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## Recombinant human interleukin-11 treatment enhances collateral vessel growth after femoral artery ligation

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### Abstract

**Objective**—We investigated the role of recombinant human interleukin-11 (rhIL-11) on *in vivo* mobilization of CD34+/VEGFR2+ mononuclear cells and collateral vessel remodeling in mouse model of hindlimb ischemia.

**Methods and Results**—We observed that treatment of Sv129 mice with continuous infusion of 200 µg/kg/day of rhIL-11 led to *in vivo* mobilization of CD34+/VEGFR2+ cells that peaked at 72 hours. Sv129 mice pre-treated with rhIL-11 for 72 hours prior to 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.

**Conclusions**—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 adjunctive treatment of patients with peripheral vascular disease.

### Keywords

Peripheral vascular disease; CD34+/VEGFR2+ cells; collateral vessel remodeling; arteriogenesis; hindlimb ischemia

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Peripheral vascular disease (PVD) is a major cause of morbidity as well as significant health care cost in the United States<sup>1</sup>. Currently, it is estimated that over 8 million Americans have PVD<sup>2</sup>, and its prevalence is expected to increase concurrently with the rise of the aging

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### Disclosures

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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, which may not be utilized 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<sup>3</sup>. Others have reported a correlation between patient outcomes and the number of intramuscularly injected CD34+ cells within the patient's mononuclear cell population<sup>4</sup>. Although these studies have demonstrated favorable safety and feasibility of cell-based therapy for treatment of patients with PVD, the need for *ex-vivo* processing of mononuclear cells prior to delivery is a major impediment to the performance of a large scale study in these patient populations—thus, creating a need for novel method of rapid *in vivo* mobilization of mononuclear cells for treatment of patients with PVD.

Human blood outgrowth cells (HBOCs) are cultured CD34+/VEGFR2+ mononuclear cells from normal 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<sup>5</sup>, thus serving as useful tools for screening the biology of vasculoprotective mononuclear cells in *in vitro* assays. We have recently reported that a 12-mer peptide ligand that binds with high affinity to HBOCs has sequence homology to human interleukin-11 (IL-11)<sup>6</sup>. This finding, coupled with reports of high expression of interleukin-11 receptor alpha (IL-11R $\alpha$ ) in highly vascular tissues<sup>7</sup>, 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 interleukin-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<sup>8, 9</sup>, protection of endothelial cells from alloinjury by upregulation of survivin<sup>10, 11</sup> and protection against endothelial cell death<sup>12</sup>. Fortuitously, rhIL-11 has already been used extensively in human patients<sup>13, 14</sup>. Since the clinical efficacy and safety parameters of rhIL-11 have been well characterized in humans, we performed a pre-clinical study to investigate the role of rhIL-11 as a potential pharmacologic 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 since these markers are present in human and mice, have been used to identify mouse progenitor cells that enhance neovascularization during hindlimb ischemia<sup>15, 16</sup>, and will allow for correlation of CD34+/VEGFR2+ cells in mice and future human studies.

## Methods

Please see online Supplemental material for methods not described here.

### Unilateral hindlimb ischemia

Unilateral femoral artery ligation was performed using 10-week old Sv129 mice as previously described<sup>17</sup>. Briefly, mice were anesthetized with 1.25% isoflurane/O<sub>2</sub> during hindlimb depilation (one 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 (50mg/

kg, IM), topical Furazolidone and Pentazocine (10mg/kg, IM) were administered. The University of North Carolina Institutional Animal Care and Use Committee approved all our animal procedures.

### Laser Doppler perfusion imaging

Laser Doppler perfusion imaging was performed as previously described<sup>17</sup>. Briefly, mice were anesthetized with 1.25% isoflurane/O<sub>2</sub> and their body temperature was strictly maintained at 37°C ± 0.5 during the entire procedure. Laser Doppler perfusion imaging (Moor Instruments Ltd, Devon, UK) 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 MoorLDIV5.0 software. Regions of interest (ROI) were drawn with respect to anatomical 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; 1–5, cyanosis or loss of nail(s) (where the score is dependent on the number of nails affected); 6–10, partial or complete atrophy of digit(s) (where the score reflects number of digits affected); 11, partial atrophy of forefoot. Hindlimb use was scored as an index of muscle function: 0, normal; 1, no toe flexion; 2, no plantar flexion; 3, dragging foot.

### Statistical Analysis

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

## Results

### HBOCs express IL-11R $\alpha$ and administration of rhIL-11 activates downstream STAT-3

We recently identified peptide ligands that bind specifically to HBOCs by screening phage display libraries in order to identify novel surface markers of vasculoprotective mononuclear cells<sup>6</sup> (see cartoon in Figure 1A). One such 12-mer-peptide ligand has sequence homology with human IL-11. This observation, coupled with reports of high expression of IL-11R $\alpha$  in highly vascular tissues<sup>7</sup>, 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 multi-parametric investigation consisting of identification of their characteristic cobblestone morphology<sup>5</sup>, acetylated LDL uptake<sup>18</sup> as well as their expression of cell surface markers such as VEGFR2, CD34 and CD31. These characteristics, along with the lack of expression of CD45 (data not shown),<sup>19</sup> suggests that these HBOCs have properties of dedifferentiated mononuclear cells<sup>20</sup>. To determine if HBOCs express IL-11R $\alpha$ , we incubated HBOCs with anti-IL-11R $\alpha$  antibody and found that HBOCs robustly express IL-11R $\alpha$  on their surface (Figure 1B). In order to determine if signaling via the IL-11R $\alpha$ /rhIL-11 (receptor/ligand) axis was intact, we stimulated HBOCs with rhIL-11 and found that rhIL-11-treated cells displayed a time-dependent phosphorylation of STAT-3 (Figure 1C)—a downstream effector molecule that support cell survival by upregulation of anti-apoptotic proteins like survivin<sup>10</sup> and upregulation of pro-angiogenic factors like VEGF<sup>21</sup>. These data not only demonstrate that HBOCs express a functional IL-11R $\alpha$  but also suggest a link between IL-11/HBOC interaction and IL-11R $\alpha$  signaling.

### Recombinant human IL-11 stimulates directed migration and tubule formation in HBOCs

To study the physiologic effects of rhIL-11 on HBOCs, we administered rhIL-11 to HBOC in a Boyden chamber. Recombinant human IL-11 administration at 25ng/ml led to optimal cell migration of HBOCs towards a concentration gradient of rhIL-11 when compared to control (Figure 2A). Similarly, treatment of HBOC with 25 ng/ml of rhIL-11 resulted in 3-fold increase in HBOC proliferation (Figure 2B). Since 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 Figures 2D and E, rhIL-11-treated HBOCs showed 11-fold increase in cumulative sprout length (Figure 2D) and 8-fold increase in sprouts/spheroids (Figure 2E) when compared to HBOCs treated with PBS control. These observations indicate that rhIL-11 enhances *in vitro* migration and proliferation of HBOCs and suggests a potential role of rhIL-11 for *in vivo* mobilization of CD34+/VEGFR2+ cells to sites of vascular remodeling.

### **Recombinant human IL-11 treatment leads to *in vivo* mobilization of CD34+/VEGFR2+ mononuclear cells**

Having already demonstrated that HBOCs express CD34+/VEGFR2+ markers *in vitro*, we next 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 delivers 200 µg/kg/day of rhIL-11 or PBS. 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 mini-pump implantation for characterization of mononuclear cells by flow cytometry. Figure 3A illustrates mononuclear cells profiled according to their forward and side scatter. Mice treated with rhIL-11 had a significantly higher percentage of cells in the R2 quadrant (1.3% of total peripheral blood mononuclear cells), which contained cells expressing both CD34+ and VEGFR2+ surface markers (Figure 3C and Supplemental Figure 1 and 2), 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 platelet and a 2.5-fold increase in monocytes, and we did not observe a significant change in red blood cells, total white blood cells, lymphocytes or granulocytes (supplemental figure 3). This data provided the basis for further physiologic characterization of rhIL-11.

### **Recombinant human IL-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 2 millimeter 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<sup>17, 22–24</sup>) and in the adductor region. Blood flow dropped to low values in both PBS- and rhIL-11-treated mice immediately after ligation (“Post-Op”) and continued to be decreased in both groups of animals 2 days later (Figure 4A, B and C), 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 time points suggests that rhIL-11 does not have an acute effect on post-occlusive reperfusion. However, when examined at 4, 6 and 8 days following 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 B and Supplemental Figure 4 A). 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 D and Supplemental Figure 4 B)<sup>17, 22, 24</sup>. Together, these observations suggest that rhIL-11 augments pre-existing collateral vessel remodeling.

### **Recombinant human IL-11 improves functional recovery and collateral vessel growth**

In order to determine the functional significance of the observed increase in reperfusion described above, we utilized a hindlimb use score (index of hindlimb muscle function) and

appearance score (index of hindlimb ischemia)<sup>17, 22, 24</sup> and discovered that rhIL-11-treated mice exhibited better hindlimb use and appearance scores than their PBS-treated counterparts (Supplemental Figure 5 A and B). To measure pre-existing 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 B), compared to PBS 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 6).

### **Recombinant human IL-11 enhances influx of CD34+/VEGFR2+ mononuclear cells into perivascular tissues**

Since 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 muscle to identify CD34+/VEGFR2+ mononuclear cells in the perivascular tissues 8 days after femoral artery ligation. As shown in Figure 5C and Supplemental Figure 7 A, mice treated with rhIL-11 displayed a 4.4-fold increase in perivascular CD34+/VEGFR2+ mononuclear cells when compared to control mice. By comparison, we only observed 2.6-fold increase in perivascular CD11b cells after rhIL-11 treatment (supplementary figure 7 B and C). 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 acute or chronic vessel occlusion, resulting in significantly lowered 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 pre-existing 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<sup>25</sup>. These methods of mononuclear cell delivery are cumbersome, invasive, and associated with clinically significant procedural complications. Therefore, identification of a simple, less invasive strategy of revascularization by *in vivo* mobilization of progenitor cells is crucial to successful treatment of patients with PVD.

In this report, 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 is consistent with other reports showing a correlation between circulating CD34+ cells and the extent of collateral vessel remodeling during cerebral<sup>26</sup> or hindlimb ischemia<sup>27</sup> in human



and augmentation of regional blood flow and neovascularization<sup>28</sup> during cerebral or hindlimb ischemia in mice<sup>29, 30</sup>.

Our histologic analysis of anterior and posterior gracilis muscle 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 (Figures 5A and B). 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 four days after femoral artery ligation (Figure 4 and Supplemental Figure 4).

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)<sup>31</sup>, activate STAT-3-dependent pathways (Supplemental Figure 8) and result in a reduction of apoptosis-mediated cell death (Supplemental Figure 9) as has been shown by others<sup>32</sup>. Since monocytes and platelets have been previously shown to be involved in collateral vessel growth<sup>33–35</sup>, 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 above cells to the observed collateral vessel growth, and we cannot exclude the possibility that the observed effects are direct effects rhIL-11. Due to 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. Pre-treatment of Sv129 mice with rhIL-11 prior to femoral artery ligation leads to an increase in collateral vessel growth and faster recovery of perfusion, which correlates with functional recovery of hindlimb use. Since rhIL-11 has been used for the treatment of other human diseases, this report suggests the possibility that rhIL-11 could be used as adjunctive treatment for patients with PVD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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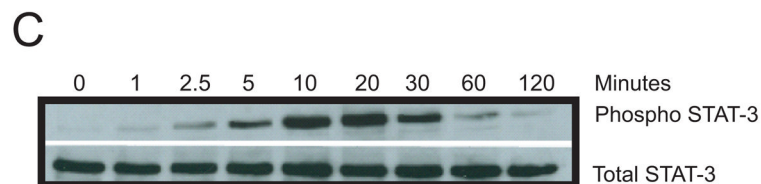
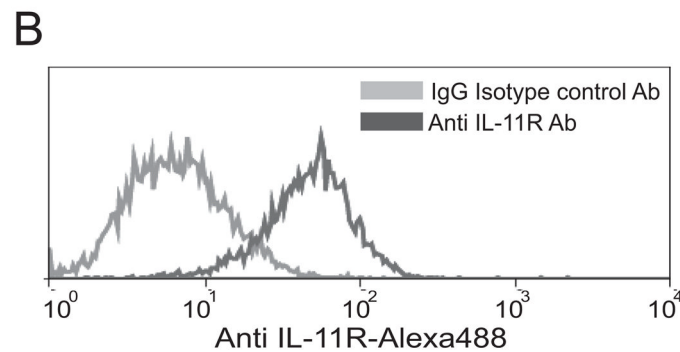
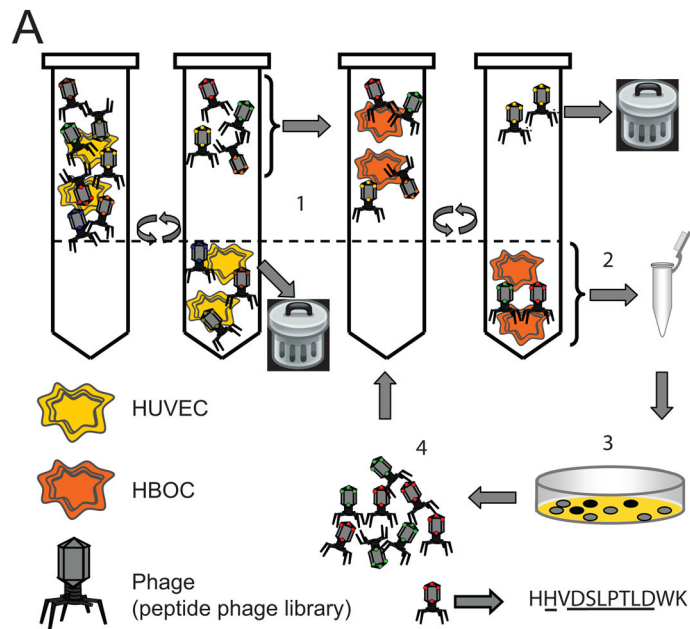
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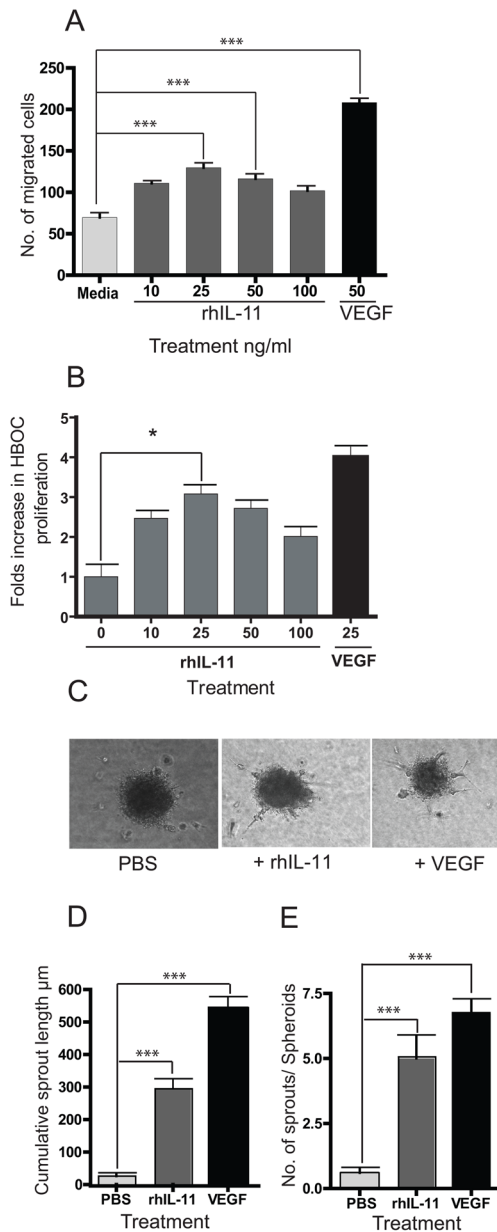




### Figure 1. HBOCs abundantly express interleukin-11 receptor-alpha

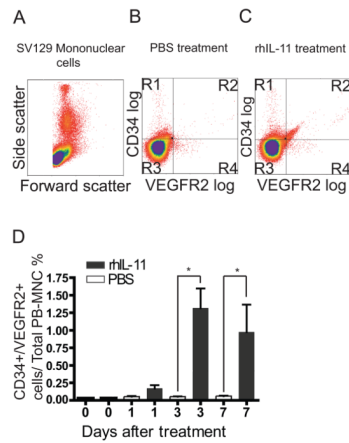
**A.** Schematic representation of the screening procedure used to identify peptide ligands that selectively bind to HBOCs. A 12-mer random peptide phage display library was screened using a two-step selection protocol. First, the library was depleted of ligands binding to common receptors by incubation with a non-target cell line (HUVEC). Second, the unbound phage pool was biopanned on HBOCs. Phage that bound to HBOC were amplified in *Escherichia coli* to the original input titer of the library and used for subsequent rounds of biopanning (steps 1 through 4). After three rounds of biopanning, HBOC-bound phage were plaque-purified for sequence analysis<sup>6</sup>. **B.** HBOCs were incubated with an anti human IL-11 receptor antibody-Alexa488 or mouse IgG isotype control antibody and analyzed by flow

cytometry. HBOCs abundantly express interleukin-11 receptor alpha. C HBOCs were treated with 25ng/ml of rhIL-11 for 1, 2.5, 5, 10, 20, 30, 60 and 120min. Cell lysates were analyzed by western blotting with anti-phospho STAT-3 (Tyr705) Ab and anti-STAT (79D7) Ab. Recombinant human IL-11 induced STAT-3 phosphorylation in HBOCs at the indicated time points.



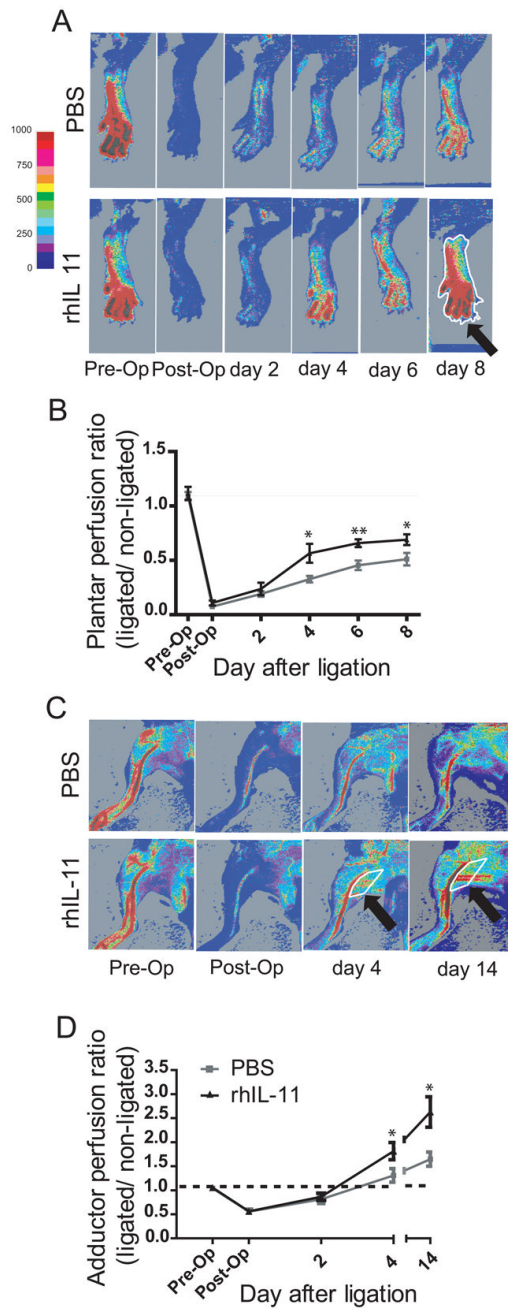
### Figure 2. Effects of rhIL-11 on migration and proliferation of HBOC

**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 to control cells. **C.** Images of collagen embedded HBOC spheroids demonstrate sprouting after 24hrs of treatment with media, rhIL-11 or VEGF. **D.** Recombinant human IL-11 treatment leads to 11-fold increase in the cumulative sprout length, compared to media-treated control. **E.** Treatment with rhIL-11 results in 8-fold increase in the number of sprouts per spheroid. Each bar in the graphs represents the mean of three independent experiments. Unpaired t-test for rhIL-11 vs PBS treated mice, two-tailed  $*= P < 0.05$ ,  $***=P < 0.0001$ .



**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 the mouse blood were analyzed by flow cytometry and profiled according to their forward and side scatter. PBS treated mice showed fewer CD34+/VEGFR2+ mononuclear cells in the R2 quadrant (**B**) compared with rhIL-11 treated mice (**C**) under the same experimental condition. **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  $\pm$  SEM of 6 mice. Unpaired student t-test, two tailed  $*= P < 0.05$ .

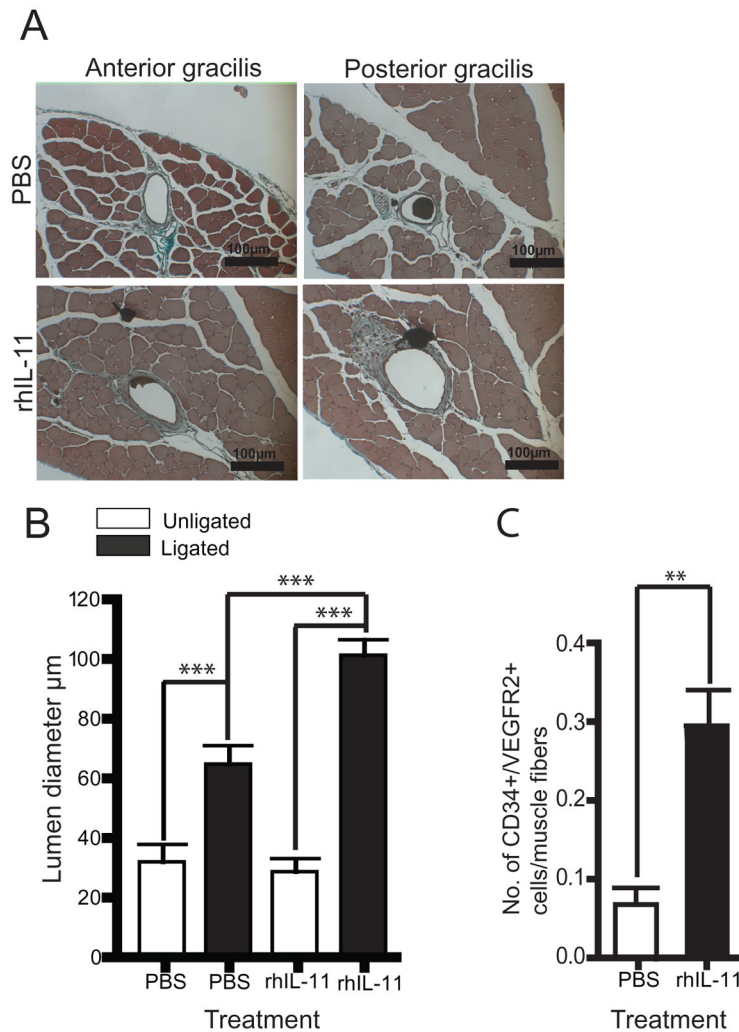


**Figure 4. Recombinant human IL-11-treated mice show faster recovery of perfusion**

Sv129 mice were pre-treated with rhIL-11 or PBS for 72 hours prior to 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 ratio of perfusion rate in ligated/non-ligated plantar foot. Recombinant human IL-11 treated mice have significantly increased perfusion rates from day 4 to day 8 after femoral artery ligation. Each time point denotes mean  $\pm$  SEM of 9 mice. Unpaired student T-test, two tailed \* =  $P < 0.05$ , \*\* =  $P < 0.01$ . Typical region of interest (ROI) used to calculate 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 day 4 and 14 in the rhIL-11-treated animal. **D.**



Graph showing ratio of perfusion rate in ligated/non-ligated adductor region. Color scale shows relation between color and units of perfusion rate.



**Figure 5. Mice treated with rhIL-11 have increased collateral vessel luminal diameter and influx of perivascular CD34+/VEGFR2+ cells**

**A.** Cyano-Masson-Elastin staining of anterior and posterior gracilis muscle 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 adductor collateral vessel compared to PBS control. Each bar depicts mean  $\pm$  SEM of 9 mice. Unpaired student t-test \*\*\*= $P < 0.0001$  ( $20\times$  magnification). **C.** Anterior and posterior gracilis muscle were stained for influx of perivascular CD34+/VEGFR2+ cells. Mice treated with rhIL-11 have 4.4-fold increase in perivascular CD34+/VEGFR2+ cells 8 days after femoral artery ligation. Each bar depicts mean  $\pm$  SEM of 8 mice. Unpaired student T-test, two tailed \*\*= $P < 0.01$ .