Online supplement

Materials and Methods

Animals

The green fluorescent protein (GFP) transgenic mice (GFP mice, background: C57BL/6), which ubiquitously express enhanced GFP protein, were a generous gift from Dr. Masaru Okabe (Osaka University, Osaka, Japan). The ROSA26 mice (background: C57BL/6), which are knock-in mice that express the lacZ gene in essentially all tissues, were purchased from Jackson Laboratory (Bar Harbor, ME). All wild-type mice (C57BL/6, male, 8- to 12-weeks-old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). They were fed a standard diet and water and were maintained on a 12-hour light and dark cycle. All experiments in this study were performed in accordance with the Shinshu University Guide for Laboratory Animals which conforms to the NIH Guidelines.

Wire-mediated vascular injury

Wire-mediated vascular injury of the right femoral artery was produced as previously described by Sata et al.\(^1\) We confirmed that this procedure induced a reproducible neointimal formation in 8- to 12-week-old C57BL/6 mice.\(^2\)

Experimental protocols

In preliminary experiments, we examined the effect of M-CSF (5, 50, and 500 µg/kg) on the number of peripheral monocytes in C57BL/6 mice, and found that the administration of 500 µg/kg of human recombinant M-CSF (kindly provided by Morinaga Milk Industry Co. Ltd., Kanagawa, Japan) significantly increased the number of peripheral monocytes; this is consistent with the previous report.\(^3\) Therefore, in the present study, we used M-CSF at a dose of 500 µg/kg·day. The mice were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium and splenectomized not
only to eliminate spleen-derived MNCs and EPCs but also to prevent the homing of bone
marrow-derived MNCs and EPCs to the spleen The animals were allowed to recover for
14 days before vascular injury was performed. Recombinant human M-CSF [500
\( \mu g/(kg \cdot day) \)] or saline (control) was administered intraperitoneally in splenectomized
mice for 10 consecutive days, starting 4 days prior to the wire-mediated vascular injury.
M-CSF treatment was well tolerated and no abnormal behavior was observed. AMD3100
(Sigma, St. Louis, MO) was administered using a micro-osmotic pump (Alzet model
1007D; Durect Corporation, Cupertino, CA) and implanted subcutaneously.

Histology and immunohistochemistry

Histology and immunohistochemistry were performed as previously
described. After brief irrigation with saline to ensure that the blood was completely
washed out in the femoral artery, mice were euthanized. The femoral arteries were
excised from each mouse; the arteries were embedded in OCT compound (Tissue-Tek;
Miles Laboratories, IN), and frozen in liquid nitrogen. Neointimal formation in the
femoral arteries was evaluated at 5 locations that were separated at a distance of 100 \( \mu m \),
with the most distal site located at the point where the wire-inserted branch first appeared.
The formation was stained with hematoxylin and eosin (HE). To quantify intima/media
(I/M) ratio, each image was digitized and analyzed under a microscope (BX-51;
Olympus, Tokyo, Japan) using NIH Image software ver. 1.63. The average of the value at
5 locations in each artery was determined.

For immunohistochemical analysis, the arterial sections were incubated with
primary antibodies against mouse CD31 (clone MEC13.3, BD Biosciences), F4/80
(clone A3-1, RDI, Flanders, NJ), \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA: clone 1A4, Sigma,
Saint Louis, MO), SDF-1\( \alpha \) (clone 79014, R&D Systems, Minneapolis, MN), M-CSF
(kindly provided by Morinaga Milk Industry Co. Ltd.), c-fms (Santa Cruz Biotechnology,
CA), GFP (MBL, Nagoya, Japan), and CD184 (CXCR4: clone 2B11, BD Biosciences).
This was followed by incubation with biotin-conjugated secondary antibodies. Next, the sections were washed and treated with avidinperoxidase (ABC kit, Vector Laboratories, Burlingame, CA). The reaction was developed using the DAB substrate kit (Vector Laboratories). The sections were then counterstained with hematoxylin. No signals were detected when irrelevant IgG (Vector Laboratories) was used instead of the primary antibody as a negative control. For immunofluorescence staining, Cy3-labeled goat/donkey anti-rat/mouse IgG (for CD31, F4/80, and α-SMA: Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) and FITC-labeled goat anti-rabbit IgG (for GFP) were used as secondary antibodies. The sections were also stained with 4',6-diamidino-2-phenylindole (DAPI: Wako Pure Chemical Industries Ltd., Osaka, Japan) for nucleic acid staining. Immunofluorescence was observed by confocal laser scanning microscopy (Leica TCS-SP2 AOSB spectral laser scanning confocal microscopy system, Heidelberg, Germany). No signals were detected when normal goat serum or irrelevant IgG was used instead of the primary antibody as a negative control. The quantification of CD31 and SDF-1α staining was independently performed in a double-blind fashion by at least 2 researchers.

Flow cytometry analysis

Blood samples were collected from the mice after M-CSF [500 μg/(kg·day)] or saline (control) had been administered for 4 consecutive days. The circulating cells were identified using nucleated cell fractionation. The nucleated cells were double-labeled as follows: (1) PerCP-conjugated anti-CD11b (Mac-1) antibody (clone M1/70, BD Biosciences, San Jones, CA) and biotin-conjugated anti-Gr-1 antibody (BD Bioscience) by a second step using FITC-conjugated streptavidin (BD Biosciences); (2) FITC-conjugated anti-CD34 monoclonal antibody (clone RAM34, BD Biosciences) and PE-conjugated anti-Flk-1 antibody (VEGFR2/KDR, clone Avas12a1, BD Biosciences); (3) PE-conjugated anti-CXCR4 antibody (clone 2B11, BD Biosciences) and
PerCP-conjugated anti-CD11b (Mac-1) antibody. (4) FITC-conjugated anti-CD34 monoclonal antibody and PE-conjugated CD14 (clone rmC5-3, BD Biosciences). The cells were examined by flow cytometry (FACSCalibur, BD Biosciences) and analyzed using CellQUEST software ver.3.3 (BD Biosciences).

Bone marrow transplantation

Whole bone marrow cells from ROSA26 or GFP mice were harvested by flushing the femurs with phosphate-buffered saline (PBS). Red blood cells were lysed with ACK buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.2) at 4°C for 20 min. The cells were washed 3 times with PBS and resuspended in 0.5 mL PBS. The recipient mice (wild-type, 8 weeks old) were lethally irradiated with a total dose of 9 Gy (MBR-155R2, Hitachi, Japan) and injected with bone marrow cells through the tail vein. To verify the reconstitution of the bone marrow after transplantation by this protocol, the GFP mice were used as donors. Flow cytometry analysis revealed that 8 weeks after bone marrow transplantation, peripheral blood cells comprised more than 98% of the GFP-positive cells.

X-gal staining

To detect lacZ reporter activity, arterial sections were fixed in acetone and stained using an X-gal Substrate Set (HistoMark, KPL, Gainthersburg, MD) according to the manufacturer’s instructions. These sections were then carefully examined under a microscope to check for blue staining.

Serum cytokine levels

Blood samples were collected after saline (control) or M-CSF [500 μg/(kg·day)] was administered for 7 consecutive days, starting 4 days prior to the vascular injury. The serum levels of the monocyte chemoattractant protein-1 (MCP-1:
CCL2), interleukin (IL)-12p70, IL-10, IL-6, and tumor necrosis factor-α (TNF-α) were assessed by using the CBA Mouse Inflammation Kit (BD Biosciences) according to the manufacturer’s instructions.

**In vitro experiments**

Peripheral blood and bone marrow cells were collected from wild-type mice, and mononuclear cells (MNCs) were isolated using Lympholyte-M (Cedarlane, Ontario, Canada) according to the manufacturer’s instructions. The peripheral or bone marrow MNCs were incubated with RPMI-1640 (Invitrogen, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS: Hyclone, Logan, UT) and β-mercaptoethanol (Sigma) in the presence or absence of M-CSF (100 ng/mL) or G-CSF (100 ng/mL) for 24 hours. These cells were then analyzed by flow cytometry.

**Statistical analysis**

Data are expressed as mean ± SEM. The unpaired two-tailed t test was used to compare the 2 groups. For comparisons between multiple groups, we determined the significance of difference between the group means by one-way analysis of variance (ANOVA) followed by the Tukey-Kramer procedure for the comparison of means. All analyses were performed using StatView software (Abacus Concepts, Inc., Berkeley, CA). Differences with values of \( P<0.05 \) were considered to be statistically significant.

**References**


Supplemental Figure Legends

Supplemental figure I. Contribution of bone marrow-derived cells in the late phase
Bone marrow-transplanted mice (GFP→C57BL/6) were developed and wire-mediated vascular injury was produced in them 8 weeks after the bone marrow transplantation. Recombinant human M-CSF [500 μg/(kg·day)] was administered for 10 consecutive days, starting 4 days prior to vascular injury. The femoral arteries were excised 21 days after the injury, and immunohistochemical staining for GFP (FITC) and endothelial cells (CD31), macrophages (F4/80), or SMCs (α-SMA) were performed. Further, nucleic acid was stained with DAPI.

Supplemental figure II. Serum levels of inflammatory cytokines
Recombinant human M-CSF [500 μg/(kg·day), n=5] or saline (control, n=4) was administered for 7 consecutive days, starting 4 days prior to vascular injury. Blood samples were obtained and serum levels of MCP-1 (A), IL-12p70 (B), IL-10 (C), IL-6 (D), and TNF-α (E) were assessed. Data are mean ± SEM (n=4–5).
Supplemental Fig. I
**Supplemental Fig. II**

(A) MCP-1 (pg/mL): Control (A) vs. M-CSF (B) with a p-value of <0.05.

(B) IL-12p70 (pg/mL): Control (A) vs. M-CSF (B) showing no significant difference (NS).

(C) IL-10 (pg/mL): Control (A) vs. M-CSF (B) with no significant difference (NS).

(D) IL-6 (pg/mL): Control (A) vs. M-CSF (B) with no significant difference (NS).

(E) TNF-α (pg/mL): Control (A) vs. M-CSF (B) with no significant difference (NS).