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Brief report

Muscle-derived Gr1^{dim}CD11b⁺ cells enhance neovascularization in an ischemic hind limb mouse model

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Gr1⁺CD11b⁺ cells are characterized as myeloid-derived suppressor cells potentially involved in angiogenesis. We demonstrate that Gr1⁺CD11b⁺ cells isolated from ischemic muscle in a hind-limb ischemic C57BL/6 mouse model play a role in vessel formation after ischemic injury. Gr1^{dim}CD11b⁺ cells, a subpopulation of Gr1⁺CD11b⁺ cells, within skeletal muscle

were increased in context of ischemia. Strikingly, astrocyte-plexus formed from muscle-derived Gr1^{dim}CD11b⁺ cells in Matrigel culture, followed by formation of isolectin and von Willebrand Factor-expressing cells, similar to that reported for angiogenesis in retina. When isolated muscle-derived Gr1^{dim}CD11b⁺ cells were injected into ischemic muscles, recov-

ery of blood flow was significantly enhanced and these cells were incorporated into vessel walls. This suggests that Gr1^{dim}CD11b⁺ cells are recruited into ischemic regions after ischemia and may be involved in angiogenesis by their capacity to generate vascular cells. (*Blood*. 2010;116(9):1623-1626)

Introduction

Tumor angiogenesis and vasculogenesis are modulated by monocytes and myeloid progenitors.^{1,2} Myeloid macrophage lineage cells with a myeloid derived suppressor cell (MDSC) phenotype of Gr1⁺CD11b⁺ are significantly increased in spleen and bone marrow (BM) of animals bearing tumors.³⁻⁶ MDSCs regulate tumorigenesis through induction of angiogenesis⁷ and suppress T cell-mediated immune responses.^{8,9} However, populations of Gr1⁺CD11b⁺ cells are morphologically heterogeneous and contain neutrophils, immature dendritic cells, monocytes and early myeloid progenitors. In our study, we found that the number of tissue-residing Gr1⁺CD11b⁺ cells was markedly increased in ischemic muscle after femoral artery dissection. Between the 2 cell populations that comprise Gr1⁺CD11b⁺ cells, Gr1^{high}CD11b⁺ and Gr1^{dim}CD11b⁺, we primarily focused on Gr1^{dim}CD11b⁺ cells because Gr1^{high}CD11b⁺ cells, mostly neutrophils, increase in number with inflammatory response after surgery. To determine whether increases in the numbers of Gr1^{dim}CD11b⁺ cells in ischemic muscle might be related to neovascularization, we evaluated whether muscle-derived Gr1^{dim}CD11b⁺ cells could differentiate into endothelial cells in vitro and if direct injection of muscle-derived Gr1^{dim}CD11b⁺ cells enhanced recovery of blood perfusion in ischemic hind limbs of C57BL/6 mice.

Methods

Mouse hind-limb ischemia model and isolation of Gr1^{dim}CD11b⁺ cells from ischemic muscle

Ischemic injury was induced in female C57BL/6 mice (The Jackson Laboratory; 10-12 weeks old) by femoral artery and vein dissection,^{10,11}

with Indiana University School of Medicine Institutional Animal Care and Use Committee approval. Single cell suspension was made from ischemic muscle using 0.2% collagenase type 2 (210 U/mg; Worthington) and 0.2% dispase (0.95 U/mg; Invitrogen) digestion.^{12,13} Isolated cell suspensions were incubated with fluorescein isothiocyanate-conjugated anti-mouse CD11b and allophycocyanin-conjugated anti-mouse Gr-1 antibody (BD Biosciences). Gr1^{dim}CD11b⁺ cells and Gr1⁻CD11b⁻ cells were sorted by MoFlo XDP Cell Sorter (Beckman Coulter).

FACS analysis

Isolated Gr1^{dim}CD11b⁺ cells were analyzed for expression of PE-CD115, F4/80 (eBiosciences), CD45 (BD Bioscience), and Ly6C (Miltenyi Biotec) antibodies using FACS Vantage (Becton Dickinson).

Real time quantitative PCR

Real-time quantitative polymerase chain reaction (q-PCR) analysis was performed using following primers: MCP-1 forward (F): GGCTCAGC-CAGATGCAGTTAA, reverse (R): CTACTCATTGGGATCATCTTGCT), SDF-1 (F:CAGCCGTGCAACAATCTGAAG, R:CTGCATCAGT-GACGGTAAACC), MIP-1 α (F:TCTTCTCAGCGCCATATGGA, R:CGTGGAATCTTCCGGCTGTA) and VEGF-A (F:ACCATGAACTT-TCTGCTCTCTTG, R: GAACTTGATCACTTCATGGGACT). Real time q-PCR was performed by MyiQ Real Time PCR Detection Systems (Bio-Rad) and relative quantification analysis was generated with Bio-Rad IQ5 software.

Intramuscular injection of muscle derived Gr1^{dim}CD11b⁺ cells and evaluation of blood flow

Isolated muscle-derived Gr1^{dim}CD11b⁺ cells or Gr1⁻CD11b⁻ cells (5×10^5 cells/mouse) were injected into ischemic adductor muscles 2 days after

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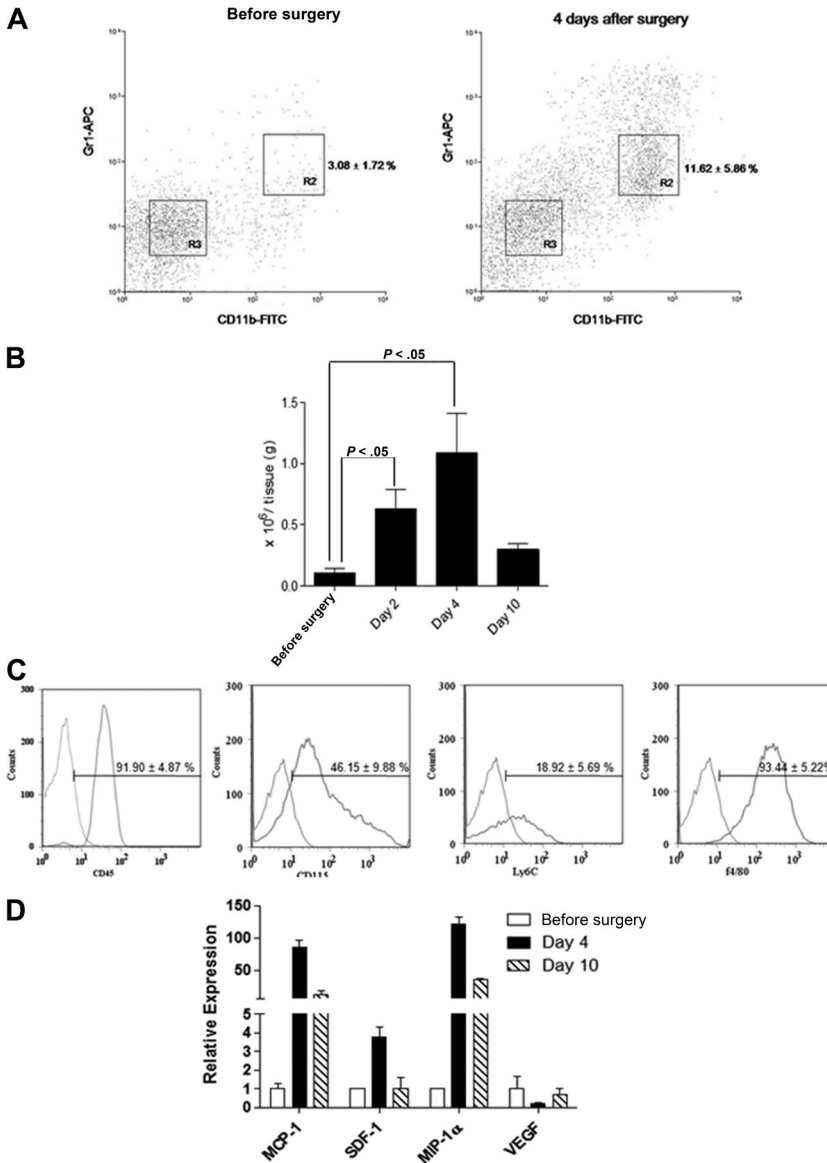


Figure 1. Ischemic muscles recruit Gr1^{dim}CD11b⁺ cells after femoral artery dissection. Cell suspensions from nonsurgically treated C57BL/6 mice and ischemic muscles of femoral artery dissected C57BL/6 mice were stained with anti-CD11b and anti-Gr1 monoclonal antibodies. (A) Representative dot plots from nonsurgically treated muscles of C57BL/6 mice and ischemic muscles of femoral artery dissected C57BL/6. Percentages of cells are shown as the mean \pm SEM. (B) Total number of Gr1^{dim}CD11b⁺/g tissue (n = 5 each). (C) Flow cytometric analysis of Gr1^{dim}CD11b⁺ cells. Curves to right in each panel indicate staining with specific antibody, and curves to left represent staining with isolated control antibodies. (D) Real-time q-PCR expression profile of MCP-1, SDF-1, MIP-1 α , and VEGF in ischemic muscle before surgery, and 4 and 10 days after surgery.

femoral vessel dissection. Blood perfusions in both ischemic and nonischemic limbs of the same mouse were evaluated using laser Doppler perfusion scanner (Moore Instruments).^{10,14}

In vitro differentiation and in vivo localization of injected Gr1^{dim}CD11b⁺ cells

For in vitro analysis, sorted muscle-derived Gr1^{dim}CD11b⁺ cells and Gr1⁻CD11b⁻ cells were separately seeded at $0.5 \times 10^5/\text{cm}^2$ onto Matrigel (BD Bioscience)-coated chamber Slide (Lab-Tek) and cultured in EGM-2 medium (Lonza). Cultured cells were stained with anti-rabbit Glial Fibrillary Acidic Protein (GFAP; Abcam), anti-rabbit von Willebrand Factor (VWF; Santa Cruz V Biotechnology), and anti-isolectin (Sigma-Aldrich) antibodies. Binding of primary antibodies was detected with Alexa 566 anti-rabbit (Molecular Probes) antibody with nuclei stained with 4',6-diamidino-2-phenylindole. To detect localization of injected cells and whether they incorporated into vessels, we isolated muscle-derived Gr1^{dim}CD11b⁺ cells from C57BL/6 (CD45.2⁺) mice and injected them into ischemic muscle of congenic CD45.1 mice (B6.SJL-Ptpr^ePep3^b/BoyJ; Jackson Immunoresearch Laboratories). Frozen tissues were stained with anti-mouse SMA (Abcam) antibody and biotin-conjugated anti-mouse CD45.2 (eBioscience). Binding of primary antibodies was detected with

Alexa 488 anti-rabbit and Alexa 555-conjugated streptavidin (Molecular Probes). Tissue sections and cultured cells stained for different antigens were photographed under a Zeiss LSM 510 Meta Microscope with AxioVision software (Carl Zeiss).

Statistical analysis

Data are given as mean plus or minus SEM. Comparisons of laser Doppler imaging index between 2 groups (Gr1^{dim}CD11b⁺ cells-injected group vs Gr1⁻CD11b⁻ cells-injected group), considering the time effect, used Bonferroni correction. A *P* value of less than .05 was considered statistically significant.

Results and discussion

Femoral artery dissection is a common model used for studying non-tumor angiogenesis.^{10,15} We assessed cells with a MDSC phenotype in a C57BL/6 hind-limb ischemic model. Infiltrated Gr1^{dim}CD11b⁺ cells within the ischemic hind limb were analyzed by flow cytometry (Figure 1). The percentage of infiltrated

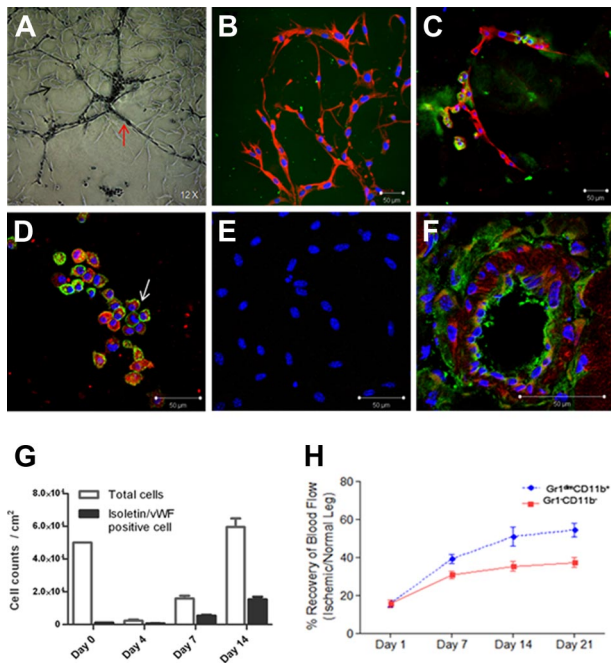


Figure 2. Cell culture and in vivo transplantation of muscle-derived Gr1^{dim}CD11b⁺ and Gr1⁻CD11b⁻ cells. (A) Images of cultured Gr1^{dim}CD11b⁺ cells 4 days after in Matrigel. (B-C) Immunofluorescence of cultured Gr1^{dim}CD11b⁺ cells with anti-rabbit GFAP antibody (red) and isolectin (green) 4 days after in Matrigel. (D) Immunofluorescence of cultured Gr1^{dim}CD11b⁺ cells with anti-rabbit von Willebrand Factor (VWF) antibody (arrow; red) and isolectin (green) 4 days after in Matrigel. (E) No isolectin and VWF expressions were observed in cultured Gr1⁻CD11b⁻ cells. (F) Red fluorescent signals indicate localization of transplanted C57BL/6 derived Gr1^{dim}CD11b⁺ cells (CD45.2). Ten days after cell injection, CD45.2 positive cells were present in the vascular wall of the vessels of Boy/J mice. (G) Growth of Gr1^{dim}CD11b⁺ cells in Matrigel culture. Total cells and isolectin/VWF double positive cells were counted at the indicated times. Quantitative analysis of hind-limb perfusion; (H) LDP index was significantly higher in the Gr1^{dim}CD11b⁺ cells (5×10^5 cells/mouse) injected group compared with the control group injected with Gr1⁻CD11b⁻ cells (5×10^5 cells/mouse) 3 weeks after cell injection. Data are mean \pm SEM; $P = .003$.

Gr1^{dim}CD11b⁺ cells (R2 fraction; Figure 1A) was markedly increased after surgery ($n = 5$ each; $3.08\% \pm 1.72\%$ before surgery vs $11.62\% \pm 5.86\%$ in ischemic muscle at day 4; $P < .05$). Isolated Gr1^{dim}CD11b⁺ cells increased in injured muscles at day 4 ($n = 5$ each; $0.10 \pm 0.06 \times 10^6/g$ tissue presurgery vs $1.13 \pm 0.32 \times 10^6/g$ tissue at day 4; $P < .05$), and dropped to noninjured muscle levels by day 10 ($0.42 \pm 0.33 \times 10^6/g$ tissue; Figure 1B). We reasoned that Gr1^{dim}CD11b⁺ cells might play a role in healing after ischemic injury. Infiltrated Gr1^{dim}CD11b⁺ cells were positive for myelocyte/monocyte (CD45, CD115 and Ly6C) and macrophage (F4/80) markers (Figure 1C). Expression of candidate chemokines implicated in directing monocyte migration were analyzed.¹⁶ MCP-1 and MIP-1 α mRNA expression was increased 85 and 121-fold in ischemic muscles 4 days after surgery with a lesser increase in SDF-1 (Figure 1D), suggesting that MCP-1 and MIP-1 α , and perhaps SDF-1 may be involved in ischemic tissue recruitment of Gr1^{dim}CD11b⁺ cells.

When new vessels are generated in retina, astrocyte-plexus is formed and endothelial cells proliferate.¹⁷⁻²⁰ Four days after Gr1^{dim}CD11b⁺ cell culture in Matrigel, astrocyte-plexus formed (black arrow; Figure 2A), followed by isolectin-positive endothelial cell formation (red arrow; Figure 2A, green; Figure 2C) as reported in retina. Astrocyte-plexus was positive for GFAP (red; Figure 2B), and NG-2 (data not shown), markers for astrocytes/

pericytes. Isolectin-positive cells (green; Figure 2D) also expressed VWF (arrow; red; Figure 2D) in their cytoplasm. Isolectin and VWF expression was not detected in cultures of control Gr1⁻CD11b⁻ cells (Figure 2E) isolated from the R3 fraction (Figure 1A). Most Gr1^{dim}CD11b⁺ cells died 4 days after culture in Matrigel and the remaining cells increased 30-fold 2 weeks after culturing (Figure 2G). Less than 3% of Gr1^{dim}CD11b⁺ cells were isolectin/VWF double positive cells on day 0. Isolectin/VWF double positive cells were slightly decreased by day 4, but increased after further culturing. It is possible that remaining cells after 4 days of culture might be endothelial precursor cells. As GFAP positive astrocyte-plexus, and isolectin/VWF-positive endothelial cells arose from Gr1^{dim}CD11b⁺ cells, we conclude that infiltrated Gr1^{dim}CD11b⁺ cells in ischemic muscles are still a heterogeneous cell population, with at least 1 subpopulation generating endothelial marker positive cells.

To evaluate whether muscle-derived Gr1^{dim}CD11b⁺ cells contribute to neovascularization, we injected ischemic muscle-derived Gr1^{dim}CD11b⁺ or control Gr1⁻CD11b⁻ cells into adductor muscles of C57BL/6 mice after femoral artery dissection. Laser Doppler perfusion (LDP) index of the Gr1^{dim}CD11b⁺ cell-injected group ($54.5\% \pm 3.6\%$ on day 21) was significantly higher than that of the Gr1⁻CD11b⁻ group ($37.4\% \pm 2.5\%$ on day 21; $p = .003$; $n = 9$ each; Figure 2H), demonstrating improved blood perfusion in the Gr1^{dim}CD11b⁺ cell-injected group. Without injection of cells, results were similar to the Gr1⁻CD11b⁻ cell group (data not shown).

To explore whether injected muscle-derived Gr1^{dim}CD11b⁺ cells directly incorporate into the vessel wall, C57BL/6 derived Gr1^{dim}CD11b⁺ cells (CD45.2) were injected into ischemic muscle of CD45.1 positive Boy/J mice after surgery. At day 10, CD45.2 positive cells were clearly present in the CD45.1 positive Boy/J vascular wall of ischemic tissues (Figure 2F). The percentage of chimeric vessels formed by injected CD45.2 Gr1^{dim}CD11b⁺ cells was $16.0\% (\pm 4.2\%)$, suggesting that Gr1^{dim}CD11b⁺ cells may directly participate in vessel formation. However, our results do not exclude a paracrine effect of injected Gr1^{dim}CD11b⁺ cells.

The results suggest that muscle-derived Gr1^{dim}CD11b⁺ cells play a positive role in ischemia-induced neovascularization, events with possible clinical implications.

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Authorship

Contribution: J.A.K., K.M., and H.E.B. designed experiments, analyzed results, and wrote and revised drafts of the manuscript; and H.-D.C., B.J., S.-J.P., T.C., and S.M.-C. performed experiments and offered important advice.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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