Recombinant Human Interleukin-11 Treatment Enhances Collateral Vessel Growth After Femoral Artery Ligation

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Objective—To investigate the role of recombinant human interleukin-11 (rhIL-11) on in vivo mobilization of $CD34^+$/vascular endothelial growth factor receptor (VEGFR) 2^+ mononuclear cells and collateral vessel remodeling in a mouse model of hindlimb ischemia.

Methods and Results—We observed that treatment of Sv129 mice with continuous infusion of 200-μg/kg rhIL-11 per day led to in vivo mobilization of $CD34^+$/$VEGFR2^+$ cells that peaked at 72 hours. Sv129 mice pretreated with rhIL-11 for 72 hours before femoral artery ligation showed a 3-fold increase in plantar vessel perfusion, leading to faster blood flow recovery; and a 20-fold increase in circulating $CD34^+$/$VEGFR2^+$ cells after 8 days of rhIL-11 treatment. Histologically, experimental mice had a 3-fold increase in collateral vessel luminal diameter after 21 days of rhIL-11 treatment and a 4.4-fold influx of perivascular $CD34^+$/$VEGFR2^+$ cells after 8 days of therapy. Functionally, rhIL-11–treated mice showed better hindlimb appearance and use scores when compared with syngeneic mice treated with PBS under the same experimental conditions.

Conclusion—These novel findings show that rhIL-11 promotes in vivo mobilization of $CD34^+$/$VEGFR2^+$ mononuclear cells, enhances collateral vessel growth, and increases recovery of perfusion after femoral artery ligation. Thus, rhIL-11 has a promising role for development as an adjunctive treatment of patients with peripheral vascular disease. **(*Arterioscler Thromb Vasc Biol*. 2011;31:306-312.)**

Key Words: blood flow ■ ischemia ■ peripheral arterial disease ■ peripheral vasculature ■ reperfusion

Peripheral vascular disease (PVD) is a major cause of morbidity and significant health care cost in the United States.[1] It is estimated that >8 million Americans have PVD,[2] and its prevalence is expected to increase concurrently with the increase of the aging population. The high rate of morbidity associated with this disease has created a need for identification of novel therapies for adjunctive treatment of patients with PVD.

Collateral vessels are preexisting arteriolar connections that may not be used to provide perfusion under normal conditions but can be recruited to bypass the site of acute or chronic vessel occlusion. Collateral vessel growth (arteriogenesis) provides a natural adaptive mechanism to lessen tissue injury caused by PVD. However, this process is insufficient in many patients, and therapies to augment it are needed. Several investigators have shown that intramuscular injection of ex vivo–processed autologous mononuclear cells leads to short-term symptomatic improvement in patients with PVD.[3] Others[4] have reported a correlation between patient outcomes and the number of intramuscularly injected $CD34^+$ cells within the patient's mononuclear cell population. Although these studies have demonstrated favorable safety and feasibility of cell-based therapy for the treatment of patients with PVD, the need for ex vivo processing of mononuclear cells before delivery is a major impediment to the performance of a large-scale study in these patient populations. This creates a need for a novel method of rapid in vivo mobilization of mononuclear cells for the treatment of patients with PVD.

Human blood outgrowth cells (HBOCs) are cultured $CD34^+$/vascular endothelial growth factor receptor (VEGFR) 2^+ mononuclear cells from healthy human subjects. These late-outgrowth cells have a slow initial rate of growth, and they exhibit tremendous proliferative capacity after several weeks in culture,[5] thus serving as useful tools for screening the biological features of vasculoprotective mononuclear cells in in vitro assays. It was recently reported that a 12-mer peptide ligand that binds with high affinity to HBOCs has

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sequence homology to human interleukin-11 (IL-11).[6] This finding, coupled with reports of high expression of IL-11 receptor α (IL-11Rα) in highly vascular tissues,[7] suggests a potential role for IL-11 as a putative ligand for in vivo mobilization of mononuclear cells and vascular remodeling.

IL-11 is a member of the IL-6 cytokine family. IL-11 is produced by a variety of tissues and has pleiotropic effects on multiple tissues, including promotion of human and murine megakaryocytopoiesis and thrombopoiesis,[8,9] protection of endothelial cells from alloinjury by upregulation of survivin,[10,11] and protection against endothelial cell death.[12] Fortuitously, recombinant human IL-11 (rhIL-11) has already been used extensively in patients.[13,14] Because the clinical efficacy and safety parameters of rhIL-11 have been well characterized in humans, we performed a preclinical study to investigate the role of rhIL-11 as a potential pharmacological agent for in vivo mobilization of $CD34^+/VEGFR2^+$ mononuclear cells and collateral vessel remodeling using a mouse model of hindlimb ischemia. We chose $CD34^+/VEGFR2^+$ cells because these markers are present in humans and mice, have been used to identify mouse progenitor cells that enhance neovascularization during hindlimb ischemia,[15,16] and will allow for correlation of $CD34^+/VEGFR2^+$ cells in mice and future human studies.

Methods

The supplemental material (available online at http://atvb.ahajournals.org) provides methods not described herein.

Unilateral Hindlimb Ischemia

Unilateral femoral artery ligation was performed using 10-week-old Sv129 mice, as previously described.[17] Briefly, mice were anesthetized with 1.25% isoflurane/O_2 during hindlimb depilation (1 day before surgery) and during hindlimb ischemia surgery. The right femoral artery was exposed through a 2-mm incision and ligated with two 7-0 sutures placed proximal to the origin of the lateral caudal femoral artery. The artery was transected and separated. The wound was irrigated with sterile saline and closed; and cefazolin (50 mg/kg IM), topical furazolidone, and pentazocine (10 mg/kg IM) were administered. The University of North Carolina (Chapel Hill) Institutional Animal Care and Use Committee approved all of our animal procedures.

Laser Doppler Perfusion Imaging

Laser Doppler perfusion imaging was performed as previously described.[17] Briefly, mice were anesthetized with 1.25% isoflurane/O_2 and their body temperature was strictly maintained at 37°C, ±0.5°C, during the entire procedure. Laser Doppler perfusion imaging of plantar foot or adductor thigh of both legs was performed before, immediately after, and at 2, 4, 6, 8, and 14 days after femoral ligation. Images were analyzed using computer software (MoorLDIV5.0). Regions of interest were drawn with respect to anatomic landmarks, and flow rate was calculated. Perfusion in the ligated leg was normalized to the unligated leg. Foot appearance was scored as an index of ischemia: 0, normal; 1 to 5, cyanosis or loss of nail(s) (the score is dependent on the number of nails affected); 6 to 10, partial or complete atrophy of digit(s) (the score reflects number of digits affected); and 11, partial atrophy of the forefoot. Hindlimb use was scored as an index of muscle function: 0, normal; 1, no toe flexion; 2, no plantar flexion; and 3, dragging foot.

All data are reported as mean±SE. Differences were subjected to unpaired t tests (2-tailed). $P<0.05$ was considered significant.

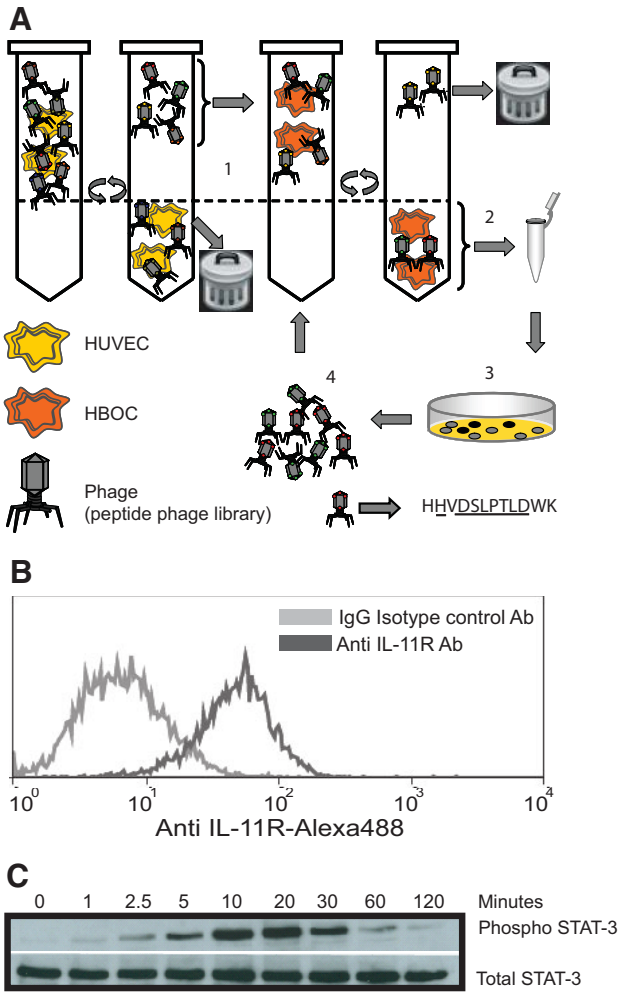

Figure 1. HBOCs abundantly express IL-11Rα. A, Schematic representation of the screening procedure used to identify peptide ligands that selectively bind to HBOCs. A 12-mer random peptide phage display library was screened using a 2-step selection protocol. First, the library was depleted of ligands binding to common receptors by incubation with a nontarget cell line (human umbilical vein endothelial cells). Second, the unbound phage pool was biopanned on HBOCs. Phages that bound to HBOCs were amplified in *Escherichia coli* to the original input titer of the library and used for subsequent rounds of biopanning (steps 1–4). After 3 rounds of biopanning, HBOC-bound phages were plaque purified for sequence analysis.[6] B, HBOCs were incubated with an anti–human IL-11R antibody–Alexa488 or mouse IgG isotype control antibody and analyzed by flow cytometry. HBOCs abundantly express IL-11Rα. C, HBOCs were treated with 25-ng/mL rhIL-11 for 1, 2.5, 5, 10, 20, 30, 60, and 120 minutes. Cell lysates were analyzed by Western blotting with anti–phosphorylated STAT-3 (tyrosine 705) antibody and anti–STAT (79D7) antibody. rhIL-11 induced STAT-3 phosphorylation in HBOCs at the indicated points.

Results

HBOCs Express IL-11Rα and Administration of rhIL-11 Activates Downstream STAT-3

We[6] recently identified peptide ligands that bind specifically to HBOCs by screening phage display libraries to identify novel surface markers of vasculoprotective mononuclear cells (Figure 1A, cartoon). One such 12-mer peptide ligand has sequence homology with human IL-11. This observation, coupled with reports of high expression of IL-11Rα in highly

vascular tissues,[7] prompted us to investigate the potential significance of the IL-11/HBOC interaction on vascular remodeling. As a first step, we confirmed the phenotype of HBOCs by multiparametric investigation consisting of identification of their characteristic cobblestone morphological features,[5] acetylated low-density lipoprotein uptake,[18] and expression of cell surface markers (eg, VEGFR2, CD34, and CD31). These characteristics, along with the lack of expression of CD45 (data not shown),[19] suggest that these HBOCs have properties of dedifferentiated mononuclear cells.[20] To determine whether HBOCs express IL-11Rα, we incubated HBOCs with anti–IL-11Rα antibody and found that HBOCs robustly express IL-11Rα on their surface (Figure 1B). To determine whether signaling via the IL-11Rα/rhIL-11 (receptor/ligand) axis was intact, we stimulated HBOCs with rhIL-11 and found that rhIL-11–treated cells displayed time-dependent phosphorylation of signal transducer and activator of transcription-3 (STAT-3) (Figure 1C), a downstream effector molecule that supports cell survival by upregulation of antiapoptotic proteins (eg, survivin)[10] and upregulation of proangiogenic factors (eg, VEGF).[21] These data demonstrate that HBOCs express a functional IL-11Rα and suggest a link between IL-11/HBOC interaction and IL-11Rα signaling.

rhIL-11 Stimulates Directed Migration and Tubule Formation in HBOCs

To study the physiological effects of rhIL-11 on HBOCs, we administered rhIL-11 to HBOCs in a Boyden chamber. rhIL-11 administration at 25 ng/mL led to optimal cell migration of HBOCs toward a concentration gradient of rhIL-11 when compared with control (Figure 2A). Similarly, treatment of HBOCs with 25-ng/mL rhIL-11 resulted in a 3-fold increase in HBOC proliferation (Figure 2B). Because cell migration and cell proliferation are cellular events that support blood vessel assembly, we performed a spheroid assay using HBOCs that were stimulated with rhIL-11 (Figure 2C) and counted the cumulative sprout length and total number of sprouts for each spheroid. As shown in Figure 2D and 2E, rhIL-11–treated HBOCs showed an 11-fold increase in cumulative sprout length (Figure 2D) and an 8-fold increase in sprouts/spheroids (Figure 2E) when compared with HBOCs treated with PBS control. These observations indicate that rhIL-11 enhances in vitro migration and proliferation of HBOCs and suggest a potential role of rhIL-11 for in vivo mobilization of $CD34^+/VEGFR2^+$ cells to sites of vascular remodeling.

rhIL-11 Treatment Leads to In Vivo Mobilization of $CD34^+/VEGFR2^+$ Mononuclear Cells

After demonstrating that HBOCs express $CD34^+/VEGFR2^+$ markers in vitro, we investigated the potential role of rhIL-11 on in vivo mobilization of $CD34^+/VEGFR2^+$ mononuclear cells by implanting Sv129 mice with osmotic pumps that continuously deliver 200-μg/kg rhIL-11 or PBS daily. This dose resulted in maximum in vivo mobilization of $CD34^+/VEGFR2^+$ mononuclear cells (data not shown). Blood was drawn 1, 3, and 7 days after minipump implantation for characterization of mononuclear cells by flow cytometry. Figure 3A illustrates mononuclear cells profiled according to

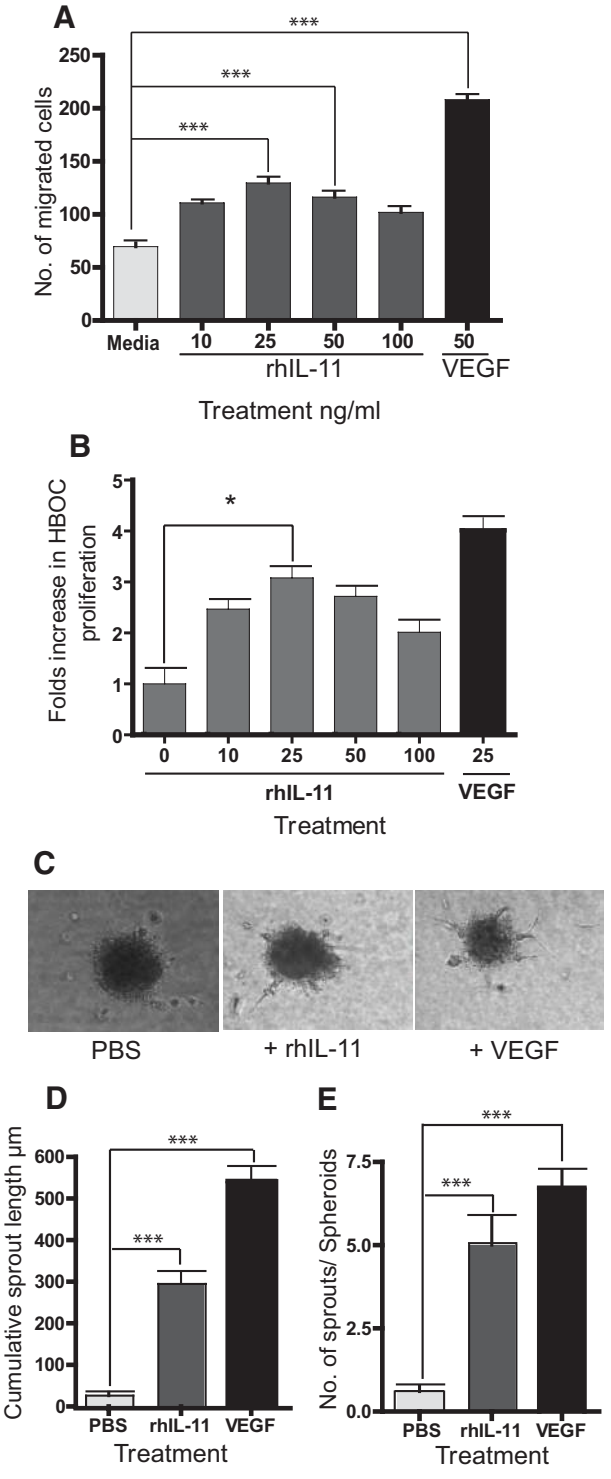

Figure 2. Effects of rhIL-11 on migration and proliferation of HBOCs. A, HBOCs were analyzed in a Boyden chamber with media, rhIL-11, or VEGF at the indicated concentrations. B, HBOC cells treated with rhIL-11 show a 3-fold increase in HBOC cell proliferation compared with control cells. C, Images of collagen-embedded HBOC spheroids demonstrate sprouting after 24 hours of treatment with media, rhIL-11, or VEGF. D, rhIL-11 treatment leads to an 11-fold increase in cumulative sprout length compared with media-treated control. E, Treatment with rhIL-11 results in an 8-fold increase in the number of sprouts per spheroid. Each bar in the graphs represents the mean of 3 independent experiments (unpaired *t* test for rhIL-11–vs PBS-treated mice, 2-tailed $^*P<0.05$ and $^{***}P<0.0001$).

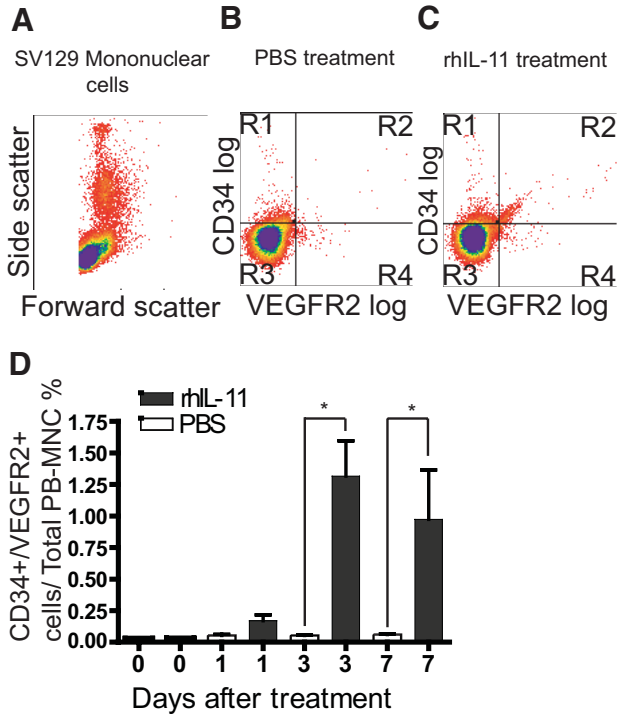

Figure 3. Mice treated with rhIL-11 have in vivo mobilization of $CD34^+/VEGFR2^+$ mononuclear cells. Sv129 mice were implanted with osmotic pumps loaded with either PBS or rhIL-11 for 3 days. A, Mononuclear cells from mouse blood were analyzed by flow cytometry and profiled according to their forward and side scatter. B and C, PBS-treated mice showed fewer $CD34^+/VEGFR2^+$ mononuclear cells in the R^2 quadrant (B) compared with rhIL-11–treated mice (C) under the same experimental conditions. D, $CD34^+/VEGFR2^+$ mononuclear cell mobilization peaked 3 days after rhIL-11 treatment, with rhIL-11–treated mice exhibiting a 20-fold increase in circulating $CD34^+/VEGFR2^+$ mononuclear cells compared with PBS control mice on day 3. Each bar represents the mean±SEM of 6 mice (unpaired Student *t* test, 2-tailed $^*P<0.05$).

their forward and side scatter. Mice treated with rhIL-11 had significantly more cells in the R^2 quadrant (1.3% of total peripheral blood mononuclear cells), which contained cells expressing both $CD34^+$ and $VEGFR2^+$ surface markers (Figure 3C, supplemental Figure I, and supplemental Figure II), compared with mice treated with PBS (Figure 3B). Seventy-two hours after rhIL-11 administration, there was a 20-fold increase in the number of circulating $CD34^+/VEGFR2^+$ cells (Figure 3D), compared with PBS-treated mice. By comparison, we observed a 2.8-fold increase in circulating platelets and a 2.5-fold increase in monocytes; we did not observe a significant change in red blood cells, total white blood cells, lymphocytes, or granulocytes (supplemental Figure III). These data provided the basis for further physiological characterization of rhIL-11.

rhIL-11 Increases Recovery of Perfusion After Femoral Artery Ligation

We used a model of hindlimb ischemia in which the right femoral artery is ligated proximal to the origin of the lateral caudal femoral artery. High-resolution infrared laser Doppler perfusion imaging with a 2-mm sampling depth was then used to measure perfusion in the plantar foot (which is dependent on collateral vessels in the thigh and is a good index of overall leg perfusion[17,22–24]) and in the adductor region. Blood flow decreased to low values in both PBS- and rhIL-11–treated mice immediately after ligation and continued to be decreased in both groups of animals 2 days later (Figure 4A through 4C), confirming successful femoral artery ligation in both animal groups. In addition, the lack of significant difference in blood flow between PBS- and rhIL-11–treated animals at these points suggests that rhIL-11 does not have an immediate effect on postocclusive reperfusion. However, when examined at 4, 6, and 8 days after ligation, a greater recovery of plantar perfusion was seen in rhIL-11–treated mice when compared with syngeneic mice treated with PBS (Figure 4A and 4B and supplemental Figure IVA). In addition, rhIL-11–treated mice exhibited a greater increased measure of perfusion in the center of the adductor region, which contains collaterals interconnecting the saphenous and popliteal artery trees with medial trees branching from the femoral and iliac arteries proximal to the lateral circumflex femoral artery (Figure 4C and 4D and supplemental Figure IVB).[17,22,24] Together, these observations suggest that rhIL-11 augments preexisting collateral vessel remodeling.

rhIL-11 Improves Functional Recovery and Collateral Vessel Growth

To determine the functional significance of the observed increase in reperfusion previously described, we used a hindlimb use score (index of hindlimb muscle function) and appearance score (index of hindlimb ischemia)[17,22,24] and discovered that rhIL-11–treated mice exhibited better hindlimb use and appearance scores than their PBS-treated counterparts (supplemental Figure VA and VB). To measure preexisting collateral vessel remodeling directly, we performed histomorphometry of the single collateral present in each of the anterior and posterior gracilis muscles at 8 and 21 days after femoral artery ligation. Mice treated with rhIL-11 showed a 1.5-fold increase in collateral vessel luminal diameter on day 8 (data not shown) and a 3-fold increase in collateral vessel luminal diameter on day 21 after femoral artery ligation (Figure 5A and 5B), compared with PBS-treated control mice, indicating that rhIL-11 plays a significant role in time-dependent collateral vessel growth. By comparison, we did not observe a difference in angiogenesis measured by isolectin B4+ cell analysis 21 days after femoral artery ligation (supplemental Figure VI).

rhIL-11 Enhances Influx of $CD34^+/VEGFR2^+$ Mononuclear Cells Into Perivascular Tissues

Because we observed a 20-fold increase in circulating $CD34^+/VEGFR2^+$ cells associated with rhIL-11 treatment (Figure 3), we hypothesized that rhIL-11–mediated collateral vessel growth is functionally linked to mobilized $CD34^+/VEGFR2^+$ mononuclear cells. To examine this further, we performed immunohistochemical analysis of the anterior and posterior gracilis muscles to identify $CD34^+/VEGFR2^+$ mononuclear cells in the perivascular tissues 8 days after femoral artery ligation. As shown in Figure 5C and supplemental Figure VIIA, mice treated with rhIL-11 displayed a 4.4-fold increase in perivascular $CD34^+/VEGFR2^+$ mononu-

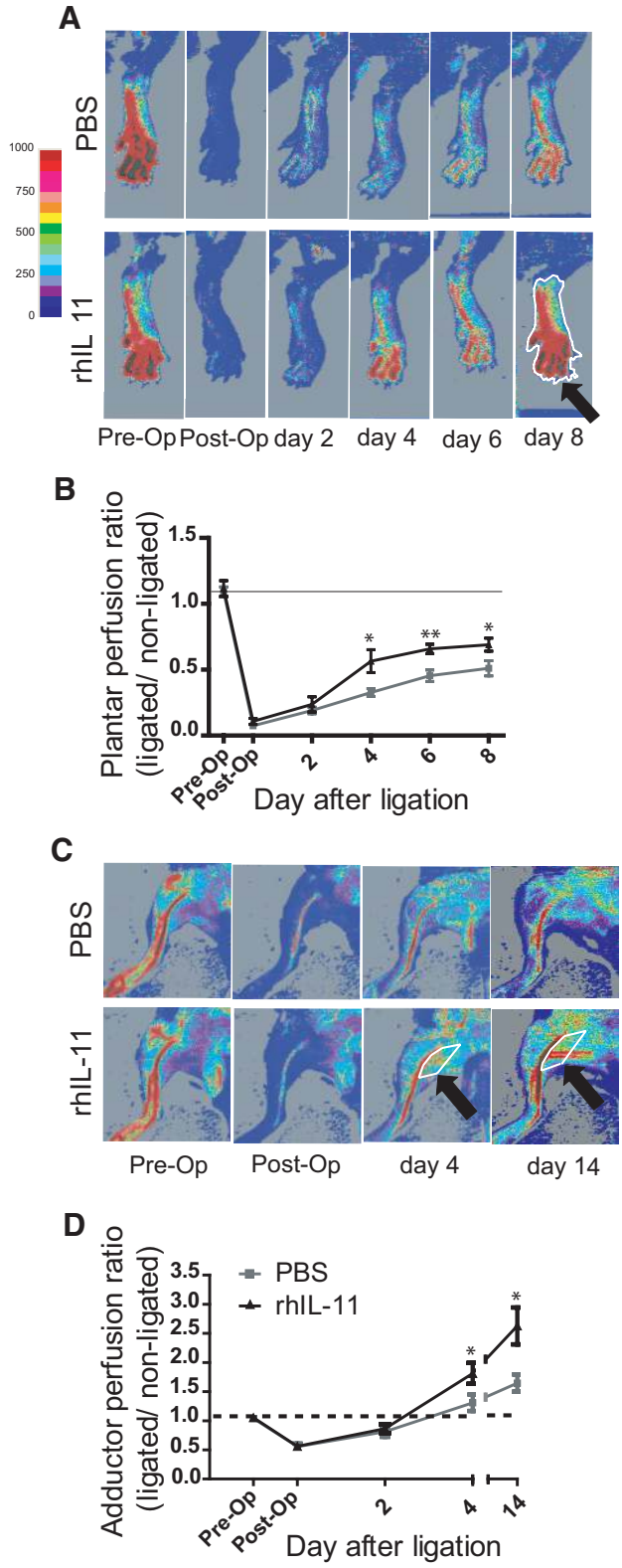

Figure 4. rhIL-11–treated mice show faster recovery of perfusion. Sv129 mice were pretreated with rhIL-11 or PBS for 72 hours before femoral artery ligation. A, Laser Doppler perfusion imaging of the plantar foot of rhIL-11–treated mice showed increased plantar perfusion and faster blood flow recovery compared with PBS control mice. B, Graph showing the ratio of perfusion rate in the ligated/nonligated plantar foot. rhIL-11–treated mice have significantly increased perfusion rates from day 4 to day 8 after femoral artery ligation. Each point denotes the mean±SEM of 9 mice (unpaired Student *t* test, 2-tailed $*P<0.05$ and $**P<0.01$). The typical region of interest used to calculate the perfusion rate is marked in white (arrow). C, Laser Doppler perfusion imaging of the adductor region. Increased perfusion through the collaterals in the superficial gracilis muscle is evident on days 4 and 14 in the rhIL-11–treated animal. D, Graph showing the ratio of perfusion rate in the ligated/nonligated adductor region. The color scale shows the relation between color and units of perfusion rate.

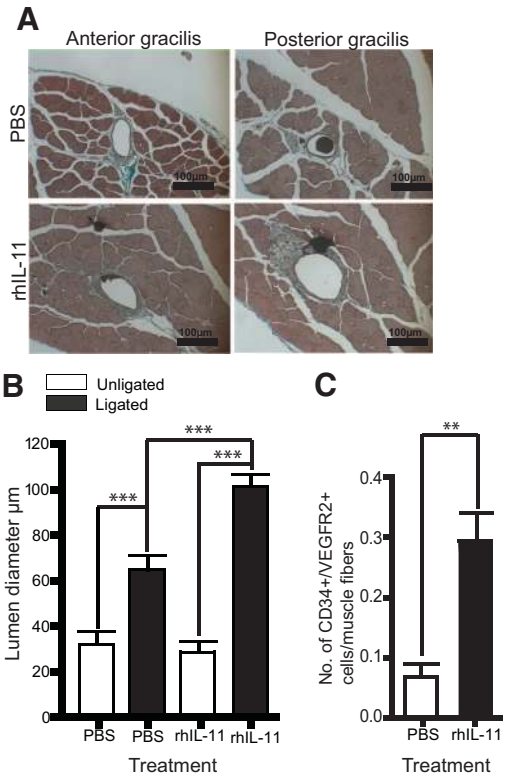

Figure 5. Mice treated with rhIL-11 have increased collateral vessel luminal diameter and an influx of perivascular $CD34^+/VEGFR2^+$ cells. A, Cyano-Masson-elastin staining of anterior and posterior gracilis muscles showing increased collateral vessel luminal diameter in rhIL-11–treated mice 21 days after femoral artery ligation. B, Graph showing that rhIL-11–treated mice have a 3-fold increase in luminal diameter of the adductor collateral vessel compared with PBS control. Each bar depicts the mean±SEM of 9 mice (unpaired Student *t* test, $***P<0.0001$; ×20 magnification). C, Anterior and posterior gracilis muscles were stained for influx of perivascular $CD34^+/VEGFR2^+$ cells. Mice treated with rhIL-11 have a 4.4-fold increase in perivascular $CD34^+/VEGFR2^+$ cells 8 days after femoral artery ligation. Each bar depicts the mean±SEM of 8 mice (unpaired Student *t* test, 2-tailed $**P<0.01$).

clear cells when compared with control mice. By comparison, we only observed a 2.6-fold increase in perivascular CD11b cells after rhIL-11 treatment (supplemental Figure VIIB and VIIC). Taken together, these data suggest that rhIL-11 enhances collateral vessel growth by augmentation of the influx of $CD34^+/VEGFR2^+$ and $CD11b^+$ mononuclear cells into perivascular tissues.

Discussion

Although native collateral vessels are functional after vessel occlusion, they are often not well developed in short- or long-term vessel occlusion, resulting in significantly de-

creased chances of clinical improvement in symptoms. The clinical utility of mononuclear cells for adjunctive treatment of patients with PVD is based on the ability of mononuclear cells to revascularize occluded vessels by growth of preexisting collaterals. Current methods of delivering mononuclear progenitor cells to patients with PVD include intramuscular injection of ex vivo–processed bone marrow–derived or apheresed peripheral blood–derived mononuclear cells into the ischemic limb.[25] These methods of mononuclear cell delivery are cumbersome, invasive, and are also associated with clinically significant procedural complications. Therefore, the identification of a simple and less invasive strategy of revascularization by in vivo mobilization of progenitor cells is crucial to successful treatment of patients with PVD.

Herein, we describe a novel role for rhIL-11 on in vivo mobilization of $CD34^+/VEGFR2^+$ mononuclear cells and augmentation of collateral vessel growth after femoral artery ligation. We observed that rhIL-11 treatment led to a 20-fold increase in circulating $CD34^+/VEGFR2^+$ mononuclear cells and that mobilized $CD34^+/VEGFR2^+$ cells home into sites of collateral vessel remodeling, resulting in collateral vessel growth and faster recovery of perfusion to ischemic limb in a manner that closely correlated with mobilization of $CD34^+/VEGFR2^+$ cells. These observations suggest that rhIL-11–mobilized $CD34^+/VEGFR2^+$ mononuclear cells, in part, participate in collateral vessel growth and are consistent with other reports showing a correlation between circulating $CD34^+$ cells and the extent of collateral vessel remodeling during cerebral[26] or hindlimb[27] ischemia in humans and augmentation of regional blood flow and neovascularization[28] during cerebral or hindlimb ischemia in mice.[29,30]

Our histological analysis of anterior and posterior gracilis muscles shows an increase in the luminal diameter of collateral vessels that was significant (1.5-fold) 8 days after femoral artery ligation and more pronounced (3-fold) 21 days after femoral artery ligation (Figure 5A and 5B). This finding complements our laser Doppler perfusion imaging data showing faster recovery of plantar and adductor perfusion in rhIL-11–treated mice as early as 4 days after femoral artery ligation (Figure 4 and supplemental Figure IV).

The mechanism of rhIL-11–mediated collateral vessel growth is an important topic of future study. Our data suggest that it is likely related to rhIL-11–induced circulating $CD34^+/VEGFR2^+$ mononuclear cells that are recruited to the perivascular ischemic region of the hindlimb, where they may release cytokines/growth factors (paracrine effect),[31] activate STAT-3–dependent pathways (supplemental Figure VIII), and result in a reduction of apoptosis-mediated cell death (supplemental Figure IX), as shown by other researchers.[32] Because monocytes and platelets are involved in collateral vessel growth,[33–35] our data suggest that $CD34^+/VEGFR2^+$ cells are candidate cells that also play a role in collateral vessel growth. We did not characterize the relative contribution of each of the previously described cells to the observed collateral vessel growth, and we cannot exclude the possibility that the observed effects are direct effects of rhIL-11. Because of these limitations, additional studies using chimeric mice with tissue and/or cellular depletion of IL-11 will be necessary for further characterization of this mechanism.

In summary, this report shows that rhIL-11 treatment leads to in vivo mobilization of $CD34^+/VEGFR2^+$ mononuclear cells in mice. Pretreatment of Sv129 mice with rhIL-11 before femoral artery ligation leads to an increase in collateral vessel growth and faster recovery of perfusion, which correlates with functional recovery of hindlimb use. Because rhIL-11 has been used for the treatment of other human diseases, this report suggests the possibility that rhIL-11 could be used as an adjunctive treatment for patients with PVD.

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Disclosures

None.

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