Material and Methods

1. Mice

IL-21 receptor knockout mice in C57BL/6 mice were initially produced in National Heart, Lung and Blood Institute (NHLBI) as previously described[1]. Briefly, the IL21R KO was initially selected from embryonic stem (ES) cells with homologous recombination, then injected into C57BL/6 blastocysts, the resulting chimeric mice were mated with C57BL/6 mice and heterozygous offspring were then interbred to generate homogenous IL-21R-KO C57BL/6 mice. In this experiment, adult homozygous IL-21 receptor knockout (Il21r-/-) mice and their wide-type (Il21r+/+, WT) littermates were bred in the University of Virginia (UVa) vivarium using breeding pairs of Il21r heterozygous (+/−) mice in a C57BL/6 background. Age and sex matched 12-20 week old WT and Il21r-/- mice were compared in the experiments. Male C57BL/6 or BALB/c mice from 12 to 16 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME) or were bred in house from mice purchased from Jackson Labs, (number as indicated for each experiment results). Animal studies were approved by the Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2. Hindlimb Ischemia, Perfusion Recovery and Necrosis Score

After anesthesia induction (ketamine 90 mg/kg and xylazine 10 mg/kg), unilateral femoral artery ligation and excision were performed on mice as described previously[2, 3]. Perfusion flow in the ischemic and contralateral non-ischemic limbs was measured as described previously with the use of a laser Doppler perfusion imaging system (Perimed, Inc,

Ardmore, PA)[2, 3]. Perfusion was expressed as the ratio of the left (ischemic) to right (non-ischemic) hindlimb and was performed on days 0, 3, 7, 14, and 21 after surgery. In mice that developed autoamputation, the perfusion ratio obtained from the limb before autoamputation was used. The extent of necrosis was scored as follows: grade I, involving only toes; grade II, extending to dorsum pedis; grade III, extending to crus; and grade IV, extending to thigh or complete necrosis.

3. Mouse IL-21 receptor Fc Chimera treatment

The mouse IL-21R-Fc used in our study was prepared in the Protein Expression Laboratory, National Cancer Institute and can neutralize IL-21[4] . . IL-21R-Fc were injected at a dose of 0.2 mg/mouse intra-peritoneal immediately and 1, 3, 5, 7, 9, 11 days after surgical HLI (n = 11); and an equivalent dose of mouse IgG (Sigma-Aldrich, St. Louis, MO) was used in the control group (n = 12). The dose and time point of treatment were determined based on previous studies[5, 6]

4. Immunofluorescence

Immunofluorescent staining was performed as described previously[2, 7]. Briefly, to co-stain IL-21R and CD31; anti-IL-21R antibody (polyclonal rabbit Ab; cat. #ab13268; Abcam, Cambridge, MA) and anti-CD31 antibody (rat anti-mouse CD31 Ab; cat: # 550274; BD Pharmingen San Jose, CA) were applied on acetone-fixed cryosections of ischemic and non-ischemic gastrocnemius muscle specimens. Sections were protein blocked (5% goat serum; Sigma) for 60 min, then primary antibodies (anti-IL-21R at 1:600) and anti-CD31 at 1:25) were applied at 4˚C overnight in blocking solution. After rinsing with Phosphate Buffered Saline (PBS), secondary reagents, which included Goat anti-rat Alexa Fluor 488

(1:100, Invitrogen) or goat anti-rabbit Alexa Fluor 555 (1:100; Invitrogen), were applied for 1h at room temperature. Sections were then rinsed with PBS and mounted with Vectashield mounting medium (Vector Lab, Burlingame, CA). Secondary antibody only, without primary antibody, was used as a negative control to assess non-specific binding. Stained sections were examined with 200X magnification, using an Olympus BX51 high-magnification microscope. The co-staining of IL-21R and α-smooth muscle actin (α-SMA, 1:50, Sigma), CD31 and STAT3 (1:100, Cell Signaling, Danvers, MA, USA), CD31 and caspase 3 (1:100, Santa Cruze Biotechnology, Santa Cruze, CA) were similar to the protocol we used for CD31 and IL-21R co-staining.

For assessment of capillary density, 21 days post-HLI, ischemic gastrocnemius muscle sections from *Il21r*-/- mice and WT littermates were analyzed by immunofluorescence with a rat anti-mouse CD31 antibody by counting 3 random high-power (magnification ×200) fields, and was expressed as the number of CD31+ cells per muscle fiber area, as described previously[2, 8].

5. Cell Culture

Pooled human umbilical vein endothelial cells (HUVEC) were purchased (Cell Applications Inc, San Diego, CA), and grown in standard endothelial cell growth medium (Cell Applications Inc) with 10% FBS. HUVECs were exposed to hypoxia (2% oxygen, BioSpherix, Lacona, NY) and serum starvation (HSS) to simulate ischemia *in-vitro*; IL-21R mRNA levels were determined by qPCR after 24h exposure to HSS conditions.

For *in-vitro* transfection studies, plasmid vectors delivering shRNA targeting IL-21R (SABiosciences, a Qiagen company, Frederick MD) or nonsense control were transfected

with Cytofect™ Endothelial Cell Transfection Kit (Cell Applications Inc, San Diego, CA) following the manufacturer's protocol. Cell viability, tube formation and apoptosis assays were assessed 48 hours after transfection.

6. Cellular Viability and Apoptosis

HUVECs were plated in a 96-well plate at a density of 1×10^4cells/well. After shRNA transfection, cells were treated with 50ng/mL recombinant human IL-21 (rhIL-21; Cell Signaling Technology; Danvers; MA) in HSS conditions for 48h, treatment with PBS was used as control. At the end of the incubation, cell viability was assessed using tetrazolium dye incorporation (BioVision, Milpitas, CA); apoptosis in cells was determined using a TUNEL assay (TiterTACS, Trevigen Gaithersburg, MD). For the apoptosis assay, TACS nuclease treated wells were used as positive control, while wells without addition of TdTs were used as negative controls. Each experiment was repeated with at least two different batches of HUVECs.

7. *In-vitro* Angiogenesis Assay

In-vitro angiogenesis assay were performed as previously described[2]. Briefly, after exposure to HSS conditions for 24h, transfected HUVECs were plated at a density of 1×10^4 cells/well on 96-well dishes which were coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) , and then exposed to HSS conditions for 6h with rhIL-21 (50ng/mL) or with vehicle alone to assess tube formation. Each condition was done in 6 wells. The degree of tube formation was determined by measuring the length of the tubes and the number of loops from each well under 40 × magnifications using the online

WimTube application module (Wimasis GmbH, Munich, Germany). Each experiment was repeated at least in two different batches of HUVECs.

8. RNA isolation, quantitative PCR, and protein analysis

Total RNA was isolated and used for real-time quantitative RT-PCR (qPCR) as previously described[7, 9]. qPCR was performed using primers for *Il21r* and hypoxanthine phosphoribosyltransferase 1(Hprt1) from Applied Biosystems (Foster City, CA). To study IL-21R mRNA levels in endothelial cells from hindlimb muscle, CD31 positive cells were isolated from ischemic and nonis-chemic C57BL/6 hindlimb muscle with Dynabeads® magnetic separation (Life Technology, Grandland, NY) using methods similar to those previously described[10], and then used for RNA isolation and qPCR. Levels of target protein was analyzed by western blotting as previously described[2, 7] using antibodies to BCL-2, BAX, p-STAT1(Y701), p-STAT3 (Y705), p-AKT (Ser473) p-ERK1/2 (Thr202, Tyr 204), STAT1, STAT3, AKT, and ERK1/2 (Cell Signaling, Danvers, MA, USA). Western blots were analyzed by Odyssey Infrared Imaging System (LI-COR Biosciences, NE) and quantified by Scion Image software. The level of cytokines includes interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1) were determine with enzyme-linked immuno assay (ELISA) by using capture antibodies and reporter antibodies (eBiosciences, San Diego, CA), based on the protocol provided by the manufacturer.

9. Flow cytometry

To quantify IL-21R level on the surface of endothelial cells, we then isolated cells from the ischemic and non-ischemic gastrocnemius muscle, using methods similar to those we recently published[10]. The isolated cells were incubated with phycoerythrin (PE)

conjugated IL-21R antibody (BD Pharmigen, San Jose, CA), endothelial cells were then sorted for analysis by Cytek FACSCalibur Benchtop Analyzers usibng CellQuest Pro Acquisition software (BD Biosciences, San Jose, CA).

10. Statistical Analysis

Statistical analysis was performed with GraphPad Prism software. An unpaired t test was used for comparison between 2 groups, and comparisons in experiments with ≥3 groups were performed with One-way ANOVA and the Tukey post hoc test. Differences in necrosis score between WT and *Il21r*-/- mice were analyzed by Mann-Whitney test. Statistical significance was set at $p<0.05$.

Reference

1. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, Morse HC, Liu CY, Schwartzberg PL, Leonard WJ. A critical role for IL-21 in regulating immunoglobulin production. *Science.* 2002;298:1630-1634.

2. Hazarika S, Farber CR, Dokun AO, Pitsillides AN, Wang T, Lye RJ, Annex BH. MicroRNA-93 Controls Perfusion Recovery After Hindlimb Ischemia by Modulating Expression of Multiple Genes in the Cell Cycle Pathway. *Circulation.* 2013;127:1818-1828.

3. Md AOD, Keum S, Hazarika S, Li Y, Lamonte GM, Wheeler F, Marchuk DA, Annex BH. A quantitative trait locus (LSq-1) on mouse chromosome 7 is linked to the absence of tissue loss after surgical Hindlimb ischemia. *Circulation.* 2008;117:1207-1215.

4. Spolski R, Wang L, Wan CK, Bonville CA, Domachowske JB, Kim HP, Yu ZX, Leonard WJ. IL-21 Promotes the Pathologic Immune Response to Pneumovirus Infection. *J Immunol.* 2012;188:1924-1932.

5. Zhang WS, Zhang D, Lee J, Sehn M, Tian Y, Lee WPA, Zheng XX. An IL-21 Receptor-Targeted Antagonizing IL-21/Fc Protein Promotes Allograft Tolerance. *Am J Transplant.* 2010;10:8-8.

6. Young DA, Hegen M, Ma HLM, Whitters MJ, Albert LM, Lowe L, Senices M, Wu PW, Sibley B, Leathurby Y, Brown TP, Nickerson-Nutter C, Keith JC, Collins M. Blockade of the interleukin-21/interleukin-21 receptor pathway ameliorates disease in animal models of rheumatoid arthritis. *Arthritis Rheum-Us.* 2007;56:1152-1163.

7. Hazarika S, Dokun AO, Li Y, Popel AS, Kontos CD, Annex BH. Impaired angiogenesis after Hindlimb ischemia in type 2 diabetes Mellitus - Differential regulation of vascular endothelial growth factor receptor 1 and soluble vascular endothelial growth factor receptor 1. *Circ Res.* 2007;101:948-956.

8. Meisner JK, Song J, Annex BH, Price RJ. Myoglobin Overexpression Inhibits Reperfusion in the Ischemic Mouse Hindlimb through Impaired Angiogenesis but Not Arteriogenesis. *Am J Pathol.* 2013;183:1710-1718.

9. Hazarika S, Angelo M, Li YJ, Aldrich AJ, Odronic SI, Yan Z, Stamler JS, Annex BH. Myocyte Specific Overexpression of Myoglobin Impairs Angiogenesis After Hind-Limb Ischemia. *Arterioscl Throm Vas.* 2008;28:2144-U2175.

10. Imoukhuede PI, Dokun AO, Annex BH, Popel AS. Endothelial cell-by-cell profiling reveals the temporal dynamics of VEGFR1 and VEGFR2 membrane localization after murine hindlimb ischemia. *Am J Physiol-Heart C.* 2013;304:H1085-H1093.