived cells were mixed and plated in a 24-well plate in fresh medium (1:1 mixture of each medium for both Lin+ blood cells and Lin− cells/MSCs), and further cultured for 48 h. For the non-contact co-culture, a trans-well system was used. Lin+ blood cells were plated in the bottom well and Lin− cells/MSCs were plated on the membrane of the top well (insert) of the trans-well (Cell Culture Insert, 0.4-μm pore size; Falcon, Corning, NY, USA) with each medium. After co-culture for 48 h, cells were harvested and used for the RNA sample preparation. The conditioned medium was collected and used for the measurement of IL-10 by ELISA as described above. Lin+ blood cells were separated from Lin− cells/MSCs, and the status of M2-Mφ marker expression in Lin+ blood cells was evaluated after the cell-to-cell-contact co-culture.

2.13. Adhesion and adhesion inhibitory assays

For adhesion assay, Lin− cells/MSCs were prepared from the P2 culture of bone marrow cells derived from non-fluorescent wild-type mice. The cells were plated on a type I collagen-coated 96-well plate and cultured until the cells just reached confluence. Lin+ blood cells were prepared from P2 culture of bone marrow cells derived from td-Tomato mice. These Lin+ blood cells were added on the pre-cultured Lin− cells/MSCs in the 96-well plate and allowed to bind for 1 h at 37 °C. The plate was washed four timed with cold PBS to eliminate the unbound Lin+ cells. The remaining Lin+ blood cells bound to Lin− cells/MSCs were visible in red under fluorescent microscopy. The number of fluorescent cells in each well was determined.

For adhesion inhibition, two different methods were performed to inhibit the ICAM-1- and VCAM-1-dependent adhesion: in one method, the separated fluorescent (td-Tomato) Lin+ blood cells were added on the non-fluorescent Lin− cells/MSCs that were pre-cultured and grown to the confluent on the type I collagen-coated 96-well dish as stated above. The neutralizing antibody for ICAM-1 or VCAM-1 was then added to the culture, and Lin+ blood cells were seeded on the Lin− cells/MSCs and then allowed to bind for 1 h at 4 °C. After washing with PBS, each well was photographed under fluorescent microscopy and the number of fluorescent Lin+ blood cells bound to Lin− cells/MSCs was determined. In the other method, the selective high affinity inhibitor for LFA-1 (integrin αLβ2), RWJ50271, which inhibits ICAM-1 binding to LFA-1, and that for VLA-4 (integrin α4β1), BIO5192, which inhibits VCAM-1 binding to VLA-4 (both from Tocris Bioscience, Bristol, UK) were added to the bone marrow co-culture at the plating time of P1 or P2, and the culture was further maintained for 7 days. Then, cells were harvested and analyzed for the expression of CD206 by flow cytometry.

2.14. Statistical analysis

The data were presented as mean ± SD (n = 3, 5 or 6). The data were statistically analyzed by Student's *t*-test, and values of $^*P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant. The results shown in all experiments were representative of at least 3 separate experiments.

3. Results

3.1. Establishment of a co-culture system of mouse bone marrow-derived Lin− cells/MSCs and Lin+ blood cells

We primarily cultured the bone marrow cells flushed out from the

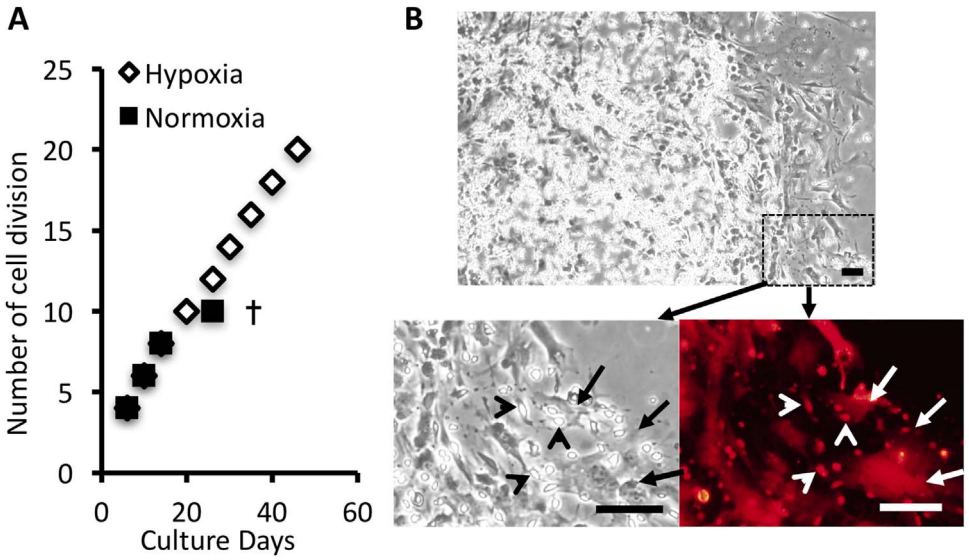


Fig. 1. Mesenchymal-like cells and blood cell-like cells are grown together in the primary culture of mouse bone marrow-derived cells under hypoxic atmosphere condition. Mouse bone marrow-derived cells were cultured using StemXVivo® (MSC Expansion Media, R & D Systems) in hypoxic (5% O_2–5% CO_2, open squares) and normoxic (20% O_2–5% CO_2, closed squares) conditions. Details of cell preparation and the culture system are described in Section 2. (A), Cumulative doubling times were plotted against cumulative culture days. A cross represents the proliferation limit of the primary bone marrow-derived cells under the normoxic condition. (B), Phase-contrast microscopy (upper and bottom-left panels) and fluorescent image (bottom, right panel) of the bone marrow-derived cell culture under hypoxic atmosphere on day 6 from passage 2 (P2) were shown. Arrows and arrowheads indicate mesenchymal-like cells, and blood cell-like cells, respectively (scale bar, 50 μm).

tibias of 2–3 week-old transgenic td-Tomato mice. The culture was carried out under conditions of normoxic or hypoxic atmosphere with the selected medium (MSC-expansion media StemXVivo, R & D Systems) and type I collagen-coated dish as described in detail in the Section 2. By using this culture condition, we derived a method of culturing mesenchymal-like cells and blood cell-like cells together in the same dish. Cumulative doubling times were plotted against cumulative culture days (Fig. 1A). Primary culture of bone marrow-derived cells did not divide after 4 passages (8 divisions) under normoxia. In contrast, cellular life span of primary culture of bone marrow-derived cells under hypoxia was increased compared to that of cells under normoxia. Phase contrast microscopy of the P2 culture (on day 6) under hypoxia showed two types of cells; smaller cells (pointed with arrowheads) were adhesive and shown to be preferably grown on or around the mesenchymal-like cells (pointed with arrows) (Fig. 1B, lower right panel).

Then, the Lin+ blood cells in the primary culture were separated from the P2 culture of bone marrow-derived cells as described in Section 2, resulting in the acquisition of Lin+ and Lin– cell groups. The separated Lin– mesenchymal-like cells grew vigorously on the type I collagen dish (Fig. 2A left), whereas the separated Lin+ blood cells barely grew (Fig. 2A, right).

Next, the cell type-specific marker expression of Lin– mesenchymal-like cells and Lin+ blood cells was determined. As shown in Fig. 2B, left panels, flow cytometry analysis revealed that Lin– mesenchymal-like cells (P2) strongly expressed MSC markers, Sca-1 at a level more than 90% and CD90 at that of ~ 71%, whereas the Lin– mesenchymal-like cells scarcely expressed CD45 and CD11b at levels less than 10%. On the other hand, Lin+ blood cells expressed blood cell marker CD45 at ~ 84%, and the Mo/Mφ marker CD11b at ~ 93%, whereas the expression of both Sca-1 and CD90 were low at levels around 12–15% (Fig. 2B, right). In addition, we confirmed that Lin– mesenchymal-like cells retained multipotency to differentiate into osteoblasts and adipocytes, showing positive staining of calcified materials by Alizarin red and that of oil droplets by Oil red, respectively (Fig. 2C, left). In contrast, Lin+ blood cells retained no differentiation ability as expected (Fig. 2C, right). From the characteristics of cell type-specific marker expression and differentiation ability, it is considered that the separated Lin– mesenchymal-like cells were composed mostly of MSCs, whereas Lin+ blood cells, which also expressed CD14 (data not shown), mostly consisted of Mo/Mφ lineage.

3.2. Cell-to-cell-contact co-culture between Lin– cells/MSCs and Lin+ blood cells promoted vigorous proliferative activity of Lin+ blood cells, and emergence of the M2-Mφ fraction

We then performed the reconstitutive co-culture using Lin– cells/MSCs and Lin+ blood cells separated from the bone marrow-derived cells of td-Tomato mouse and GFP mouse, respectively, to visualize the growth of both cells in the co-culture (Fig. 2D). At 12 h after the reconstitution, Lin– cells/MSCs (red) and Lin+ blood cells (green) were scattered over the dish. Intriguingly, the Lin+ blood cells (green), particularly near or on the Lin– cells/MSCs (red), were likely promoted to vigorously grow at 120 h after the reconstitution (Suppl. online Video 1). Next, we investigated the status of M2-polarization of the bone marrow-derived cell culture by using flow cytometry analysis. Although Lin+ blood cells directly separated from freshly obtained bone marrow without any culture did not express the M2-Mφ marker CD206 (Fig. 2E, left), around 70% of Lin+ blood cells separated from the P1 culture of bone marrow-derived cells expressed CD206 (Fig. 2E, middle). Moreover, the ratio of cell number of CD206-positive Lin+ blood cells separated from the P2 (Fig. 2E, right)-, and the P3 (data not shown)-culture increased up to 85% and 90% of the cell number of the total separated Lin+ blood cells, respectively. These results suggest that culturing mouse bone marrow-derived cells under hypoxia could successfully expand the M2-Mφ fraction.

3.3. Lin– cells/MSCs secreted macrophage colony-stimulating factor (M-CSF), whereas Lin (+) blood cells expressed M-CSF receptor (c-fms)

In our culture system of bone marrow-derived cells, Lin– cells/MSCs seemed to affect Lin+ blood cells to induce emergence of M2-Mφs. In order to identify Lin– cells/MSCs-derived factors, and to clarify the interaction between Lin– cells/MSCs and Lin+ blood cells, we first searched candidates of the cytokine–cytokine receptor axis expressed in Lin– cells/MSCs and Lin+ blood cells, by using a primer array for “cytokine–cytokine receptor interaction (mouse)”. Differentially expressed genes between the separated Lin– cells/

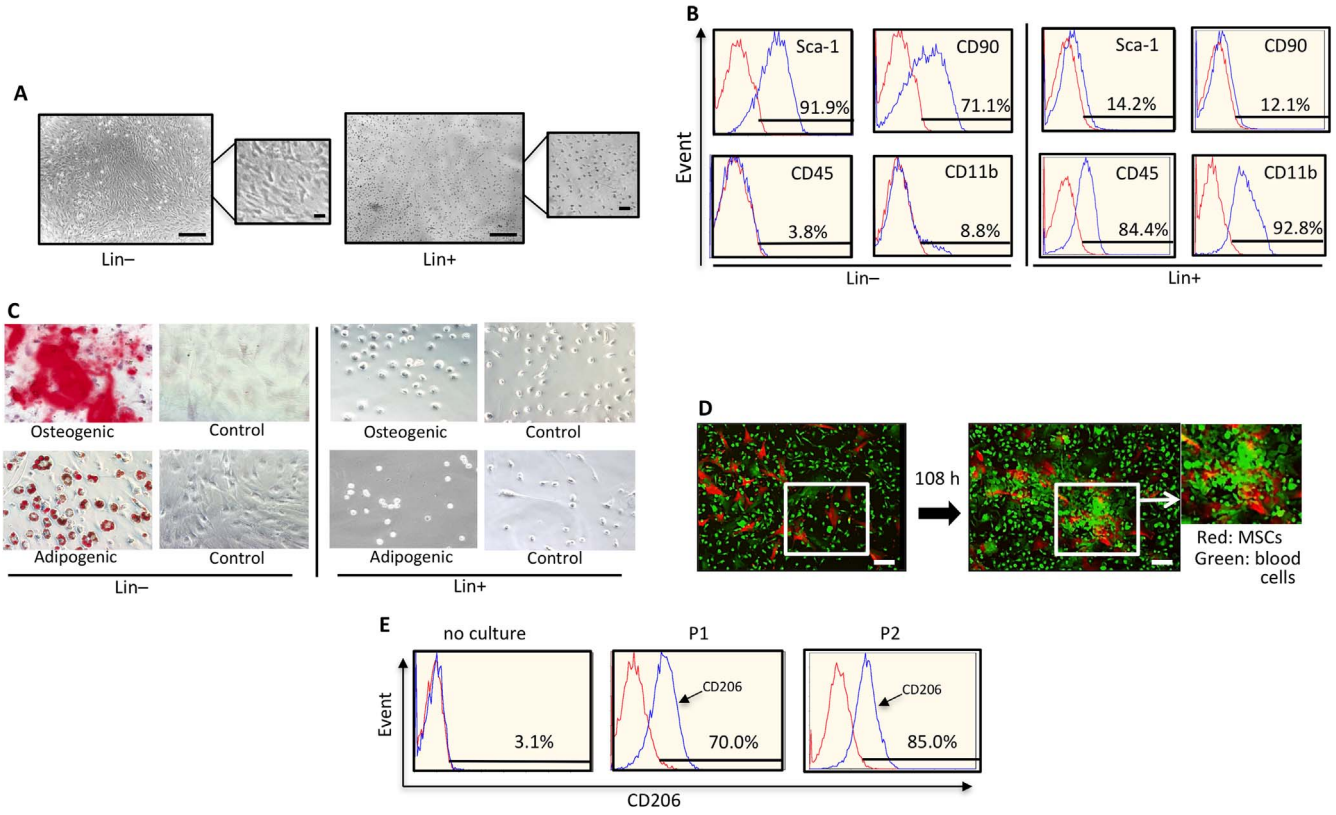
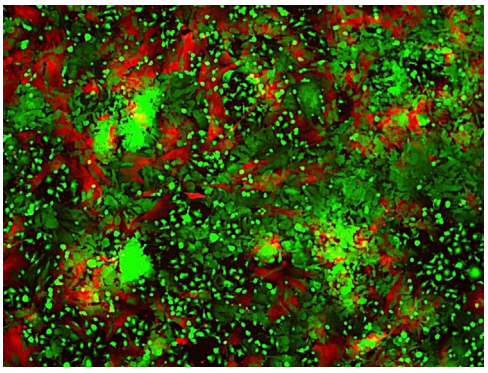


Fig. 2. The separated lineage-negative and lineage-positive cells possess the characteristics of mesenchymal stem cells and monocytes/macrophages, respectively, and the monocytes/macrophages differentiated into M2-macrophages during the co-culture of these cells. Bone marrow-derived cells were cultured until P2. The cells were harvested and then separated by MACS system. (A), The obtained lineage negative (Lin–) (left panels) and lineage positive (Lin+) cells (right panels) were re-plated again and subsequently cultured for 1 week, and then photographed. (B), Lin– and Lin+ cells were characterized for the expression of mesenchymal stem cell-, and blood cell-marker proteins on cell surfaces by flow cytometry, respectively. (C), The differentiation abilities of both cells to osteoblasts and adipocytes respectively, were examined by culturing for 2 (for osteogenic) or 1 (for adipogenic) week in each differentiation medium containing differentiation inducers as described elsewhere [32]. Cells were then fixed with 4% paraformaldehyde and stained with Alizarin red-S or Oil red-O. (D), The Lin– cells separated from P2 bone marrow-derived cell culture of td-Tomato mouse (red) and the Lin+ cells separated from that of GFP mouse (green) were equivalently combined and re-plated on one dish and cultured. Fluorescent images at 12 h and 120 h after the re-plating are shown (scale bar, 50 μm). A magnified image of squared area at 120 h is shown in the right small panel. (E), CD206 expressions in Lin+ cells separated from freshly harvested bone marrow (left panel), and separated from the P1 (middle), and the P2 (right) cultures of bone marrow-derived cells were examined by flow cytometry.

MSCs and Lin+ blood cells were identified (Table 1). Two types of axis of M-CSF–M-CSF receptor and SDF-1α/CXCL12–CXCR4 that seemed to relate to the interactions between the two types of cells in our bone marrow cell culture system were found. In addition, we tried to identify these soluble factors in the conditioned media from co-culture, single culture of Lin– cells/MSCs, and that of Lin+ blood cells by using SDS-PAGE and LC-MS/MS analysis. From these analyses, M-CSF and SDF-1 were detected with high scores in the conditioned medium of single culture of Lin– cells/MSCs and co-culture (Table 2 and Supplementary material Fig. 1). Because M-CSF is known to generate an activated Mφ population *in vitro*, we focused on the M-CSF-M-CSF receptor axis. We also confirmed the protein expression of M-CSF and M-CSFR in Lin– cells/MSCs and Lin+ blood cells, respectively, by using immunofluorescence staining (Supplementary material Fig. 2).

3.4. M-CSF secreted from Lin– cells/MSCs promoted expansion of the pre-M2-Mφ fraction belonging to Lin+ blood cells in the co-culture consisting of Lin– cells/MSCs and Lin+ blood cells

To investigate how M-CSF affects the expansion of M2-Mφ fraction among the bone marrow-derived cell culture, we first examined effect of an inhibitor of the M-CSF receptor, GW2580, on the ratio of CD206-positive cell number to total cell number of the P2 culture of bone marrow-derived cells. As shown in Fig. 3A, GW2580 (0.5 μM) clearly decreased the ratio of cell number of CD206-positive M2-Mφ to the total cell number of the P2 culture (36.8% to 3.2%), suggesting that the M-CSF secreted from Lin– cells/MSCs was an indispensable factor for the expansion of the CD206-positive cell fraction in this culture. In addition, we confirmed that GW2580 (0.5 μM) itself did not affect the cell viability of Lin+ blood cells separated from the P2 culture for at least 72 h (Supplementary material Fig. 3) and of RAW264.7 Mo/Mφ cells for 120 h (data not shown). We also found that M-CSF (10 ng/ml) significantly upregulated the cell viability of the Lin+ blood cells separated from the P2 culture at 72 h after administration and in a dose-dependent manner (Fig. 3B). Intriguingly, the conditioned medium from Lin– cells/MSCs also upregulated the cell viability of the separated Lin+ blood cells, and GW2580 completely inhibited the M-CSF- or conditioned medium-induced upregulation of cell viability of the separated Lin+ blood cells (Fig. 3C). In addition, an immunocytofluorescence analysis revealed that M-CSF did not affect the number of CD206-positive cells (green) in the re-plated P2 culture of bone marrow-derived cells, whereas M-CSF significantly increased the number of CD206-negative cells stained only in blue (DAPI) in the re-plated P2 culture (Fig. 3D). Moreover, pre-treatment of the separated Lin+ blood cells with M-CSF (50 ng/ml) for 5 (data not shown) or 12 days before the subsequent reconstituted co-culture with human MSC line UE7T-13 significantly increased the number of CD206-positive cells in the reconstituted co-culture (Fig. 3E), indicating that M-CSF secreted from Lin– cells/MSCs promoted expansion of the pre-M2-Mφ fraction belonging to Lin+ blood cells in the co-culture consisting of Lin– cells/MSCs and Lin+ blood cells.



Video S1. Lineage negative cells/mesenchymal stem cells (red) and lineage positive blood cells (green) were separated from the bone marrow-derived cells of tandem-Tomato mouse and GFP mouse, respectively, and reconstitutively co-cultured as described in the Section 2. The video was edited with the photographs taken by using the time-lapse function of the fluorescent microscope (BZ-X700, Keyence, Osaka, Japan). Photographs were taken every 30 min from the time point at 12 h to that at 120 h after the start of co-culture. A video clip is available online. Supplementary material related to this article can be found online at doi:10.1016/j.yexcr.2017.07.014.

3.5. Cell-to-cell contact between Lin+ blood cells and Lin− cells/MSCs promoted the differentiation of blood cells to CD206-positive M2-Mφs

To investigate whether cell-to-cell contact between Lin+ blood cells and Lin− cells/MSCs contributes to the M2-Mφ polarization of Lin+ blood cells, Lin− cells/MSCs and Lin+ blood cells (ratio of cell number 1:10) separated from the P2 culture of bone marrow-derived cells were reconstitutively co-cultured with cell-to-cell-contact, and non-contact (trans-well culture) conditions. After performing the non-contact co-culture, Lin+ blood cells in the bottom well were analyzed for mRNA expression and cytokine secretion. However, after performing the cell-to-cell-contact co-culture, Lin+ blood cells were separated from Lin− cells/MSCs. Then, the mRNA expression status of M2-Mφ markers in the separated Lin+ blood cells was investigated. CD206 mRNA expression in Lin+ blood cells was clearly increased in the cell-to-cell-contact co-culture as compared to monaural culture or non-contact co-culture (Fig. 4A). IL-10 mRNA was significantly upregulated in both the non-contact and cell-to-cell-contact co-cultures, although the upregulated level in the non-contact co-culture was relatively lower than that in the cell-to-cell-contact co-culture. In addition, IL-10 secretion was significantly upregulated in the cell-to-cell-contact co-culture as compared to monaural and non-contact co-cultures (Fig. 4B).

Table 2
Identified proteins in the conditioned medium of MSC and each Mascot Score (MS) and function.

	MS	Function
Connective tissue growth factor (CTGF)	321	fibroblast differentiation,
Inhibin beta A chain	245	erythroid differentiation, insulin secretion
Macrophage colony-stimulating factor (M-CSF)	165	macrophage growth, differentiation
Stromal cell-derived factor 1 (SDF-1)	92	lympho-monocytic chemoattactant, angeogenesis
Pigment epithelium-derived factor (SDF-3)	335	neurotrophic activity, anti-angeogenesis
C-C motif chemokine 9 (CCL9)	44	inflammation
Insulin-like growth factor-binding protein 4, 5, 7	106, 208, 1060	cell adhesion
LG3 fragment from Perlecan	698	anti-angiogenesis, anti-apoptosis (MSC)

Details were described in Section 2.

3.6. Cell-to-cell adhesion mediated by ICAM-1 between Lin− cells/MSCs and Lin+ blood cells is likely to promote the M2-Mφ polarization of Lin+ blood cells

Because Lin− cells/MSCs expressed the typical adherence factors ICAM-1 and VCAM-1, and the expression level of ICAM-1 was more than 100-fold higher than that of VCAM-1 (Supplementary material Fig. 4), we focused on ICAM-1. The addition of neutralizing antibody for ICAM-1 to the culture medium inhibited the binding of Lin+ blood cells with Lin− cells/MSCs grown on a 96-well plate to ~ 70% of non-added control (Fig. 5A). Moreover, an addition of an inhibitor for LFA-1/

Table 1
Genes whose expression level is more than 5-fold in comparison between Lin+ and Lin−cells.

Genes				Fold change
Higher expression in Lin− (MSC-like) cells	Ligand	*Tgfb2*	transforming growth factor, beta 2	7.01
		Tgfb3	transforming growth factor, beta 3	6.23
		Vegfc	vascular endothelial growth factor C	6.15
		Cxcl12	chemokine (C-X-C motif) ligand 12	6.11
		Cx3cl1	chemokine (C-X3-C motif) ligand 1	5.43
		Mcsf	macrophage colony-stimulating factor	5.31
	Receptor	*Pdgfra*	platelet derived growth factor receptor, alpha polypeptide	7.01
		Pdgfrb	platelet derived growth factor receptor, beta polypeptide	6.96
		Lepr	leptin receptor	6.96
		Kit	KIT proto-oncogene receptor tyrosine kinase	6.77
		Ifnar1	interferon (alpha and beta) receptor 1	6.32
		Ghr	growth hormone receptor	6.06
		Bmpr1b	bone morphogenetic protein receptor, type 1B	5.82
		Egfr	epidermal growth factor receptor	5.21
Higher expression in Lin+ (blood cell-like) cells	Ligand	*Ccl6*	chemokine (C-C motif) ligand 6	13.36
		Ccl12	chemokine (C-C motif) ligand 2	8.63
		Ccl8	chemokine (C-C motif) ligand 8	7.52
	Receptor	*Ccr2*	chemokine (C-C motif) receptor 2	22.78
		Cx3cr1	chemokine (C-X3-C motif) receptor 1	12.21
		Mcsfr	macrophage colony-stimulating factor receptor	11.63
		Gcsfr	granulocyte colony-stimulating factor receptor	11.31
		Il2rg	interleukin 2 receptor, gamma chain	6.41
		Il6ra	interleukin 6 receptor, alpha	5.82
		Cxcr4	chemokine (C-X-C motif) receptor 4	5.70

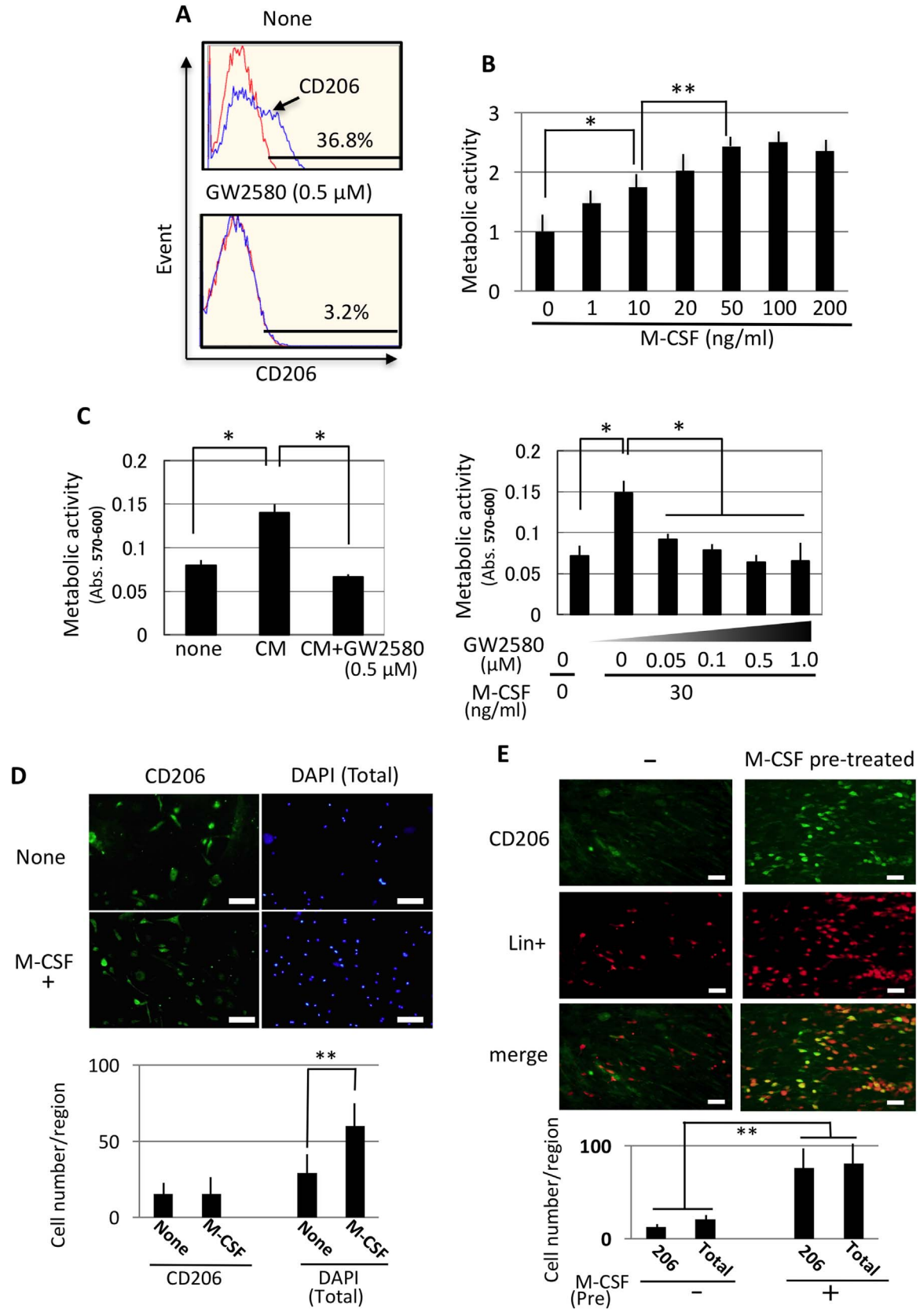

A
None
CD206
36.8%
GW2580 (0.5 μM)
3.2%
Event
CD206
B
Metabolic activity
M-CSF (ng/ml)
0 1 10 20 50 100 200
C
Metabolic activity (Abs. 570-600)
none
CM
CM+GW2580 (0.5 μM)
GW2580 (μM)
M-CSF (ng/ml)
0 0 0.05 0.1 0.5 1.0
0 30
D
CD206
DAPI (Total)
None
M-CSF +
Cell number/region
CD206
DAPI (Total)
E
–
M-CSF pre-treated
CD206
Lin+
merge
Cell number/region
206
Total
M-CSF (Pre)

Fig. 3. Macrophage colony-stimulating factor secreted from lineage negative (Lin–) cells/mesenchymal stem cells (MSCs) promoted expansion of pre-M2-Mφ fraction belonging to lineage positive (Lin+) blood cells in the co-culture consisting of Lin–cells/MSCs and Lin+ blood cells. (A), Freshly prepared bone marrow-derived cells were plated on the dish and cultured for 7 days. Then, the culture was added with (lower panel) or without (upper panel) 0.5 μM GW2580. Cultures were further continued until P1. Then, cells were harvested and analyzed for CD206 expression by flow cytometry. (B), Lineage positive (Lin+) blood cells were separated from the P2 culture of bone marrow-derived cells. Lin+ blood cells (1×10^4 cells) were cultured in a 96-well Poly-D-lysin dish with increasing amount of M-CSF in high-glucose D-MEM containing decreased concentration of FBS (2%) for 4 days. WST assay was then performed. Cell viability (metabolic activity; $Abs._{450-690}$) is shown as relative ratios to each non-treated control as 100%. The data are represented as the mean ± SD (n = 4). (C), Cell viability was examined as described in (B) except that the conditioned medium (CM) of lineage negative (Lin–) cells/mesenchymal stem cells (MSCs) at the 50% concentration of total volume or 30 ng/ml M-CSF was administrated 2 h after the cell plating, and further cultured for 3 days. In some cases, M-CSF receptor inhibitor GW2580 (0.5 μM) was added to the culture 30 min before the addition of Lin– cells/MSCs-CM or M-CSF. Data are represented as the mean ± SD (n = 6). (D), P2-cultured bone marrow-derived cells were harvested, and cells (5×10^3) were re-plated onto a type-I collagen-coated 8-well chamber plate and cultured with or without 50 ng/ml of M-CSF for 6 days. Cells were analyzed for CD206 expression (green) by immunofluorescent staining as described in Section 2. DAPI was used to stain all cell nuclei (blue) (scale bar, 50 μm). The CD206-positive- and total cells in six separate regions of the well were counted. (E), Lin+ blood cells (red) (2×10^4 cells) separated from the freshly prepared bone marrow-derived cells of td-Tomato mouse were plated on the chamber slide, and cultured with or without 30 ng/ml of M-CSF for 12 days. Then, human MSCs UE7T-13 (7.2×10^4 cells) were seeded into each well and co-cultured for a further 3 days. Cells were fixed and CD206 expression (green) was examined by immunofluorescent staining (scale bar, 50 μm). The CD206-positive (green)-, and total Lin+ blood (red)-cells in six separate microscopic fields of the well were counted. (B)–(E), The values of $^*P < 0.05$ and $^{**}P < 0.01$ were considered statistically significant.

ICAM-1 receptor, Rwj50271, to the culture medium in P2 co-culture dose dependently attenuated CD206 expression in the Lin+ blood cells from P2 co-culture (Fig. 5B). We also examined the possible implication of VCAM-1 in the adhesion of Lin+ blood cells to Lin– cells/MSCs and subsequent M2-Mφ polarization. The binding between these cells was inhibited in the presence of the neutralizing antibody for VCAM-1, although the degree of the inhibition was a little low (~ 76% of control), as compared with the neutralizing antibody for ICAM-1 (data not shown). However, an inhibitor for VLA-4/VCAM-1 receptor, BIO5192, in the medium did not affect CD206 expression in the Lin+ blood cells from the P2 co-culture (data not shown). These results suggest that ICAM-1-mediated cell adhesion between Lin– cells/MSCs and Lin+ blood cells is likely to promote the M2-Mφ polarization of Lin+ blood cells.

4. Discussion

We found that M2-Mφs stably proliferated in the existence of Lin– cells/MSCs in the MSC-expansion Media (StemXVivo®, R & D Systems) under the hypoxic condition (5% O_2—5% CO_2) (Figs. 1 and 2). Intriguingly, we also utilized another type of MSC-expansion Media (Mesencult-XF™, Stem Cell Technologies Inc., Cambridge, MA, USA) for the primary culture of mouse bone marrow-derived cells. However, Mesencult-XF™ did not increase M2-Mφs under the condition both of hypoxia and normoxia (20% O_2—5% CO_2) (data not shown). Although, there is still room to investigate what kinds of soluble factors in StemXVivo® affect Lin– cells/MSCs or pre-M2-Mφs to induce the resultant survival and increase of M2-Mφs in the primary culture of mouse bone marrow-derived cells, our unique hypoxic co-culture system with Lin– cells/MSCs and Lin+ blood cells increased M2-Mφ fraction without the addition of any growth factor or cytokine.

We investigated the mechanisms of the propagation of M2-Mφs in our co-culture system consisting of Lin+ blood cells and Lin– cells/MSCs: primer array analysis revealed that Lin– cells/MSCs expressed M-CSF, whereas Lin+ blood cells expressed the M-CSF receptor in our co-culture system (Table 1). In addition, LC/MS/MS analysis confirmed that Lin– cells/MSCs secreted M-CSF protein in our co-culture system (Table 2 and Supplementary material Fig. 1). Importantly, the M-CSF receptor inhibitor, GW2580, clearly decreased the ratio of cell number of CD206-positive cells to the total cell number in the P2 culture of bone marrow-derived cells, suggesting that the M-CSF secreted from Lin– cells/MSCs must be indispensable for the expansion of M2-Mφ fraction in this P2 culture (Fig. 3A). In addition, M-CSF, or the conditioned medium from Lin– cells/MSCs, significantly induced upregulation of cell viability of the separated Lin+ blood cells, and GW2580 clearly abrogated this upregulation (Fig. 3B and C, respectively). We also found that the number of CD206-positive cells was not affected by M-CSF treatment in this P2 culture, whereas the number of CD206-negative cells was increased by M-CSF treatment (Fig. 3D). Moreover, pre-treatment of the separated Lin+ blood cells with M-CSF increased the number of CD206-positive cells under the condition of the re-constitutive co-culture with human MSC line UE7T-13 as compared to the non-pre-treated Lin+ blood cells (Fig. 3E). These results strongly suggest that M-CSF secreted from Lin– cells/MSCs increased pre-M2-Mφs such as M0 (zero)-Mφs and un-differentiated Mo in Lin+ blood cells, and the other soluble- or insoluble-factors other than M-CSF subsequently enabled the expanded pre-M2-Mφs to differentiate into M2-Mφs in our culture system.

We thought that adherence factors between Lin+ blood cells and Lin– cells/MSCs might also relate to the M2-Mφ polarization of pre-M2-Mφs in this culture system, since the cell-to-cell-contact co-culture consisting of Lin– cells/MSCs and Lin+ blood cells promoted M2-Mφ polarization, showing elevated expressions of CD206 as compared to

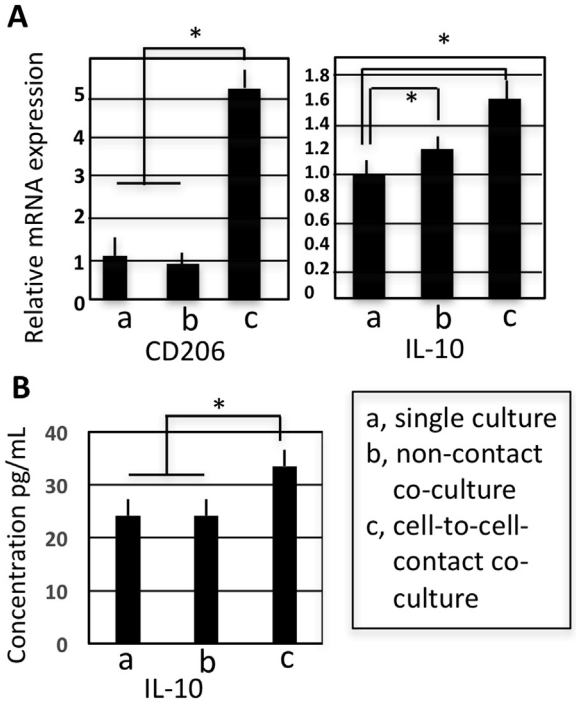


Fig. 4. Cell-to-cell contact between lineage negative cells/mesenchymal stem cells and lineage positive (Lin+) blood cells promoted M2-macrophage polarization of Lin+ blood cells. (A, B), For cell-to-cell-contact co-culture, 3.7×10^5 of lineage positive (Lin+) blood cells and 3.7×10^4 of lineage negative (Lin–) cells/mesenchymal stem cells (MSCs) separated from the P2 culture of bone marrow-derived cells were reconstituted and re-plated on a 24-well plate and cultured for 48 h in 1:1 mixture of medium for each cell culture. For non-contact co-culture, using trans-well, Lin+ blood cells (3.7×10^5) were plated in the bottom well, and Lin– cells/MSCs (3.7×10^4) were plated on the membrane of the top well (insert) of the trans-well with each medium. Monaural culture included 3.7×10^5 of Lin+ blood cells as described in the Section 2. (A), After co-culture for 48 h, cells were harvested and used for the RNA preparation. qRT-PCR analysis for CD206 and IL-10 was performed using GAPDH as an internal control. (B), The conditioned medium from cell-to-cell-contact-, and non-contact-*co*-culture as described in (A) was also collected and used for the measurement of IL-10 by ELISA. Data are represented as the mean ± SD (n = 3). The values of $^*P < 0.05$ were considered statistically significant. a, single culture; b, non-contact co-culture; c, cell-to-cell-contact co-culture.

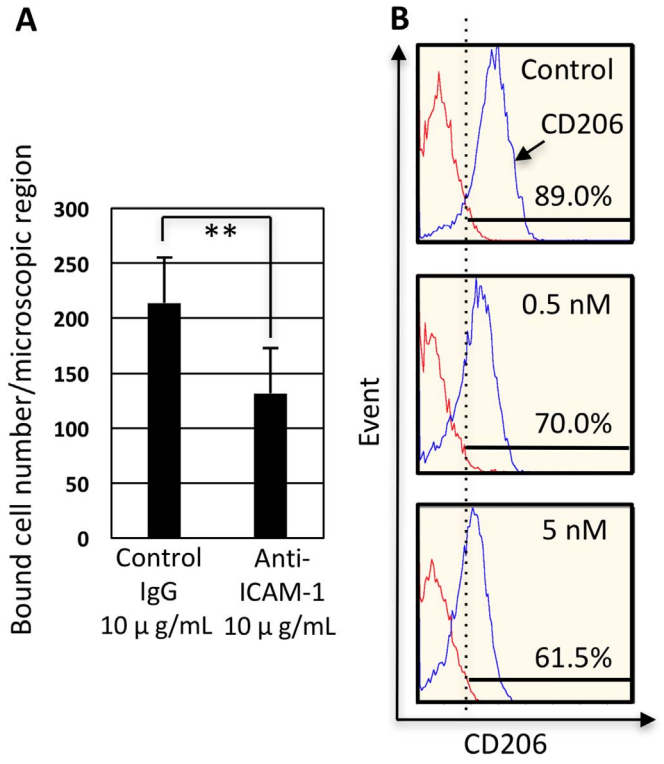


Fig. 5. Inhibition of ICAM-1—ICAM-1 receptor axis suppressed the adhesion between lineage negative cells/mesenchymal stem cells and lineage positive (Lin+) blood cells and the following M2-macrophage polarization of the Lin+ blood cells. (A), Lineage negative (Lin−) cells/mesenchymal stem cells (MSCs) were separated from the P2 culture of non-fluorescent wild-type mouse bone marrow-derived cells. The Lin− cells/MSCs were plated into each well of a type I collagen-coated 96-well plate and cultured until the cells just reached confluence. On the other hand, lineage positive (Lin+) blood cells were separated from P2 culture of tandem-Tomato mouse bone marrow-derived cells. These Lin+ blood cells were seeded onto confluent Lin− cells/MSCs in the 96-well plate as described above. Some cells were pre-treated with 10 μg/ml hamster anti-ICAM-1 antibody for 30 min before seeding. For control, cells were pre-treated with the same concentration of hamster IgG (SantaCluz). Lin+ blood cells were allowed to bind to Lin− cells/MSCs for 1 h at 37 °C. After washing three times with cold PBS, the remaining Lin+ blood cells bound to Lin− cells/MSCs were counted using the images taken under fluorescent microscopy. Data are represented as average ± SD from six separate regions of the well. The value of $^{**}P < 0.01$ was considered statistically significant. (B), An inhibitor of LFA-1 (ICAM-1 receptor), RWJ50271 (0.5 and 5 nM), was added to the P2 culture of bone marrow-derived cells. Seven days after culture, cells were harvested, and Lin+ blood cells were separated from Lin− cells/MSCs, and the cell surface expression of CD206 was evaluated by flow cytometry. The representative data of 3 independent experiments are shown.

monaural or non-contact co-culture with trans-well (Fig. 4). Intriguingly, the addition of a neutralizing antibody against adhesion molecule ICAM-1 to the culture medium inhibited the binding between Lin− cells/MSCs and Lin+ blood cells (Fig. 5A). Moreover, addition of an inhibitor for LFA-1/ICAM-1 receptor, Rwj50271, to the culture medium in the P2 co-culture partially attenuated CD206 expression in the Lin+ blood cells from P2 co-culture (Fig. 5B), suggesting that ICAM-1 expressed on Lin− cells/MSCs and its receptor LFA-1 expressed on Lin+ blood cells (Supplementary material Fig. 4) partially mediate their adhesion and contribute to the subsequent M2-Mφ polarization of Lin+ blood cells. However, more detailed studies of the adherence factor(s) are needed, and the signaling mechanisms after the adherence of these cells for M2-Mφs polarization remain to be clarified. In addition, we do not exclude the possible effect of the soluble factor(s) secreted by Lin− cells/MSCs or Lin+ blood cells during the adhesive co-culture of these cells. To date, some MSC-originated secretory factors such as PGE2 or TGF-β that relate to M2-Mφ activation have been reported [13,19,20]. However, we found that the PG synthesis inhibitor, indomethacin, or the TGF-β receptor inhibitor, SB431542, in the P2 culture of bone marrow-derived cells did not affect the status of M2-Mφs polarization (data not shown).

Together, our results show that M-CSF secreted from Lin− cells/MSCs functions to increase the number of the pre-M2-Mφs, and cell-to-cell contact between the pre-M2-Mφs and Lin− cells/MSCs functions to promote the subsequent M2-Mφ polarization from the pre-M2-Mφs in co-culture of Lin− cells/MSCs and Lin+ blood cells derived from mouse bone marrow. However, in order to prove the participation of adherence factors other than ICAM-1/LFA-1, or soluble factors for the M2-Mφ polarization and the following signaling mechanism in our co-culture system, more detailed experiments are necessary.

The propagation of M2-Mφs by MSC in our co-culture of Lin− cells/MSCs and Lin+ blood cells stably occurred under hypoxia. In human body, hypoxic environment was sometimes appeared in the ischemic lesions of solid tumors [33] or in chronically inflamed tissues [34]. Particularly, in a solid tumor, various cells, including fibroblasts, cancer-associated fibroblasts (CAFs), MSCs, endothelial cells and various immune cells such as macrophages/tumor-associated macrophages (TAMs), neutrophils and lymphocytes, are found in the tumor stroma [35]. These stromal cells create a tumor microenvironment, where they interrelate with tumor cells and with each other to affect the tumorigenic features. Interestingly, in these cells, TAMs, being educated by tumor cells, have M2-Mφ-like characteristics. Therefore, TAMs are interested in their role of preventing tumor cells from the immune system (immune evasion) and helping the tumor growth and expansion in human malignancies [36]: M2-TAMs are most likely propagated also by MSCs in the tumor-associated microenvironment. Thus, the interplay between M2-TAMs and MSCs seemed to have a unique role under the hypoxic condition such as the tumor-associated microenvironment in human body.

Actually, we examined the effect of culture between under hypoxia and normoxia on the expression status of M-CSF and ICAM-1, respectively, by real time RT-PCR analysis. In this experiment, some dishes of the P2 co-culture of bone marrow-derived cells grown under usual hypoxia were transferred to the normoxic condition on day 3 and cultured for further 4 days under normoxia before the separation of Lin− cells/MSCs and Lin+ blood cells. The mRNA expression levels of M-CSF and ICAM-1 in the separated Lin− cells/MSCs, using β-actin as an internal control, were not significantly changed by the culture under normoxia for 4 days as compared with the culture under usual hypoxia (data not shown). In addition, the expression of M-CSF receptor and ICAM-1 receptor LFA-1 mRNAs in the separated Lin+ blood cells was not significantly changed between hypoxia and normoxia (4 days) cultures (data not shown). Considering these findings, another signaling axis (ligand/receptor axis) other than M-CSF/M-CSFR or ICAM-1/LFA-1 system that we have not found until now may be implicated in the propagation of M2-Mφ in our co-culture system under hypoxia.

Conclusively, our original *ex vivo* primary culture system of bone marrow-derived cells under hypoxia could be clinically applicable for the treatment for regenerating or restoring injured tissues with high efficiency; anti-inflammatory M2-Mφs obtained in our culture system will be a useful tool for cytotherapy against inflammatory diseases.

5. Conclusion

We found that M-CSF secreted from MSCs functions to increase the number of the pre-M2-Mφs. In addition, cell-to-cell contact between the pre-M2-Mφs and MSCs partially mediated by ICAM-1/LFA-1 functions to promote the subsequent M2-Mφ polarization from the pre-M2-Mφs. Thus, we successfully established a co-culture system consisting of mouse bone marrow-derived Lin+ blood cells and MSCs under hypoxic conditions (5% O_2—5% CO_2), which facilitated an expansion of M2-Mφ fraction among Lin+ blood cells.

Disclosure of potential conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2017.07.014.

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