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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.