Loss of Interleukin-21 Receptor Activation in Hypoxic Endothelial Cells Impairs Perfusion Recovery After Hindlimb Ischemia


Objective—Surgical hindlimb ischemia (HLI) in mice has become a valuable preclinical model to study peripheral arterial disease. We previously identified that the different phenotypic outcomes after HLI across inbred mouse strains is related to a region on the short arm of mouse chromosome 7. The gene coding the interleukin-21 receptor (IL-21R) lies at the peak of association in this region.

Approach and Results—With quantitative real-time polymerase chain reaction, we found that a mouse strain with a greater ability to upregulate IL-21R after HLI had better perfusion recovery than a strain with no upregulation after HLI. Immunofluorescent staining of ischemic hindlimb tissue showed IL-21R expression on endothelial cells (ECs) from C57BL/6 mice. An EC-enriched fraction isolated from ischemic hindlimb muscle showed higher IL-21R levels than an EC-enriched fraction from nonischemic limbs. In vitro, human umbilical vein ECs showed elevated IL-21R expression after hypoxia and serum starvation. Under these conditions, IL-21 treatment increased cell viability, decreased cell apoptosis, and augmented tube formation. In vivo, either knockout Il21r or blocking IL-21 signaling by treating with IL-21R-Fc (fusion protein that blocks IL-21 binding to its receptor) in C57BL/6 mice resulted in less perfusion recovery after HLI. Both in vitro and in vivo modulation of the IL-21/IL-21R axis under hypoxic conditions resulted in increased signal transducer and activator of transcription 3 phosphorylation and a subsequent increase in the B-cell lymphoma leukemia-2/BCL2–associated X protein ratio.

Conclusion—Our data indicate that IL-21R upregulation and ligand activation in hypoxic ECs may help perfusion recovery by limiting/preventing apoptosis and favoring cell survival and angiogenesis through the signal transducer and activator of transcription 3 pathway. (Arterioscler Thromb Vasc Biol. 2015;35:00-00. DOI: 10.1161/ATVBAHA.115.305476.)

**Key Words:** peripheral arterial diseases ■ receptors, interleukin 21

Although rates of death from ischemic heart disease in the United States have fallen over the past decades, the prevalence of peripheral arterial disease (PAD) has actually increased and PAD will become an even greater healthcare problem in the years ahead as the major drivers for PAD are advancing age, smoking, and diabetes mellitus. The primary problem in PAD is reduced blood flow to the leg, and because total occlusions in ≥1 more of the major inflow arteries to the leg(s) are common in patients with PAD, the magnitude of distal blood flow becomes dependent on the number and extent of collateral blood vessels that connect to the distal microvasculature. Current medical therapies used to treat PAD include antiplatelet agents, statins to lower cholesterol, antihypertensive therapy with angiotensin-converting enzyme inhibitors or angiotensin 2 receptor blocker or β-blockers, blood glucose control, and smoking cessation. However, there are no agents that have shown the ability to improve leg blood flow in patients with PAD and thus no medical therapies are available to directly treat the primary problem of reduced blood flow. New treatment paradigms are needed for PAD.

The response that follows surgically induced hindlimb ischemia (HLI) in mice has been widely used to test agents to treat PAD. Genetic factors modify the clinical outcomes when PAD is present. Our laboratory and others have shown that C57BL/6 mice recover from HLI much better than BALB/c mice. We previously showed that a quantitative trait locus that spans a 31 million base pair region on the short arm of mouse chromosome 7 (termed LSq-1) was sufficient

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*These authors contributed equally to this article.

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to determine the extent of perfusion recovery and tissue loss after HLI, and using the outcome additional inbred mouse strains, gene blocks within this region were identified. The interleukin-21 receptor (IL-21R), whose modulation is being clinically studied in a variety of diseases, lies at or near the peak of the strength of association across this region based on locations and on the NCBI37/mm10 genome (Figure I in the online-only Data Supplement).

IL-21 is a type I cytokine whose receptor is 1 of 6 receptors that forms a heterodimeric receptor complex with the common \( \gamma \) chain (\( \gamma_c \)). IL-21 has pleiotropic actions and regulates the differentiation and function of lymphoid and myeloid cells. Because of its ability to regulate immune responses, enhancing or inhibiting the action of IL-21 or IL-21R has been demonstrated to have therapeutic effects on a wide range of diseases. To the best of our knowledge, there is no information for a role of IL-21R in PAD, but phase I and II clinical trials investigating the effects of administering IL-21 as an anticancer agent or blocking IL-21R in autoimmune disease are underway. Here, we present data suggesting that IL-21R upregulation and activation induce a protective effect in hypoxic endothelium, and modulation of IL-21R needs to be studied in PAD.

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Results**

**IL-21 Receptor mRNA Level Varies Greatly Between C57BL/6 and BALB/c in Ischemic Muscle Tissue**

Although C57BL/6 and BALB/c mice have different outcomes after HLI, the degree of perfusion recovery was comparable at an early (day 3) time point after HLI. We first studied 5 mice from each strain at day 3 post HLI by quantitative real-time polymerase chain reaction, \( \text{Il21r} \) mRNA level was much more highly upregulated in ischemic hindlimb muscles from C57BL/6 mice (\( \approx 39 \)-fold) than from BALB/c (\( \approx 1.7 \)-fold) mice (Figure 1A). Furthermore, the \( \text{Il21r} \) upregulation after ischemia in C57BL/6 mice was also found in earlier (day 1 post-HLI) and later (days 7 and 21, post-HLI) time points (Figure II in the online-only Data Supplement).

We sought to determine whether endothelial cells (ECs) contribute to the \( \text{Il21r} \) elevation after HLI, CD31\(^+\) cells

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**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AKT</td>
<td>protein kinase B</td>
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<tr>
<td>BAX</td>
<td>BCL-2-associated X protein</td>
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<tr>
<td>BCL-2</td>
<td>B-cell lymphoma leukemia-2</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>ERK1/2</td>
<td>extracellular signal–regulated kinases1/2</td>
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<tr>
<td>HLI</td>
<td>hindlimb ischemia</td>
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<tr>
<td>HSS</td>
<td>hypoxia and serum starvation</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>IL-21</td>
<td>interleukin-21</td>
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<tr>
<td>IL-21R</td>
<td>interleukin-21 receptor</td>
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<tr>
<td>PAD</td>
<td>peripheral arterial disease</td>
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<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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**Figure 1.** Interleukin-21 receptor (IL-21R) levels vary greatly between C57BL/6 and BALB/c mice after hindlimb ischemia (HLI). A, Three days after HLI, ischemic gastrocnemius muscle (IGA) from C57BL/6 mice showed upregulation (\( \approx 39 \)-fold) of \( \text{Il21r} \) mRNA level compared with nonischemic gastrocnemius muscle (NIGA). BALB/c mice also showed \( \text{Il21r} \) mRNA level upregulation (\( \approx 1.7 \)-fold) after HLI. However, the fold changes in BALB/c mice were significantly smaller than in C57BL/6 mice (\( P<0.001; n=5 \) per group). B, \( \text{Il21r} \) mRNA level of CD31\(^+\) cell isolated from IGA and NIGA, IL-21R level in CD31\(^+\) cells showed a significant increase in IGA compared with NIGA from C57BL/6 mouse, but did not show statistical difference from BALB/c mouse. KO indicates \( \text{Il21r} \) knockout; and NS, no statistical significance.
were isolated from C57BL/6 hindlimb\textsuperscript{23} and showed a higher IL-21R mRNA expression (≈159-fold, Figure 1B) in CD31\textsuperscript{+} cells from the ischemic side. Immunofluorescence of the ischemic muscle from C57BL/6 mice at day 3 post HLI showed numerous examples of co-staining of IL-21R with CD31 (Figure 1C). Moreover, flow cytometry showed that CD31\textsuperscript{+} fraction from ischemic hindlimb muscle had higher IL-21R protein level than the CD31\textsuperscript{+} fraction from nonischemic hindlimb muscle in C57BL/6 mice, but CD31\textsuperscript{+} fraction from BALB/c mice did not show a difference of IL-21R between ischemic and nonischemic muscle (Figure 1D). We also examined the levels of IL-21 protein by Western blotting using lysates from ischemic muscle from BALB/c and C57BL/6 mice, but did not find any difference (Figure III in the online-only Data Supplement). Thus, differences between C57BL/6 and BALB/c were at the level of the IL-21 receptor, not its ligand.

**IL-21R Activation Has Prosurvival Effects in ECs Under Hypoxia Serum Starvation Conditions**

The association of better outcomes after HLI in C57BL/6 mice compared with BALB/c mice and greater IL-21R expression in EC isolated from C57BL/6 ischemic muscle led us to look for an in vitro correlate. Using methods similar to those previously described,\textsuperscript{10} in vitro ECs (human umbilical vein endothelial cells [HUVECs]) showed ≈10 fold IL-21R expression (\textit{P}<0.05) when exposed to hypoxia serum starvation (HSS; Figure 2A). Under HSS conditions that induced IL-21 receptor upregulation, treatment of HUVEC with IL-21 (50 ng/mL) increased cell viability (Figure 2B), reduced cell apoptosis (Figure 2C), and enhanced tube formation (Figure 2D). The IL-21 effects on HUVEC viability, apoptosis, and tube formation were inhibited when IL-21R expression was knocked down using small hairpin RNA. All the above data are representative of 2 to 3 separate batches of HUVECs, n=8 to 12 samples per group. EV indicates empty vector; NS, no statistical significance; and Sh-IL21R, IL-21R knockdown using small hairpin RNA.

\textbf{Figure 2.} Interleukin-21 receptor (IL-21R) expression and the effect of IL-21 treatment in human umbilical vein endothelial cells (HUVECs). \textbf{A}, After 24 hours of hypoxia and serum starvation (HSS), quantitative real-time polymerase chain reaction showed ≈10-fold increase in IL21R mRNA expression when compared with HUVECs cultured under normoxia (normoxia indicates cells cultured under normal condition). \textbf{B}, More viable cells were detected after IL-21 (50 ng/mL) treatment for 24 hours in HSS conditions. Cell viability assay is based on the cleavage of the tetrazolium salt to formazan by cellular mitochondrial dehydrogenase. \textbf{C}, In situ terminal dUTP nick end-labeling (TUNEL) showed that IL-21 (50 ng/mL) treatment for 24 hours decreases HUVEC cell apoptosis in HSS conditions. \textbf{D}, HUVECs were plated on matrigel with reduced growth factor and incubated for 10 hours in HSS conditions with basal or treatment medium, IL-21–treated HUVECs showed enhanced tube formation, which was quantified as total length of the cords per visual filed, and total loops per visual field as represented by the bar graph. The IL-21 effects on HUVECs viability, apoptosis, and tube formation were inhibited when IL-21R expression was knocked down using small hairpin RNA. All the above data are representative of 2 to 3 separate batches of HUVECs, n=8 to 12 samples per group. EV indicates empty vector; NS, no statistical significance; and Sh-IL21R, IL-21R knockdown using small hairpin RNA.
apoptosis (Figure 2C), and enhanced endothelial tube formation in Matrigel models (Figure 2D). The specificity of these effects induced by IL-21 was demonstrated by the inhibition of survival benefit when using human IL21R small hairpin RNA (Figure 2B–2D; Figure IV in the online-only Data Supplement) and IL-21R-Fc fusion protein, respectively. Collectively, these data indicate a proangiogenic/antiapoptotic effect from activation of the IL-21/IL-21R axis in vitro in the setting of HSS. However, under normoxic conditions, IL-21 treatment did not change the survival of HUVECs (Figure V in the online-only Data Supplement).

Vascular smooth muscle cells and myocytes are also important cell types for the recovery from HLI. However, in a cultured, immortalized myocyte cell line (C2C12), IL-21R was expressed at a low level and did not show any difference in expression under hypoxic conditions (Figure VI in the online-only Data Supplement). Similarly, in cultured vascular smooth muscle cells, IL-21R was not detectable either in normoxic or hypoxic conditions. By immunofluorescence of ischemic gastrocnemius muscle, IL-21R and α-smooth muscle actin showed little overlap (Figure VII in the online-only Data Supplement).

IL-21 Treatment Regulates the Signal Transducer and Activator of Transcription 3 Pathway in ECs Under HSS

Signal transducer and activator of transcription (STAT) 1, STAT3, protein kinase B (AKT), and extracellular...
signal–regulated kinases1/2 (ERK1/2) pathways are activated by IL-21 in various cell types under different conditions. 19,25 To test whether these signaling pathways were activated by IL-21 treatment under HSS conditions, we performed Western blot analysis on protein lysates from HUVECs. After 24-hour exposure to HSS conditions, which induces IL-21R expression, HUVECs were treated with 50 ng/mL IL-21 for a series of time points. IL-21 induced peak increase of STAT3 phosphorylation 15 minutes after treatment, but did not show significant changes in STAT1, AKT1, or ERK1/2 phosphorylation at any time point (Figure VIII in the online-only Data Supplement). The IL-21–induced STAT3 phosphorylation was abolished by either IL-21R small hairpin RNA or Janus kinase 3 inhibitor (tofacitinib, 5 μmol/L; Figure 3A and 3B), which indicated that IL-21 activates STAT3 pathways via IL-21R and Janus kinase 3 in hypoxic ECs.

The ratio of B-cell lymphoma leukemia-2 (BCL-2)/BCL-2–associated X protein (BAX) expression has been reported to be regulated by STAT3 phosphorylation.26 To investigate whether BCL-2 or BAX expression was modulated in our experiment, HUVECs were treated with IL-21 under HSS conditions, which showed increased ratio of BCL-2/BAX 24 hour after treatment compared with vehicle treatment by Western blot analysis (Figure 3C). The specificity of the IL-21 effects was demonstrated by blunted BCL-2/BAX changes when using IL-21R small hairpin RNA (Figure 3B–3D).

We next investigate whether STAT3 phosphorylation induced by IL-21 signaling is required for the survival effects of IL-21 treatment in hypoxic ECs. And found that STAT3 inhibition blunted IL-21 induced cell viability, tube formation (Figure IX in the online-only Data Supplement), and BCL-2/BAX increase (Figure 3D). To determine whether the increase of BCL-2 level induced by IL-21 signaling is required for the protective effects in ECs under HSS, we reduced BCL-2 level using small interfering RNA (Figure X in the online-only Data Supplement). With BCL-2 knockdown, HUVECs incubated in IL-21 did not show significant change in cell viability and tube formation (Figure IX in the online-only Data Supplement).

### Table. Severity of Necrosis in Mice After Hindlimb Ischemia

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Mice With Any Necrosis</th>
<th>Necrosis Grade</th>
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<tr>
<td></td>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>Il21r (+/+)</td>
<td>9</td>
<td>2/9 (22)</td>
<td>2/9 (22)</td>
</tr>
<tr>
<td>Il21r (−/−)</td>
<td>12</td>
<td>5/12 (42)</td>
<td>3/12 (25)</td>
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Necrosis grade is from 0 through 4, as described in Methods. Values are expressed as number of mice affected in each category/total number of mice in the category (%). Distribution is to the right. Interleukin-21 receptor knockout mice showed a higher necrosis rate compared with the wild-type littermates after HLI, although not statistically different.
These data show that IL-21–mediated STAT3 phosphorylation and BCL-2 increase contribute to cell survival and tube formation in hypoxic ECs. Immunofluorescence staining shows that STAT3 and Caspase 3 were expressed in the ECs in the ischemic muscle tissue from HLI mouse model (Figure XI in the online-only Data Supplement).

Removal of IL-21 Ligand or Knockout of IL-21R Impairs Perfusion Recovery and Increases Tissue Loss, In Vivo, After HLI

We monitored perfusion recovery after HLI in Il21r−/− mice compared with age-matched and sex-matched wild-type (WT) littermates and found that Il21r−/− mice showed attenuated perfusion recovery starting at day 14 and continued through day 21 after HLI (Figure 4A). Consistent with poorer perfusion recovery, Il21r−/− mice tended to show higher, although not statistically different, rates of tissue loss (Table). Ischemic muscle tissue from Il21r−/− mice, 21 days after HLI, showed a lower capillary density than WT littermates (Figure 4C).

To determine whether IL-21R in leukocytes plays a role in the perfusion recovery after HLI, we measured the levels of several cytokines produced by leukocyte in the ischemic muscle tissue. Compared with WT mice, Il21r−/− mice do not show significant difference in the levels of monocyte chemoattractant protein-1 (protein level, 8.9±2.6 versus 8.6±1.1 pg/μg total protein), IL-6 (protein level, 1.2±0.4 versus 0.8±0.2, n=5 pg/μg total protein), and vascular endothelial growth factor-A (mRNA level by quantitative real-time polymerase chain reaction; \( P=0.53 \)). These may suggest that IL-21R knockout does not affect leukocyte secretion of certain cytokines.

The above data demonstrated that the IL-21R plays a role in perfusion recovery after HLI. To determine whether IL-21 ligand is also required, neutralizing IL-21R-Fc fusion protein was injected into WT C57BL/6 mice.27 Similar to the results seen with Il21r−/− mice, neutralization of IL-21 resulted in impaired perfusion recovery compared with vehicle-treated mice (Figure 4B).

We then sought to determine if the IL-21 pathways found in HUVEC under HSS are present in muscle tissue in vivo. Consistent with the in vitro results, knockout of IL-21R decreased STAT3 phosphorylation 1 day after HLI when compared with tissue from WT mice (Figure 5A). However, Il21r−/− mice did not show any statistical difference in the phosphorylation of the other 3 proteins. Furthermore, on day 7 after HLI, as assessed by Western blotting, Il21r−/− mice showed a lower BCL-2/BAX ratio in the ischemic muscle tissue than did WT littermates (Figure 5B).

Discussion

Our study describes a new role for the IL-21 receptor–ligand axis in modulating perfusion recovery after HLI via receptor activation in hypoxic ECs after HLI. IL-21 is known to play a key role in innate and adaptive immunity and signals via a heterodimeric receptor formed by IL-21R and \( \gamma_c \) and is known to regulate at least 4 pathways. 19,25 Three of these pathways (STAT3, ERK1/2, and AKT-1) are known to enhance cell survival and angiogenesis. BCL-2/BAX indicates B-cell lymphoma leukemia-2/BCL-2–associated X protein.

Figure 6. A schematic of proposed interleukin-21 (IL-21)/IL-21 receptor (IL-21R) signal in endothelial cells. A, From Castermans et al,22 under normoxic conditions with the presence of basic fibroblastic growth factor (b-FGF), IL-21 activates the signal transducer and activator of transcription 1 (STAT1) pathway, which results in angiostatic effects. B, From our study, under HSS conditions, IL-21R is upregulated; IL-21 promotes STAT3 signaling, leading to increased BCL-2/BAX ratio, and results in increased endothelial cell survival and angiogenesis. BCL-2/BAX indicates B-cell lymphoma leukemia-2/BCL-2–associated X protein.
survival and angiogenesis.\textsuperscript{28–30} However, STAT1 activation can induce EC apoptosis and inhibit proliferation.\textsuperscript{31} We began this study with the knowledge that the IL21 receptor was possibly implicated as influencing the extent of recovery after HLI based on prior quantitative trait locus analysis, but Castermans et al\textsuperscript{32} described that IL-21 ligand–mediated receptor activation inhibited basic fibroblast growth factor (b-FGF)–induced proliferation of cultured mouse ECs and decreased microvesSEL density within the tumors from a EG7 tumor-bearing mouse model, with the angiostatic properties being mediated through increased phosphorylation of STAT1 and decreased phosphorylation of STAT3. In totality, our study demonstrates that the effect of IL-21 on angiogenesis is likely to be context- and condition-dependent, with angiostatic effects being dominant when normal oxygen tensions are present and cytokines are abundant versus angiogenesis occurring when ECs are hypoxic and the receptor is upregulated and activated (Figure 6). Previous studies have shown that IL-21 receptor activation has proapoptotic effects on B cells when B-cell receptor signaling is absent; however, in the presence of B-cell receptor signaling, IL-21 induces cell proliferation and differentiation.\textsuperscript{33} Thus, there is precedent for differential effects by IL-21, depending on the context.

STAT3 is a transcription factor that is activated by several cytokines and growth factors. On activation, STAT3 becomes phosphorylated on tyrosine residue (Y705) and forms homodimers that translocate to the cell nucleus, where they modulate the transcription of target genes.\textsuperscript{34} STAT3 activation is found in ischemic tissue from a spectrum of ischemic diseases, including stroke and myocardial infarction, and functions as a protective factor to improve the recovery of these diseases.\textsuperscript{34,35} Interestingly, increased STAT3 phosphorylation was also found in the ischemic hindlimb muscle tissue when compared with muscle tissue from the healthy leg in our study (Figure V in the online-only Data Supplement). Recent studies demonstrate that STAT3 activation improves tissue recovery from ischemia by increasing the expression of the antiapoptotic gene BCL-2 and by decreasing expression of proapoptotic gene BAX.\textsuperscript{26,36} A higher BCL-2/BAX ratio is related to better perfusion recovery from HLI.\textsuperscript{37,38} Consistent with these findings, our data suggest that loss of IL-21/IL-21R signaling results in impaired perfusion recovery after HLI correlated with reduction of BCL-2/BAX ratio and inhibition of STAT3 activation (Figure 6).

ECs are a major cell type involved in the perfusion recovery after HLI. Our data showed an elevated level of IL-21R in EC fractions from the ischemic hindlimb tissue when compared with the ECs from nonischemic hindlimb tissue, in the mouse strain with good perfusion recovery. Consistent with this, elevated IL-21R expression was also found in cultured ECs when cultured in HSS conditions, which mimic the in vivo ischemic condition. IL-21 treatment of ECs in the conditions with IL-21R elevation results in improved cell survival, tube formation, and reduced cell apoptosis. The protective effects on ECs in HSS conditions are related to IL-21–induced BCL-2/BAX increase via STAT3 activation. For myocytes and vascular smooth muscle cell in vitro, IL-21R was expressed in low to undetectable levels and did not show a change with hypoxia. These data suggest that elevated IL-21R levels in EC in ischemic muscle are adaptive, and IL-21R activation in ECs contributes to the perfusion recovery after HLI.

Vascular endothelial growth factor is a potent stimulant for the induction of EC migration, proliferation, repair, and survival via the AKT or ERK1/2 pathways.\textsuperscript{39,40} However, the use of vascular endothelial growth factor in human trials has been largely unsuccessful in the treatment of PAD.\textsuperscript{41,42} This suggests that modulation of the AKT and ERK1/2 pathways may not be beneficial in PAD treatment. We have shown that IL-21–induced angiogenesis requires STAT3 rather than AKT or ERK1/2, and exploring this pathway may be promising as an approach to identify new treatment options for PAD treatment provided the receptor is present and accessible to ligand.

Our data have other potential clinical implications. The role of IL-21 in autoimmune disease is broad, contributing to development of type 1 diabetes mellitus, systemic lupus erythematosus, and experimental allergic uveitis in animal models,\textsuperscript{15,18,21,33} suggesting both humoral and cellular immunologic contributions to autoimmune disease but none of these would exclude the treatment of the majority of patients with PAD. Interestingly, antagonists of IL-21 are also being evaluated for treating autoimmune diseases or after organ transplantation. The data from our study may prove to be important to understand potential safety issues if the patients treated in these trials have, or develop, PAD.

Currently, listings at http://www.clinicaltrials.gov show that recombinant IL-21 is currently being tested in at least 12 different clinical trials mostly being used in various malignancies.\textsuperscript{37} Further studies of IL-21 treatment for HLI mouse model may provide the opportunity to allow for the rapid assessment of IL-21 to treat PAD. Collectively, our data indicate that IL-21R upregulation in response to HLI may well be adaptive, and that IL-21 signaling in ECs may contribute to improved perfusion recovery after HLI by activating the STAT3 pathway and increasing the BCL-2/BAX ratio. Thus, IL-21R is a potential target for pharmacologic modulation in PAD.

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### Disclosures

W.J. Leonard and R. Spolski are inventors on National Institutes of Health patents related to IL-21. The other authors report no conflicts.

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2. Owens CD, Conte MS. Medical management of peripheral arterial disease: bridging the “gap”? *Circulation*. 2012;126:1319–1321. doi: 10.1161/CIRCULATIONAHA.112.129692.


Peripheral arterial disease (PAD), caused by atherosclerosis that impairs blood flow to the lower extremities, is a major health problem. Currently, there are no medical therapies for PAD that have the ability to increase perfusion and correct the problem of impaired blood flow. In this study, we have shown that interleukin-21 receptor (IL-21R) expression is adaptively upregulated after hindlimb ischemia. Under ischemic conditions, either IL-21 ligand or receptor inhibition contributes to impaired perfusion recovery after experimental hindlimb ischemia (widely used as preclinical PAD model) through signal transducer and activator of transcription 3 pathway. Because of its potent effects in modulating endothelial cells, interleukin-21 (IL-21) was recently shown to be a potential therapeutic target in peripheral arterial disease (PAD) with significant efficacy in improving hindlimb perfusion in mice. However, the in vivo role of IL-21/IL-21R in PAD and potential therapeutic targets remains to be elucidated.

Significance

Peripheral arterial disease (PAD), caused by atherosclerosis that impairs blood flow to the lower extremities, is a major health problem. Currently, there are no medical therapies for PAD that have the ability to increase perfusion and correct the problem of impaired blood flow. In this study, we have shown that interleukin-21 receptor (IL-21R) expression is adaptively upregulated after hindlimb ischemia. Under ischemic conditions, either IL-21 ligand or receptor inhibition contributes to impaired perfusion recovery after experimental hindlimb ischemia (widely used as preclinical PAD model) through signal transducer and activator of transcription 3 pathway. Because of its potent effects in modulating endothelial cells, interleukin-21 (IL-21) was recently shown to be a potential therapeutic target in peripheral arterial disease (PAD) with significant efficacy in improving hindlimb perfusion in mice. However, the in vivo role of IL-21/IL-21R in PAD and potential therapeutic targets remains to be elucidated.
Loss of Interleukin-21 Receptor Activation in Hypoxic Endothelial Cells Impairs Perfusion Recovery After Hindlimb Ischemia

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). 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. 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.

4. Immunofluorescence

Immunofluorescent staining was performed as described previously. 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 previously2, 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 $1 \times 10^4$ cells/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 TdT’s 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 $1 \times 10^4$ 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 x 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 non-ischemic C57BL/6 hindlimb muscle with Dynabeads® magnetic separation (Life Technology, Grandland, NY) using methods similar to those previously described, 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, Tyr204), 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 determined 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. 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 using 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

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Supplemental Figures.

**Supplemental Figure I.** Modified from Dokun et al (Circ 2008), which shows the boundaries of Lsq-1 with the peaks of effects on necrosis and perfusion recovery. The exact coordinates of IL21R is 125.603252 --- 125.633570, which places the gene at/near the peak of this QTL. Genomic locations are based on the NCBI37/mm10 genome assembly.
Supplemental Figure II. To investigate whether IL-21R elevation after HLI in the time point other than 3 day, we checked the expression of IL-21R in ischemic and non-ischemic hindlimb muscle from C57BL/6 mice 1, 7, and 21 days after HLI. At each time point, IL-21R level was higher in the ischemic side than non-ischemic side. IGA indicates ischemic gastrocnemius muscle, NGA indicates non-ischemic gastrocnemius muscle. N=4~7/group. Data represent mean ± SEM.
**Supplemental Figure III.** IL-21 level in ischemic hindlimb muscle 3 days after HLI is not statistically different between Balb/c mice and C57BL/6 mice. BC, BALB/c mice; B6, C57BL/6 mice; IGA, ischemic gastrocnemius muscle; NGA, nonischemic gastrocnemius muscle. N=6/group. Data represent mean ± SEM.
Supplemental Figure IV. *In-vitro* knockdown of IL-21R in HUVECs. HUVECs were transfected with 1 µg/well (6 well dishes) of plasmid vector delivering shRNA targeting IL-21R or nonsense control. 24 hours after transfection, HUVECs were exposed to HSS conditions for an additional of 24 hours. Then *Il21r* mRNA level was measured with qPCR, shRNA knocked down expression of *Il21r* mRNA by 75 ± 13%. Data represent mean ± SEM. Data are representative of three experiments.
Supplemental Figure V. Under normoxia condition, IL-21 treatment did not change the viability of HUVECs.
Supplemental Figure VI. Expression of IL-21R mRNA was quantitated using qPCR in an immortalized myocyte cell line (C2C12) under normoxic and HSS conditions. Relative expression of IL-21R in C2C12 cells was not statistically different between these conditions. Data represent mean ± SEM; representative data from three different experiments using different passages of C2C12 cells.
Supplemental Figure VII. Immunofluorescence of ischemic gastrocnemius muscle, IL-21R (green), α-SMA(red) and merged from Wild Type (WT) C57BL/6 mice; co-staining of α-SMA and IL21R cannot be visualized in the ischemic muscle.
Supplemental Figure VIII. IL-21 affects STAT3 phosphorylation and BCL-2 BAX ratio in a time-dependent manner. (A) After exposed to HSS condition for 24h to induce IL-21R upregulation, HUVECs were treated with IL-21 for 5, 15, 30 and 60 minutes. Cell lysate were collected to measure STAT1, STAT3, AKT and ERK1/2 phosphorylation by Western Analysis. STAT3 showed a peak phosphorylation 15 minutes after IL-21 treatment; however, the other protein did not show a significant phosphorylation at any of the selected time point. (B) IL-21 stimulated BCL-2/BAX ratio increase at a time-dependent manner. HUVECs were treated with/without IL-21 for indicated time point, and showed a peak increase of BCL-2/BAX ratio compared to cells with IL-21 treatment for 24h.
**Supplemental Figure IX.** STAT3 inhibitor (S3I-201) blunted IL-21 induced cell viability (A) and tube formation (C) increase. When BCL-2 expression were knocked-down by SiRNA, IL-21 treatment did not show significant increase of cell viability (B) or tube formation (D) for HUVECs under HSS condition.
Supplemental Figure X. *In-vitro* knockdown of BCL-2 in HUVECs. HUVEC were transfected with BCL-2 SiRNA or its negative control. 24h after transfection, *bcl*-2 mRNA level were quantified by qPCR, which showed that *bcl*-2 mRNA were knocked down ~70% by SiRNA. Data represent mean ± SEM.
Supplemental Figure XI. Immunofluorescence of ischemic gastrocnemius muscle from C57BL/6 mouse. (A) Co-localization of STAT3 (green) and CD31 (red) can be found, as pointed by arrows. (B) Co-localization of Caspase 3 (green) and CD31 (red) can be found, as shown with arrows.
**Supplemental Figure XII.** STAT3 phosphorylation and BCL-2/BAX in ischemic and non-ischemic gastrocnemius muscle after HLI. (A) To determine whether STAT3 is activated after HLI, protein was isolated from ischemic and non-ischemic gastrocnemius muscle 1 day after HLI for western blotting of p-STAT3 and STAT3. The p-STAT3/STAT3 ratio was used to represent the degree of STAT3 activation, which showed that ischemic muscle has significantly higher STAT3 activation (p=0.02). n = 3/group, Data represent mean ±SEM. (B) BCL-2/BAX ratio is decreased in the ischemic gastrocnemius muscle after HLI when compared to non-ischemic gastrocnemius muscle. NGA = non-ischemic gastrocnemius muscle, IGA = ischemic gastrocnemius muscle.