

Functional disruption of $\alpha 4$ integrin mobilizes bone marrow–derived endothelial progenitors and augments ischemic neovascularization

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The cell surface receptor $\alpha 4$ integrin plays a critical role in the homing, engraftment, and maintenance of hematopoietic progenitor cells (HPCs) in the bone marrow (BM). Down-regulation or functional blockade of $\alpha 4$ integrin or its ligand vascular cell adhesion molecule-1 mobilizes long-term HPCs. We investigated the role of $\alpha 4$ integrin in the mobilization and homing of BM endothelial progenitor cells (EPCs). EPCs with endothelial colony-forming activity in the BM are exclusively $\alpha 4$ integrin-expressing cells. In vivo, a single dose of anti- $\alpha 4$ integrin antibody resulted in increased circulating EPC counts for 3 d. In hindlimb ischemia and myocardial infarction, systemically administered anti- $\alpha 4$ integrin antibody increased recruitment and incorporation of BM EPCs in newly formed vasculature and improved functional blood flow recovery and tissue preservation. Interestingly, BM EPCs that had been preblocked with anti- $\alpha 4$ integrin ex vivo or collected from $\alpha 4$ integrin-deficient mice incorporated as well as control cells into the neovasculature in ischemic sites, suggesting that $\alpha 4$ integrin may be dispensable or play a redundant role in EPC homing to ischemic tissue. These data indicate that functional disruption of $\alpha 4$ integrin may represent a potential angiogenic therapy for ischemic disease by increasing the available circulating supply of EPCs.

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Abbreviations used: Ab, antibody; β -gal, β -galactosidase; BMMNC, bone marrow mononuclear cell; BS, *Bandeiraea simplicifolia*; circEPC, circulating endothelial progenitor cell; EC, endothelial cell; EPC, endothelial progenitor cell; FN, fibronectin; HLI, hindlimb ischemia; HPC, hematopoietic progenitor cell; ICAM, intercellular cell adhesion molecule; LAD, left anterior descending; LV, left ventricle; MI, myocardial infarction; PB, peripheral blood; VCAM, vascular cell adhesion molecule.

A compelling body of evidence indicates that endothelial progenitor cells (EPCs) of BM origin play a critical role in postnatal physiological and pathophysiological vasculogenesis (1, 2) and hold great potential to modulate the course of ischemic disease (3, 4) and tumor biology (5). In the BM, the adhesion interaction between stem/progenitor cells and the stromal microenvironment is essential in the homing, retention, and migration of hematopoietic stem cells and in hematopoiesis (6–10). Both $\alpha 4$ integrins, $\alpha 4\beta 1$ and $\alpha 4\beta 7$, have been shown to play key roles in these processes via interactions

with ligands expressed on the surface of endothelial and stromal cells or in the extracellular matrix (6, 11–15).

During mobilization of hematopoietic progenitor cells (HPCs), expression of $\alpha 4$ integrin is substantially down-regulated (16, 17). Early hematopoietic cytokines that induce the transition of quiescent primitive HPCs into the synthetic, or S phase, of the cell cycle have also been shown to markedly reduce $\alpha 4$ integrin expression and the binding of HPCs to the extracellular matrix (16, 18). The formation of a highly proteolytic microenvironment in the BM and the subsequent proteolytic cleavage of vascular cell adhesion molecule (VCAM)-1, a major ligand of $\alpha 4$ integrins, have been found

G. Qin and M. Ii contributed equally to this work.

The online version of this article contains supplemental material.

to be critical steps in growth factor- or chemokine-induced mobilization of BM stem cells (19). In addition, direct antibody blockade of either VCAM-1 or $\alpha 4$ integrin mobilizes long-term repopulating HPCs in rodents and primates (7, 20, 21). Moreover, conditional knockout of $\alpha 4$ integrin in mice leads to a redistribution of the hematopoietic stem cell pool between the BM and the peripheral blood, favoring movement to the peripheral blood (22, 23).

Although the role of $\alpha 4$ integrin in HPC homeostasis has been established, its role in the mobilization, tissue homing, and function of BM EPCs has not yet been defined. In the current study, we present evidence that functional blockade of $\alpha 4$ integrin significantly mobilizes EPCs from the BM into the peripheral circulation, augments functional neovascularization, and enhances tissue preservation after ischemic injury.

RESULTS

BM colony-forming EPCs express $\alpha 4$ integrin

We first performed flow cytometry analysis and found that the majority of isolated bone marrow mononuclear cells (BMMNCs) expressed $\alpha 4$ integrin (Fig. 1 A). To investigate whether BM $\alpha 4$ integrin-positive populations contain primitive EPCs in the steady-state, we used FACS to obtain equal numbers of $CD45^+ \alpha 4^+$ and $CD45^+ \alpha 4^-$ cells from the BMMNCs, then conducted a two-step EPC colony culture assay. The EPC colonies were identified by double staining for DiI-acLDL uptake and isolectin B4-FITC binding (Fig. 1 B, left). Primitive EPCs with colony-forming potential were exclusively $\alpha 4$ integrin-positive (Fig. 1 B, right). Moreover, flow cytometric analysis of BMMNCs using triple staining for $\alpha 4$ (or $CD45$) with two surrogate EPC markers, Sca-1 and Flk-1, demonstrated that only $\alpha 4^+$ or $CD45^+$ populations, not $\alpha 4^-$ or $CD45^-$ cells, contain Sca-1 and Flk-1 double-positive cells (Fig. 1, C and 1D). These results are in agreement with the EPC colony assay (Fig. 1 B) and further suggest a possible role of $\alpha 4$ integrin in EPC homeostasis in the BM.

Functional blockade of $\alpha 4$ integrin increases circulating EPCs

To investigate whether blockade of $\alpha 4$ integrin mobilizes BM EPCs to the peripheral blood (PB), we injected PS/2, a monoclonal $\alpha 4$ integrin-blocking antibody (Ab) i.v. into wild-type mice. After 24 h, peripheral blood mononuclear cells (PBMNCs) were isolated, and flow cytometry analysis was performed using the EPC markers Sca-1 and Flk-1. We found a substantial increase in circulating Sca-1⁺Flk-1⁺ double-positive cells in the Ab-treated mice compared with control IgG-treated mice (Fig. 2 A). The circulating EPCs (circEPCs) were also evaluated by EPC culture assay using isolated PBMNCs. The Ab treatment significantly increased the number of circEPCs, indicated by the increase in adherent cells double positive for DiI-acLDL uptake and isolectin B4 binding after culture (Fig. 2 B). In fact, we detected a higher level of circEPCs for up to 3 d after a single injection of the anti- $\alpha 4$ Ab in the time course study (Fig. 2 C).

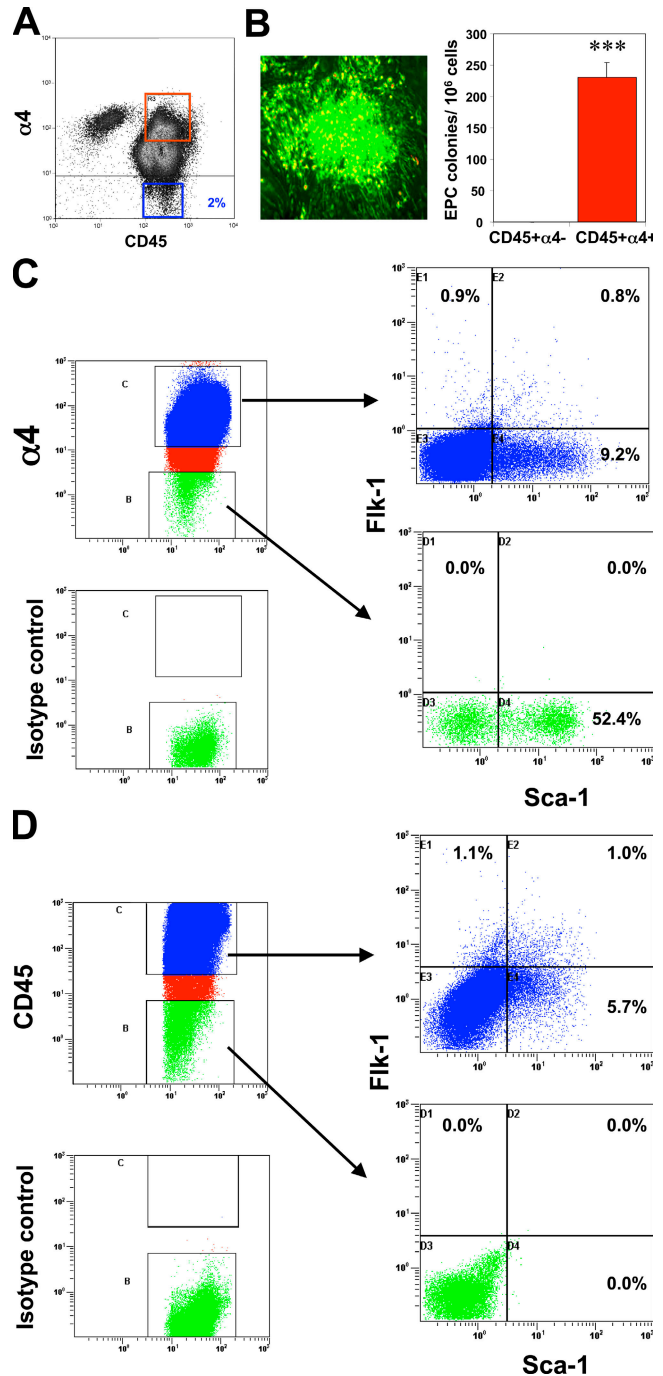


Figure 1. Colony-forming EPCs express $\alpha 4$ integrin in the BM.

(A) Flow cytometric analysis of $\alpha 4$ integrin expression in mouse BMMNCs. (B) Colony-forming EPCs were evaluated with a two-step endothelial cell differentiation culture starting with 5×10^6 $CD45^+ \alpha 4^+$ or $CD45^+ \alpha 4^-$ FACS-sorted BMMNCs (A, red and blue frame, respectively). The left panel shows a typical colony of EPCs double positive for DiI-acLDL uptake (red) and isolectin B4-FITC binding (green), appearing yellow on merged images. The right panel shows the counts of double-positive colonies grown from $CD45^+ \alpha 4^+$ and $CD45^+ \alpha 4^-$ BMMNCs, respectively ($n = 3$ per group; ***, $P < 0.001$). (C and D) Flow cytometric analysis of stem cell antigen-1 (Sca-1) and FMS-like kinase (Flk-1) expression in $\alpha 4^+$ vs. $\alpha 4^-$ BMMNC or in $CD45^+$ vs. $CD45^-$ BMMNC, respectively.

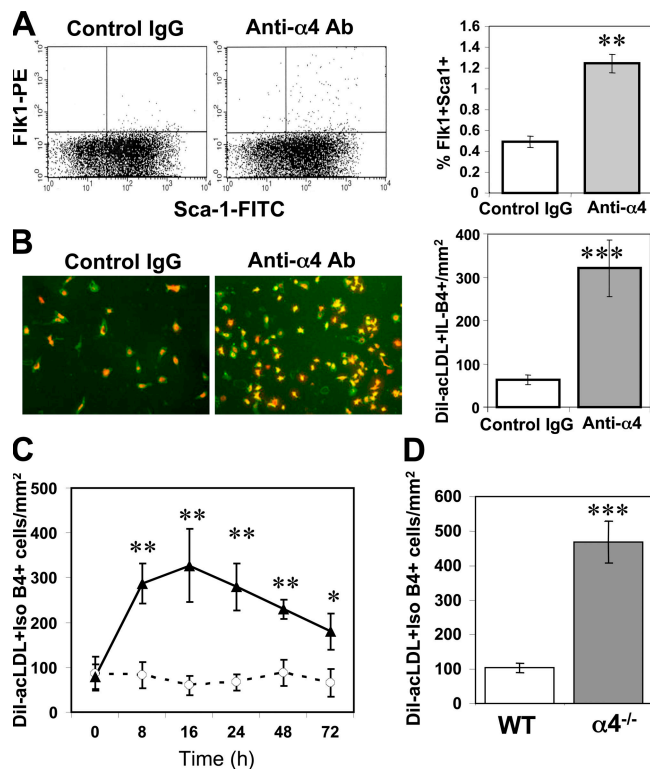


Figure 2. Single dose of anti- α 4 integrin Ab significantly increases circEPCs. (A) Flow cytometric analysis for the Sca-1⁺Flk-1⁺ cells in PBMNCs isolated at 24 h after i.v. injection of 200 μ g anti- α 4 integrin Ab ($n = 7$ per group; **, $P < 0.01$). (B) EPC culture assay using PBMNCs isolated at 24 h after i.v. injection of 200 μ g anti- α 4 integrin Ab. EPCs were cultured for 4 d then identified as adherent cells double positive for Dil-acLDL uptake (red) and isolectin B4-FITC binding (green) (left, original magnification, 200 \times) (right, ***, $P < 0.001$). (C) EPC culture assay using PBMNCs isolated at various time points after a single i.v. injection of 200 μ g anti- α 4 integrin Ab (O, control IgG; \blacktriangle , anti- α 4 integrin Ab; $n = 4$ per group at each time point; **, $P < 0.01$; *, $P < 0.05$). (D) EPC culture assay using PBMNCs from α 4 integrin conditional knockout mice or WT controls ($n = 4$; ***, $P < 0.001$).

We also performed the EPC culture assay using PBMNCs from conditional α 4 integrin knockout mice, those in which $\sim 97\%$ of the BMMNCs had lost α 4 integrin expression after induction of cre expression (22). The knockout mice demonstrated a significantly greater number of circEPCs compared with their WT littermates (Fig. 2 D). These data indicate that specific disruption of the α 4 integrin molecule increases the number of circEPCs.

Blockade of α 4 integrin dissociates adherent BM EPC from VCAM-1 or BM stroma ex vivo

Because VCAM-1 has been shown to be a major ligand of stem cell α 4 integrin in the BM, we investigated whether α 4 integrin-blocking Ab interferes with the adhesion interaction between α 4 integrin and VCAM-1, postulating that such interference could contribute to anti- α 4 integrin Ab-induced EPC mobilization. We applied freshly isolated BMMNCs to

immobilized recombinant VCAM-1 in cell culture plates. The anti- α 4 integrin Ab not only blocked BMMNC adhesion to VCAM-1 when added before the cells, but also competed with this adhesion in a dose-dependent manner when added after the cells (Fig. 3 A, top). Cells suspended after the addition of Ab were harvested and seeded for an EPC colony-forming assay. The number of EPC colonies grown from the suspended cells was proportionate to the total number of suspended cells (Fig. 3 A, bottom), suggesting that VCAM-1 supports α 4 integrin-mediated EPC attachment and that α 4 integrin-blocking Ab fosters EPC release. We also performed adhesion assays using another α 4 integrin ligand, fibronectin (FN), and another extracellular matrix molecule, intercellular cell adhesion molecule (ICAM)-1, in parallel with VCAM-1. FN and ICAM-1 conferred lower levels of BMMNC adhesion compared with VCAM-1 (Fig. 3 B). The anti- α 4 Ab, however, did not significantly compete or block adhesion of BMMNCs to FN or ICAM-1 (Fig. 3 B).

Because other α 4 integrin ligands in the BM in addition to VCAM-1 and fibronectin may be involved in α 4 integrin-dependent adhesion between EPCs and the stroma, we performed an additional adhesion assay using single-layer stromal cells grown from total mouse BM. Again, anti- α 4 integrin Ab significantly blocked and competed the adhesion of EPCs to the BM stroma in a dose-dependent manner (Fig. 3 C), suggesting that the Ab may release BM EPCs from α 4 integrin-mediated attachment in the BM.

In vivo blockade of α 4 integrin increases level of circEPCs in the setting of ischemia

Because tissue ischemia has been shown to induce EPC mobilization (24), we examined the effect of α 4 integrin blockade on the level of circEPCs after ischemia. We surgically induced hind limb ischemia (HLI) by excision of the left femoral artery in mice and randomized them to receive immediate anti- α 4 integrin Ab or control IgG twice per week for 3 wk. As shown in Fig. 4, anti- α 4 integrin Ab significantly increased the degree and the duration of HLI-induced EPC elevation in the peripheral circulation.

In vivo blockade of α 4 integrin augments BM EPC-mediated neovascularization after ischemia and improves recovery of functional blood flow

We used two mouse ischemia models, Tie2/LacZ BM transplantation plus hindlimb ischemia (Tie2/LacZ-BMT+HLI) and Tie2/GFP BM transplantation plus myocardial infarction (MI) (Tie2/GFP-BMT+MI), to investigate whether BM EPC mobilization induced by α 4 integrin blockade affects angiogenesis. In the Tie2/LacZ-BMT+HLI model, we reconstituted the BM of WT mice with BMMNCs genetically marked with Tie2/LacZ, which allows for easy detection of BM-derived cells with immunofluorescent staining for β -galactosidase (β -gal). After surgical induction of HLI, the mice received periodic injections with either anti- α 4 integrin Ab or control IgG. As shown in Fig. 5 A, the Ab-treated mice exhibited accelerated blood flow recovery compared

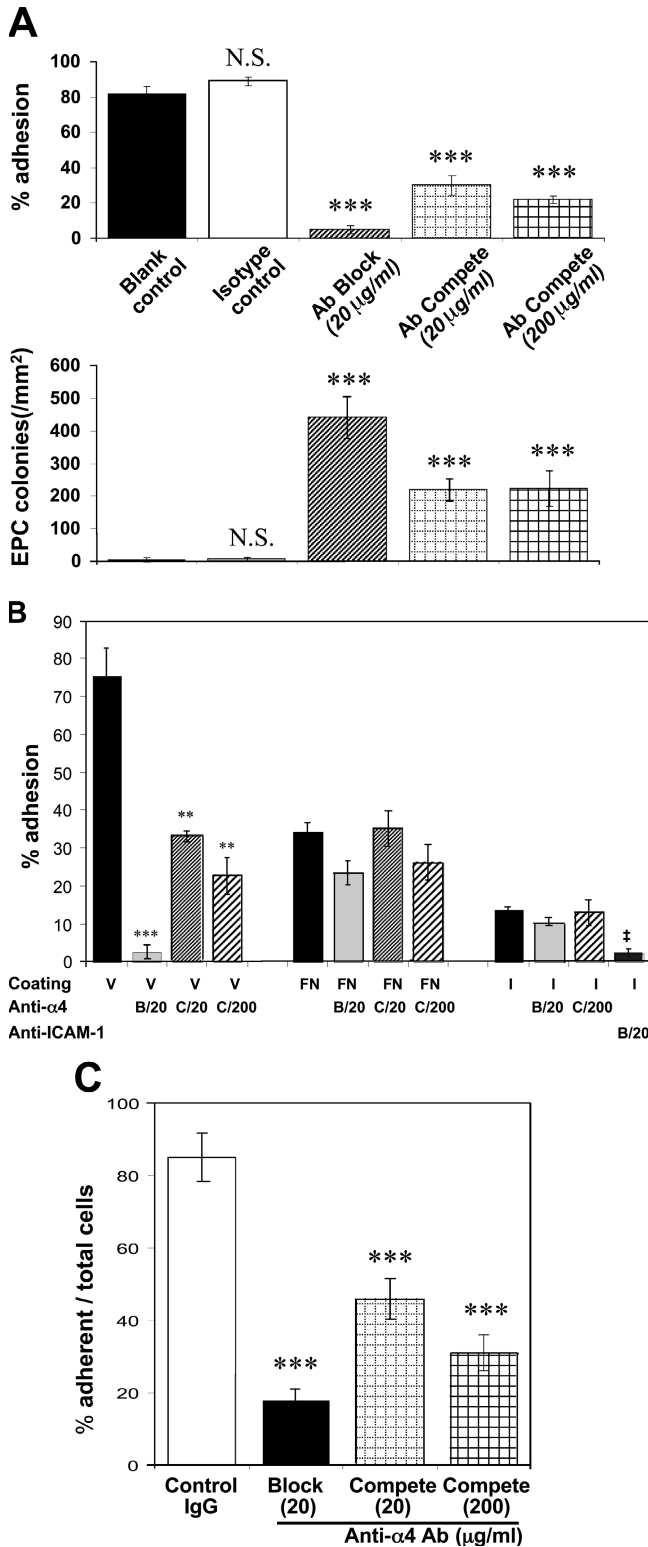


Figure 3. Anti- $\alpha 4$ integrin Ab blocks and competes for BM EPC adhesion to VCAM-1 and BM stroma. (A, top) Adhesion of freshly isolated mouse BMMNCs to the immobilized VCAM-1 was quantified with crystal violet microtiter plate. The Ab was added either before (Ab block) or 15 min after (Ab compete) the application of cells. The total incubation time of the Ab with cells was 30 min. (A, bottom) EPC colony assay using

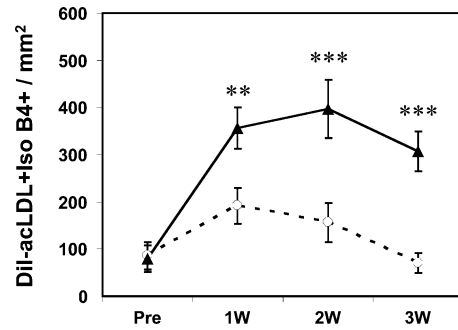


Figure 4. Anti- $\alpha 4$ integrin Ab augments EPC mobilization after ischemic injury. FVB/NJ mice received surgically induced left HLI and were immediately randomized to receive injections of $\alpha 4$ integrin-blocking Ab or control IgG twice per week. Five mice in each group were killed at each data point (presurgery, 1, 2, or 3 wk post-HLI surgery), and PBMNC EPC culture assays were performed. (○, control IgG; ▲, anti- $\alpha 4$ integrin Ab; **, $P < 0.01$; ***, $P < 0.001$).

with the IgG-treated animals, when assessed on days 7, 11, and 14. In addition, there was a significantly greater number of endothelial cells (ECs) of BM origin in the ischemic limb in the Ab-treated mice compared with the IgG-treated mice, when examined 14 d after induction of HLI (Fig. 5, B and C). The overall capillary density was also significantly higher in the Ab-treated mice (Fig. 5 C). Ab treatment conferred better long-term preservation of muscle tissue, as assessed by the ratio of muscle weight in ischemic limbs to normal limbs at 60 d after induction of HLI (Fig. 5 D). The endothelial identity of the ischemic tissues was further confirmed by immunofluorescent staining for another independent endothelial marker, CD31, along with β -gal (Fig. 5 E).

We further evaluated the EPC-mediated proangiogenic effect of $\alpha 4$ integrin blockade in the Tie2/GFP-BMT+MI model. MI was induced by permanent ligation at the middle of the left anterior descending (LAD) coronary artery. Again, Ab-treated mice exhibited a significantly greater number of BM-derived ECs in the infarcted heart, indicated by a greater number of cells staining positive for both Tie2-driven GFP and *Bandeiraea simplicifolia* (BS) lectin I-Rhodamine (Fig. 6, A and B). Interestingly, we also detected significantly more preexisting capillaries that survived

the suspension cells that resulted from each treatment in the top (**, $P < 0.001$ compared with blank control or isotype control; $n = 3$ per treatment). (B) Similar adhesion assay using different extracellular matrix or antibodies (V, VCAM-1; I, ICAM-1; FN, fibronectin; B/20 or B/200, block with 20 or 200 $\mu\text{g/ml}$ Ab, respectively; C/2, C/20, or C/200 compete with 2, 20, or 200 $\mu\text{g/ml}$, respectively; $n = 3$ per treatment; **, $P < 0.01$; ***, $P < 0.001$ compared with VCAM-1 coating without Ab group; †, $P < 0.01$ compared with ICAM-1 coating without Ab group. (C) Adhesion of isolated BMMNCs on ex vivo cultured monolayer BM stroma. Quantification was performed by counting the number of adherent cells per square unit and expressed as a percentage (***, $P < 0.001$ compared with isotype control).

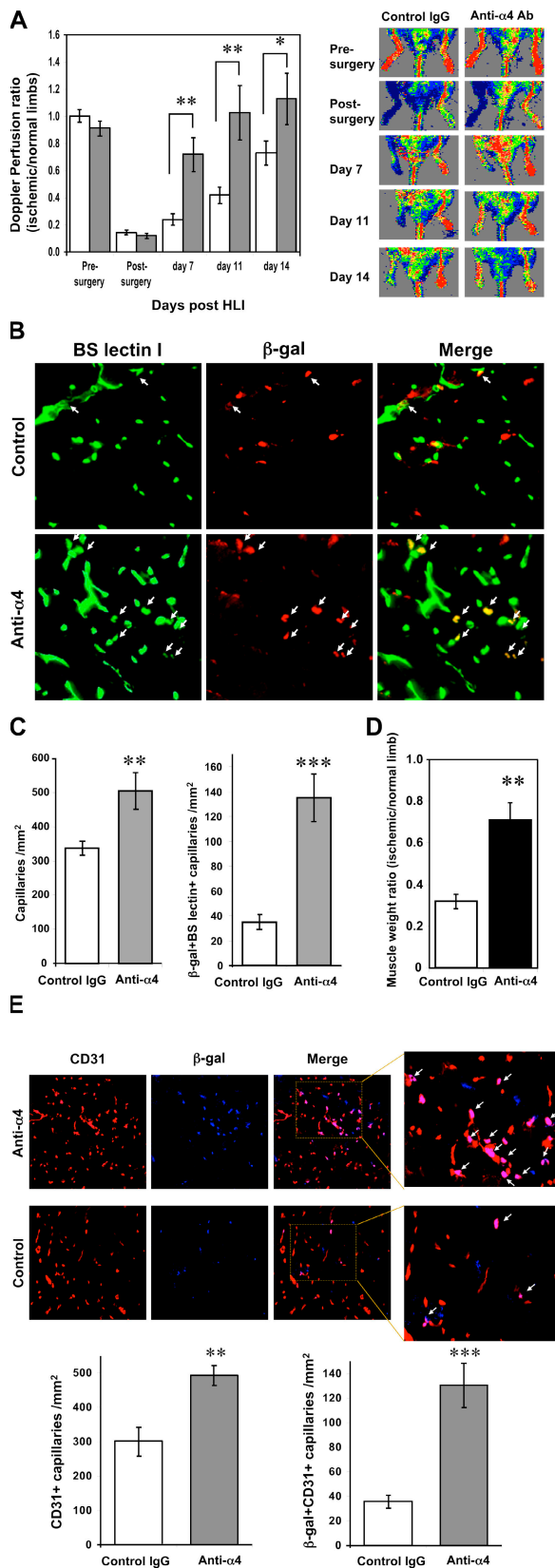


Figure 5. Tie2/LacZ-BMT+HLI mouse model. Tie2/LacZ BM-transplanted recipient FVB/NJ mice received surgically induced left HLI and

(BS lectin I–Rhodamine⁺GFP⁻) in the infarcted area (Fig. 6, A and C) and a significantly higher capillary density in the periinfarct area in the Ab-treated mice compared to controls (Fig. 6 D). $\alpha 4$ integrin blockade significantly reduced both the left ventricular fibrosis area (Fig. 6, E and F) and left ventricular dilation (Fig. 6, E and G) when examined 2 wk after infarction, suggesting a favorable effect of $\alpha 4$ integrin blockade on the remodeling of the infarcted murine heart.

Functional disruption of $\alpha 4$ integrin does not impair BM EPC homing or incorporation into neovasculature

$\alpha 4$ integrin may play an important role in leukocyte recruitment during tissue inflammation (25). The recruitment of EPCs to ischemic tissue is a key feature in EPC-mediated vasculogenesis, but the role of $\alpha 4$ integrin has not been clear. Therefore, the increased number of BM EPCs in the ischemic neovasculature after systemic blockade of $\alpha 4$ integrin was intriguing. To investigate whether $\alpha 4$ integrin plays a role specifically in EPC recruitment to ischemic tissue, we designed an in vivo EPC tissue homing assay. Isolated BMMNCs were pretreated ex vivo with either $\alpha 4$ integrin–blocking Ab or control IgG, labeled with DiI, and directly injected into the peripheral circulation of mice that had undergone surgical HLI and splenectomy without irradiation. This experimental design was used to minimize sequestering of EPCs (in the spleen) thereby providing the best opportunity to examine the impact of $\alpha 4$ blockade on tissue homing. Interestingly, BMMNCs pretreated with $\alpha 4$ integrin–blocking Ab were as well represented as BMMNCs pretreated with control IgG in the neocapillaries formed in the ischemic limb. We found

were randomized to receive injections of $\alpha 4$ integrin–blocking Ab or control IgG twice per week. On day 14, 10 mice from each group received i.v. injections of BS lectin I–FITC, which identifies vasculature, and were killed. (A) Laser Doppler Perfusion Image showing recovery of blood flow after surgery, expressed as the ratio of perfusion in ischemic limbs to normal limbs (left panel □ control IgG; ■ anti- $\alpha 4$ integrin Ab. **, $P < 0.01$; *, $P < 0.05$). On the right are representative Laser Doppler Perfusion Images at various time points. (B) Representative fluorescent microscope fields of capillaries (BS lectin I–FITC⁺, green) and BM-derived EPCs (anti- β -gal–Rhodamine staining⁺, red) at ischemic area (original magnification, 400 \times). Arrows indicate BS lectin 1 and β -gal double positive capillaries. (C) Overall capillary density (BS lectin I–FITC-positive only) (left, $n = 6$ limbs per group; **, $P < 0.01$) and BM EPC–derived capillary density (BS lectin I–FITC and β -gal–Rhodamine double positive) (right, $n = 6$ limbs per group; ***, $P < 0.001$) in the ischemic area. (D) The rest of the 10 mice in each group were killed on day 60. The wet muscular tissue of the lower limbs was isolated and weighed. Tissue preservation was expressed as the ratio of muscle weight in ischemic limbs to normal limbs (**, $P < 0.01$). (E) Immunofluorescent double staining for another endothelial marker, CD31 (red) and β -gal (blue) was performed on ischemic limbs at day 14 post-HLI (top) (original magnification, 200 \times). Arrows indicate CD31 and β -gal double positive capillaries. Quantification of CD31⁺ capillary density (bottom left, $n = 6$ limbs per group; **, $P < 0.01$) and CD31 + β -gal + double-positive BM EPC–derived capillary density (pink) (bottom, $n = 6$ limbs per group; ***, $P < 0.001$).

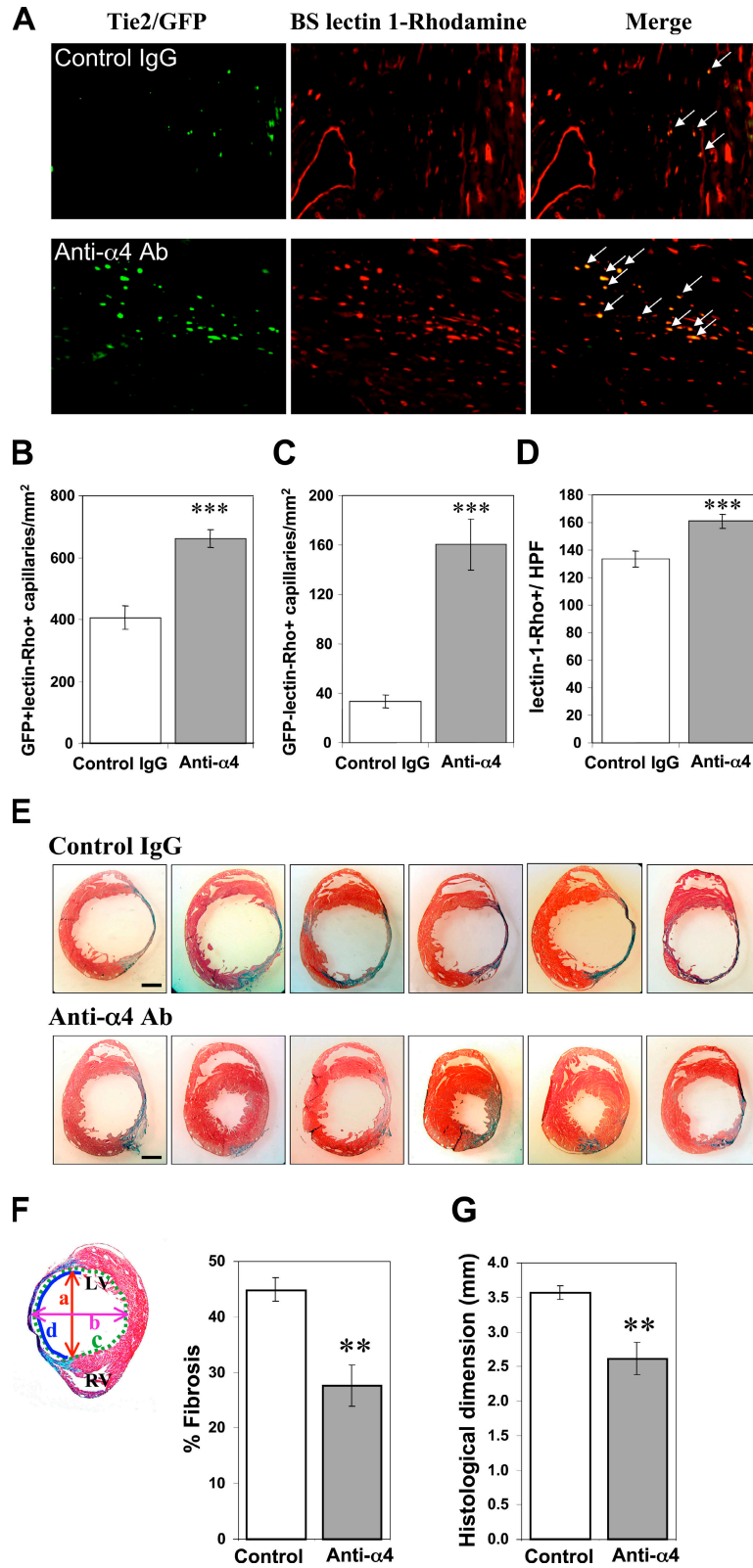


Figure 6. Tie2/GFP–BMT+MI mouse model. Myocardial infarction was induced by ligation of the LAD in Tie2/GFP BM-transplanted mice. Mice were then randomized to receive i.v. injections of anti- $\alpha 4$ integrin Ab or control IgG twice weekly ($n = 10$ per group). On day 14, the mice re-

ceived i.v. injections of BS lectin I–Rhodamine and were killed. The infarcted hearts were sectioned in a bread loaf fashion and pathohistological analysis was performed. Shown here are sections obtained at the level of 2 mm below the LAD ligation suture from each animal. (A) Representative

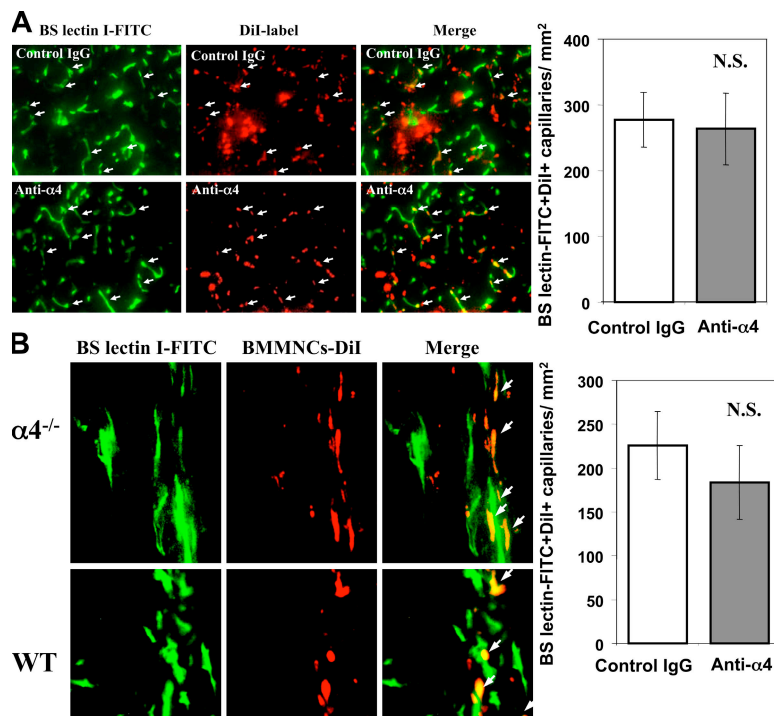


Figure 7. Functional disruption of $\alpha 4$ integrin does not alter EPC tissue homing properties. (A) Equal numbers (15×10^6) of BMMNCs were pretreated with either anti- $\alpha 4$ integrin Ab or control IgG, labeled with DiI, and injected into the peripheral circulation of mice with surgically induced HLI and splenectomy. On day 7, the mice were injected with BS lectin I-FITC and killed. Pathological analysis was conducted on the ischemic limb tissues. Capillaries in which ECs derived from injected BM EPCs were incorporated are BS lectin I-FITC and DiI double positive (top, the representative fluorescent microscopy; original magnification, $200\times$) (bottom, quantification of the densities of double-positive capil-

laries; $n = 6$ per group). (B) Background-matched, splenectomized WT recipient mice that had MI induced by LAD ligation received i.v. injections of 10^6 BMMNCs from either $\alpha 4$ integrin conditional knockout mice or WT littermates. On day 7, the recipients were injected with BS lectin I-FITC and killed. The ischemic cardiac tissues were sectioned. Neovascular tissue containing ECs derived from the injected BM EPCs appear BS lectin I-FITC and DiI double positive (top, representative fluorescent microscopy; original magnification $400\times$) (bottom, quantification of the densities of double-positive capillaries; $n = 6$ per group). Arrows indicate double positive capillaries in both panels.

similar numbers of DiI-labeled and control EPCs incorporated in the neovasculature (Fig. 7 A) and equal numbers of labeled cells from the two groups circulating in the PB. Because an equal number of BMMNCs were injected into the two groups of mice, these data indicate that the ex vivo $\alpha 4$ integrin blockade did not affect EPCs homing or incorporation into neovasculature, and further support the importance of the mobilization

effect of $\alpha 4$ integrin blockade on changes in ischemic tissue repair. Similar results were also obtained in mice without splenectomy.

To further confirm this observation and to overcome certain limitations of Ab blockade, such as unanticipated effects in nontarget tissues, BMMNCs were isolated from $\alpha 4$ integrin conditional knockout mice. WT litter mates served as controls. We injected these BMMNCs into background-matched WT mice that had undergone surgical MI and splenectomy. Again, loss of $\alpha 4$ integrin did not impair incorporation of the injected BM EPCs into the neovasculature of infarcted cardiac tissue (Fig. 7 B), consistent with the results obtained from the in vivo homing assay using $\alpha 4$ integrin-blocking Ab. These results confirm that loss of $\alpha 4$ integrin does not impair EPC homing to ischemic tissue.

DISCUSSION

In this study, we demonstrated that blockade of $\alpha 4$ integrin promotes mobilization of BM EPCs to the peripheral circulation and promotes functional neovascularization after ischemia. Several lines of evidence support this conclusion. First, primitive, colony-forming EPCs in isolated BMMNCs are

fluorescent microscopy of the infarct areas (original magnification, $200\times$; BM-derived ECs, double positive for GFP [green] and BS lectin I-Rhodamine [red; appear yellow in the merged pictures and are indicated with arrows]). (B) Density of the capillaries with incorporated BM-derived ECs in the ischemic areas ($n = 6$ per group; ***, $P < 0.001$). (C) Preexisting survival capillary density in the infarct areas (BS lectin I-Rhodamine⁺ only) ($n = 6$ per group; ***, $P < 0.001$). (D) Capillary density in the periinfarct area ($n = 6$ per group; ***, $P < 0.001$). (E) Representative Masson's Trichrome staining of hearts after MI. The blue color represents fibrosis or scar which appears reduced in the hearts from anti- $\alpha 4$ Ab treatment group. Bar, 1 mm. (F) Quantification of area of fibrosis ($d/c \times 100\%$) confirms a reduction in LV fibrosis after MI in anti- $\alpha 4$ Ab-treated animals ($n = 12$ per group; **, $P < 0.01$). (G) Histological dimensions ($(a+b)/2$) ($n = 12$ per group; **, $P < 0.01$).

exclusively $\alpha 4$ integrin-expressing cells. Second, anti- $\alpha 4$ integrin Ab blocks and competes with the adhesive interaction between BM EPCs and immobilized VCAM-1 or BM stroma *ex vivo*. Third, systemic administration of anti- $\alpha 4$ integrin Ab or conditional knockout of $\alpha 4$ integrin in the BM significantly increases circEPCs. Fourth, after ischemic injury, anti- $\alpha 4$ integrin Ab fosters homing of BM-derived EPCs to the neovasculature at ischemic tissue and augments recovery of blood flow and tissue preservation. Our study establishes for the first time that $\alpha 4$ integrin plays an important role in EPC mobilization and that functional disruption of $\alpha 4$ integrin-mediated EPC lodgment in the BM causes a shift toward a distribution of EPCs that is more favorable for neovascularization.

Anti- $\alpha 4$ integrin blocking Ab has previously been shown to mobilize BM HPCs (26). These cells, in turn, have been shown to contribute to neovascularization at ischemic sites by secreting a spectrum of growth factors and supporting the establishment of EPCs (2, 27). Because Tie2 expression has also been found in a subset of HPCs (28, 29), it is possible that the potent proangiogenic effect of anti- $\alpha 4$ integrin Ab treatment observed in our study may have resulted from the combined mobilization of circEPCs and HPCs.

Granulocytes, macrophages, and lymphocytes have been shown to secrete various pro- and antiangiogenic factors in ischemic tissue and play complex roles in recovery after ischemia (30, 31). It has also been shown that $\alpha 4$ integrin plays a role in cytokine-induced leukocyte-endothelium interactions (32–34) and that blockade of $\alpha 4$ integrin inhibits inflammatory cell recruitment (25, 35–37). Consistent with these prior observations, we noted a decrease in F4/80-positive cells in the ischemic limbs of the Ab-treated mice (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20050459/DC1>). Although the mechanism of EPC migration to ischemic tissue has been under intensive investigation, the role $\alpha 4$ integrin plays in this process is not yet elucidated. Some evidence suggests that $\alpha 4$ integrin may be unimportant for stem/progenitor cell tissue homing and migration, because $\alpha 4$ integrin levels are substantially down-regulated on mobilized stem cells in PB (16, 17, 38), whereas activated, migrating inflammatory cells express abundant $\alpha 4$ integrin (39). It has recently been shown that circEPCs isolated from human PB express low levels of $\alpha 4$ integrin (40) and that neutralizing Ab to VLA-4 significantly inhibits adherence of BM CD34⁺, but not mobilized PB CD34⁺ stem cells, to stromal cells, suggesting the existence of alternative cell adhesion molecules that mediate circulating stem cell binding (41). In addition, it has been reported that $\alpha 4$ integrin is necessary for the homing and lodgment of stem/progenitor cells to BM, but not to spleen (7, 22, 42). However, $\alpha 4$ integrin may play a role in soluble VCAM1-induced migration and angiogenesis in HUVECs (43), as recently reported.

In the current study, we found that neither *ex vivo* blockade nor genetic knockout of $\alpha 4$ integrin prevents EPC homing to ischemic tissue, suggesting that adhesion activity of

$\alpha 4$ integrin is not essential for homing of circEPCs to ischemic tissue in the setting of acute ischemia. Coincidentally, it has recently been shown that $\beta 2$ integrin may play a more prominent role in the homing of EPCs to ischemic skeletal muscle (44).

Despite the fact that several other $\beta 1$ integrins are known to be essential to various angiogenesis processes, the direct role of $\alpha 4\beta 1$ integrin in angiogenesis and vasculogenesis remains largely obscure (45–47). Limited studies in experimental models suggest that $\alpha 4$ integrin may play a role in angiogenesis induced by TNF- α and soluble VCAM-1 but not by basic fibroblast growth factor (43, 48). Studies currently underway in our lab suggest that TNF- α signaling is indeed required for ischemic angiogenesis (49). Unfortunately, with the exception of TNF- α , the stimuli for angiogenesis during tissue ischemia are not well understood at this time. Nevertheless, the effect of anti- $\alpha 4$ integrin treatment on local angiogenesis warrants further investigation.

A VLA-4-dependent mechanism has previously been shown to play an important role in mononuclear leukocyte emigration during early atherosclerosis (50), neointimal formation after vessel injury (51), and neutrophil-mediated cardiac myocyte dysfunction (52). Blockade of $\alpha 4$ integrin has been shown to attenuate atherosclerosis (53) and reduce postinjury intimal hyperplasia (54, 55) and neoadventitial formation (56) in animals. Our study indicates that $\alpha 4$ integrin blockade enhances the mobilization of EPCs and EPC-mediated neovascularization. These data suggest a novel therapeutic strategy for stimulating therapeutic neovascularization in acute and chronic ischemia. Moreover, the rapidity and durability of EPC mobilization induced by a single dose of anti- $\alpha 4$ Ab compare favorably with currently available agents such as G-CSF (57) and may make this approach a practical addition to the therapeutic armamentarium.

MATERIALS AND METHODS

Antibodies. The antimurine $\alpha 4$ integrin mAb PS/2 was purified from cultured hybridoma cells (American Type Culture Collection) using Montage Antibody Purification kits. The antimurine ICAM-1 blocking Ab was purchased from R & D Systems. All other Abs and isotype controls were purchased from BD Biosciences. A second mAb against a different recognition site of $\alpha 4$ integrin was used to confirm the specificity of the purified PS/2 Ab with flow cytometry analysis. The isotype control IgG of PS/2, rat IgG2b, was dialyzed to remove sodium azide when used *in vivo*.

Animals. Male FVB/NJ and background-matched Tie2/LacZ or Tie2/GFP transgenic mice were purchased from the Jackson Laboratories. The conditional $\alpha 4$ integrin knockout mice (*Mx.cre⁺ $\alpha 4^{flox/flox}$*) and control littermates (*$\alpha 4^{flox/flox}$*) were generated as described previously (22). The mice were maintained and operated following protocols proved by the Caritas St. Elizabeth's Institutional Animal Care and Use Committee.

Flow cytometry analysis and FACS sorting. Mouse BMMNCs or PBMNCs were isolated with density-gradient centrifugation (58). Flow cytometry analysis and FACS sorting of the isolated BMMNCs or PBMNCs were performed as previously described (58).

BMMNC EPC colony assay and PB circEPC culture assay. Taking advantage of the late growth and high proliferative properties of primitive EPCs, we developed a two-step EPC colony assay. The isolated mouse BMMNCs were cultured in 0.1% vitronectin/gelatin-coated plates in EBM-2 complete medium (EBM-2 basal medium supplemented with the cytokine cocktail in endothelial growth medium-2-microvascular; SingleQuots, Clontech, Inc.) (59). To evaluate BM colony-forming EPCs, 5×10^6 FACS-sorted CD45⁺α4⁻ or CD45⁺α4⁺ cells were cultured in a 6-well plate for 4 d and then resplit into a 10-cm plate, cultured for another 7 d. DiI-acLDL (1:500) was added, and the cells were incubated for 4 h. The cells were then washed with PBS, fixed in 1% PFA, and stained with isolectin B4-FITC (1:200). Cell colonies double positive for DiI-acLDL uptake and isolectin B4-FITC binding were counted. The numbers of EPC colonies reflected the number of primitive EPCs in the initial sorted cell fractions.

To count circEPCs, an EPC culture assay was performed as previously described (59). In brief, the PBMNCs isolated from a 500-μl sample of PB were cultured in vitronectin-coated 4-well chamber slides in EBM-2 complete media. On day 4 of the culture, DiI-labeled acLDL was added to the media. After incubating 4 h, the cells were fixed in 1% paraformaldehyde and counterstained with isolectin B4-FITC. Double-positive cells were counted as EPCs, the number of which reflected the number of initial circEPCs in the 500-μl sample.

Adhesion assay. 96-well tissue culture plates were coated with 10 μg/ml recombinant murine VCAM-1 or ICAM-1 (R & D Systems), or 50 μg/ml rat plasma fibronectin (Sigma-Aldrich). Freshly isolated BMMNCs (5×10^5) were added to each well. Antibodies were added either just before addition of the BMMNCs to block adhesion, or 15 min after the addition of cells to compete with adhesion. The cells and antibodies were coincubated in a 5% CO₂ incubator at 37°C for 30 min. After incubation, nonadherent and loosely attached cells were removed by tapping each plate and gently washing the wells three times with Dulbecco's phosphate-buffered saline. Cells in the group with 100% attachment were not washed. Attached cells were fixed in 5% glutaraldehyde, stained with 0.1% crystal violet, and solubilized in 10% acetic acid. A microplate reader was used to measure the absorbance at 564 nm. The background crystal violet staining level was subtracted from readings, and the values were expressed as the percentage of attachment. To examine the effect of α4 integrin-blocking Ab on BMMNC adhesion to BM stromal cells, a single layer of BM stroma was prepared as previously described (60). Adherent cells were counted during microscopic examination, and the result expressed as the ratio of the number of adherent cells in each experimental group to the number in the 100% attachment group.

Mouse Tie2/LacZ-BMT+HLI model. This procedure was performed as previously described (61–63). See supplemental Materials and methods, available at <http://www.jem.org/cgi/content/full/jem.20050459/DC1>, for details.

Mouse Tie2/GFP-BMT+MI model. BM transplantation and quantification of engraftment were performed (see supplemental Materials and methods) using Tie2/GFP mice as donors. Myocardial infarction was induced in recipient mice under artificial ventilation by permanent ligation of the middle of the left anterior descending (LAD) coronary artery. Mice were randomized to receive i.v. injection of either 200 μg of anti-α4 integrin Ab or control IgG, twice weekly starting on day 1. On day 14, the mice were injected with 50 μl of BS lectin I-Rhodamine (Vector Laboratories) at the apex of the left ventricle (LV), and after 5 min the cardiac vasculature was perfused with 4% PFA through the right carotid artery with distal aortic arch clamped. Cardiac tissue was fixed for 1 h in 4% PFA, incubated in 30% sucrose solution overnight, snap frozen in liquid nitrogen, and preserved at -80°C. Serial cryosectioning was performed starting at 1 mm below the suture (used to ligate the LAD) moving toward the apex, with three consecutive sections per 1 mm to allow for quantitative pathohistological analysis at each level (see next paragraph). Three sections per ischemic heart and 9 fields per section (6 fields in the infarct border zone, 3 fields in the infarct area) were examined with BS lectin I-Rhodamine⁺ to quantify total capillary

density or with Rhodamine⁺GFP⁺ to determine BM EPC-derived capillary density. Masson's Trichrome staining was performed as previously described (64). The fibrosis area was calculated as the ratio of the length of fibrotic area to the length of LV inner circumference (Fig. 5 E, d/c), and the LV dimension was quantified histologically (Fig. 5 E, (a+b)/2). All surgical procedures and patho/histological analysis was performed by investigators blinded to treatment assignment.

In vivo BM EPC homing to ischemic tissue. BMMNCs were isolated from donor WT mice. Equal numbers of the cells were either blocked with anti-α4 integrin Ab or treated with control IgG. Cells were then labeled with DiI cell tracer and washed thoroughly. Recipient mice underwent excision of the left femoral artery to induce HLI and underwent splenectomy without irradiation. Mice were then randomized to immediately receive by tail vein injection either 15×10^6 BMMNCs that had been treated with anti-α4 integrin Ab or 15×10^6 cells that had been treated with control IgG. The circulating DiI-labeled cells in the PB were monitored on days 1, 3, and 7 by flow cytometry or fluorescent analysis of PBMNCs. No difference was found between the two groups of recipient mice (unpublished data). On day 7, the mice were injected with BS lectin I-FITC and killed. The capillaries derived from injected BMMNCs, which were double positive for BS lectin I-FITC and DiI, were examined microscopically and quantified. In another similar independent experiment, BMMNCs isolated from α4 integrin conditional knockout mice (~97% of cells deficient for α4 integrin) or control WT littermates were used. 10×10^6 cells were injected i.v. into background-matched recipient mice that had undergone surgical MI and splenectomy. On day 7, the mice were injected with BS lectin I-FITC and killed. The ischemic cardiac tissue was processed as described before, and the neocapillaries derived from the injected BM EPCs in the ischemic area were quantified.

Statistics. Data are presented as average ± SEM. Comparison between two means was performed with an unpaired Student's *t* test. Comparisons of more than two means were performed using ANOVA with Fisher PLSD and Bonferroni Dunn Post Hoc analysis. Statistical significance was assigned if *P* < 0.05.

Online supplemental material. Supplemental materials and methods describe mouse Tie2/LacZ-BMT and the hindlimb ischemia model. Fig. S1 depicts hindlimb mouse tissue injected with control IgG and anti-α4 Ab. Online supplemental material is available at <http://jem.org/cgi/content/full/jem.20050459/DC1>.

The authors gratefully acknowledge the assistance of Mickey Neely in the preparation of this manuscript. This work was supported by National Institutes of Health grants (HL-53354, HL57516, HL-63414, HL-80137, and HL-66957 to D.W. Losordo) and American Heart Association grant (0430135N to G. Qin).

The authors have no conflicting financial interests.

Submitted: 2 March 2005

Accepted: 8 December 2005

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