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Endothelial interleukin-21 receptor up-regulation in peripheral artery disease

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Abstract

In most patients with symptomatic peripheral artery disease (PAD), severe stenosis in or occlusion of the major blood vessels that supply the legs make the amount of distal blood flow dependent on the capacity to induce angiogenesis and collateral vessel formation. Currently, there are no medications that improve perfusion to the ischemic limb, and thus directly treat the primary problem of PAD. A recent report from our group in a pre-clinical mouse PAD model showed that interleukin-21 receptor (IL-21R) is up-regulated in the endothelial cells from ischemic hindlimb muscle. We further showed that loss of IL-21R resulted in impaired perfusion recovery in this model. In our study, we sought to determine whether IL-21R is present in the endothelium from ischemic muscle of patients with PAD. Using human gastrocnemius muscle biopsies, we found increased levels of IL-21R in the skeletal muscle endothelial cells of patients with PAD compared to control individuals. Interestingly, PAD patients had approximately 1.7-fold higher levels of circulating IL-21. These data provide direct evidence that the IL-21R pathway is indeed up-regulated in patients with PAD. This pathway may serve as a therapeutic target for modulation.

Keywords

endothelial cells; IL-2R; interleukin-21 receptor; ischemia; PAD; peripheral artery disease

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Declaration of conflicting interests

The author(s) declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: WJL and RS are inventors on NIH patents related to IL-21.

Introduction

Peripheral artery disease (PAD) is a significant health problem that affects more than 8 million Americans.[1–4] PAD is caused by atherosclerotic narrowing in the arteries that supply blood to the lower extremity. In most patients with advanced PAD, blood flow to the lower extremity becomes dependent on ischemia-induced angiogenesis.[5,6] While current medical treatments for patients with PAD are directed toward limiting the complications of systemic atherosclerosis, there are no therapies that improve perfusion to the ischemic limb and directly treat the primary problem of PAD.[5] With millions of patients suffering from PAD, there is an urgent need to develop new therapeutic approaches.

The vascular response that follows surgically induced hindlimb ischemia (HLI) in mice is widely used in pre-clinical models of PAD.[7,8] We recently reported that interleukin-21 receptor (IL-21R) expression is significantly up-regulated in ischemic muscle in a mouse strain that demonstrates good perfusion recovery after HLI. Furthermore, using mRNA analysis from total muscle and an endothelial fraction, as well as flow cytometry and immunohistochemistry, the up-regulation was localized to endothelial cells in the distal hindlimb and loss of function of either IL-21R (IL-21R$^{-/-}$ vs wild-type (WT) controls) or IL-21 ligand (IL-21R-Fc chimera vs control antibody) reduced the extent of perfusion recovery.[9] These data suggest that IL-21R up-regulation and activation contributes to perfusion recovery after HLI.[9,10]

IL-21 plays a key role in innate and adaptive immunity signaling via the heterodimer of IL-21R and the common cytokine receptor γ chain (γc).[11] Recombinant IL-21 has been studied in at least 12 human clinical trials, and IL-21-specific antibodies are entering human studies to limit transplant rejection.[11,12] Our study was designed to determine whether IL-21R is up-regulated in the ischemic muscle tissue in PAD patients, particularly in the endothelium, and thus could potentially serve as a target for ligand therapy. We further sought to determine whether circulating levels of IL-21 ligand were different between patients with PAD when compared to age- and gender-matched healthy patients.

Materials and methods

The inclusion criteria for PAD patients were the same as previously described[13,14]: ankle–brachial index (ABI) <0.90 at rest, exercise limited by claudication, a 20% decrease in ABI after exercise or angiographic evidence of PAD. Patients with PAD were excluded if their medical history indicated critical limb ischemia, severe peripheral neuropathy, revascularization within 3 months, unstable angina, severe coronary vessel disease (>70% stenosis of at least one vessel), or diabetes. Age- and gender-matched controls were free of symptomatic coronary disease as indicated by their physical exam, history and ECG tracing. Control subjects had ABI values above 1.00 and no symptoms of intermittent claudication. Female participants were postmenopausal. All studies were performed under research protocols approved by Duke University and University of Virginia Institutional Review Boards.

Muscle biopsy and immunofluorescence

For the IL-21R tissue expression study, 16 muscle biopsies were randomly selected (PAD = 9, age- and gender-matched healthy controls = 7; age range = 48–80 years; male/female ratio of approximately 1 in each group) from a biorepository available through prior NIH-funded studies at Duke University Medical Center.[14] Skeletal muscle biopsy procedures were performed as described previously.[9,15] Briefly, biopsy samples were obtained from the medial side of the gastrocnemius muscle. The biopsy site was anesthetized with a 2% lidocaine solution, and a 1.0-cm incision was made through the skin and gastrocnemius fascia. A modified Bergstrom needle technique was inserted 10–15 mm and used to obtain 40–50 mg of skeletal muscle. Samples were embedded in optimum cutting temperature compound. Frozen sections (7 μm) were cut using a Leica CM-1950 cryostat and placed on positively charged slides. The slides were stored at −80°C until they were needed.

Quantifying IL-21R expression in muscle samples by western blot analysis would have been the preferred method; however, there is no anti-human IL-21R antibody suitable for use in western blot. The immunofluorescence procedures used were performed as previously described.[9] Briefly, muscle specimen cryosections were fixed with acetone and then blocked with 5% donkey serum (cat. no. 017-000-121; Jackson-Immuno Research Laboratories, West Grove, PA, USA) for 60 minutes. After that, anti-IL-21R antibody (1:100; polyclonal rabbit Ab; cat. no. ab5980; Abcam, Cambridge, MA, USA), anti-CD31 antibody (1:100; mouse anti-human CD31/PECAM-1 antibody (JC/70A); cat. no. MA5-13188; Thermo Fisher Scientific, Rockford, IL, USA) and/or anti-phospho-STAT3 (1:50; goat anti-Tyr 705; cat. no. sc-7993; Santa Cruz Biotechnology, Dallas, TX, USA) were applied at 4°C overnight in a blocking solution. To test the specificity of the IL-21R antibody, an IL-21R blocking peptide was incubated with the IL-21R primary antibody (blocking peptide:antibody = 10:1 molar ratio). After rinsing with phosphate-buffered saline (PBS), secondary antibodies including Alexa Fluor 555 donkey-anti-rabbit (1:200; cat. no. A-31572; Invitrogen, Carlsbad, CA), Alexa Fluor 488 donkey-anti-mouse (1:200; cat. no. A21202; Invitrogen) and Alexa Fluor 647 donkey-anti-goat (1:200; cat. no. A-21447; Invitrogen) were applied for 1 hour at room temperature. Sections were then rinsed with PBS and mounted with Prolong Gold anti-fade reagent with DAPI mounting medium (cat. no. P36935; Life Technologies, Eugene, OR, USA). Secondary antibodies alone (without primary antibody) were used as negative controls to assess non-specific binding. Slides without primary or secondary antibody were used to assess for auto-fluorescence from tissue sections. Stained sections were examined with 200× magnification using an Olympus BX51 high-magnification microscope and Zeiss LSM 510-META/FCS confocal microscope. Each individual fluorescence channel was imaged with the same setting; pictures were then merged from each individual channel to determine co-staining.

IL-21R+ staining was determined by signal shown in the slides incubated with both IL-21R primary and appropriate secondary antibodies, but not shown in the slides incubated with secondary antibody alone. We quantitatively assessed the IL-21R levels by counting IL-21R + staining at three random high-power (200× magnification) fields in muscle sections. Total IL-21R expression in the muscle biopsies was expressed as the number of IL-21R+ cells per total nuclei in all cell types in the tissue. To identify IL-21R expression in endothelial cells,

cells co-stained with both IL-21R and CD31 were counted and expressed as the percentage of IL-21R and CD31 double positive cells per total CD31+ cells. The images were analyzed using Image-Pro Plus 7.0 software by an observer (A.C.) who was blinded to the staining and grouping of muscle specimens.

Enzyme-linked immunosorbent assay (ELISA)

For the IL-21 ligand level study, 20 PAD patients and 20 healthy controls from the University of Virginia Hospital were included (Table 1). Plasma was isolated from the whole blood sample, and plasma IL-21 levels were determined using an enzyme-linked immunosorbent assay (ELISA) (cat. no. 433808; Biolegend, San Diego, CA, USA) in accordance with the manufacturer's instructions. All studies were performed under research protocols approved by the University of Virginia Institutional Review Boards.

Statistical analysis

The groups were age- and sex-matched for both skeletal muscle biopsy and plasma IL-21 quantitation. Differences between PAD patients and controls were determined using the Student's *t*-test. All data are presented as mean ± SEM. For all tests, *p*-values <0.05 were considered significant.

Results

IL-21R expression is higher in the endothelium from PAD muscle biopsy specimens

In previous work we demonstrated that IL-21R expression is increased in distal ischemic leg muscle compared to non-ischemic leg muscle in mice with good perfusion recovery following experimental HLI.[8] Results from this study indicate an approximately threefold higher ratio of IL-21R+/nucleus in muscle specimens from PAD patients (0.18 ± 0.02, $n = 9$) compared with muscle from the healthy control limbs (0.06 ± 0.01, $n = 7$, $p<0.001$) (Figure 1A). Endothelial cells in muscle specimens from PAD patients had approximately a fivefold higher percentage of IL-21R+ staining (35.4 ± 9.4, $n = 9$) than endothelial cells from healthy controls (6.9 ± 2.2, $n = 7$, $p = 0.016$) (Figure 1B). This demonstrates that IL-21R is up-regulated in endothelial cells and muscle biopsy tissue from the most symptomatic leg in PAD patients compared to control subjects.

STAT3 phosphorylation is detected in IL-21R+ endothelial cells

We previously showed that endothelial cells from distal mouse muscle tissue and in vitro cultures have up-regulated IL-21R expression under ischemic conditions.[9] Under ischemic conditions, ligand binding to IL-21R subsequently activates the STAT3 signaling pathway which protects endothelial cells from apoptosis.[9] Having shown that IL-21R is up-regulated in endothelial cells from human PAD ischemic muscle samples, we sought to determine whether STAT3 is subsequently activated (phosphorylated) in these samples. We co-stained phosphorylated STAT3, CD31 and IL-21R, which showed STAT3 phosphorylation in IL-21R+ endothelial cells (Figure 2).

Blood IL-21 ligand level is higher in PAD patients

To date, there is no data on IL-21 levels in human samples from PAD patients compared to controls. Using a new cohort to ensure use of fresh plasma samples, we found that PAD patients had a higher plasma concentration of IL-21 compared to healthy controls (66.50 ± 8.05 pg/mL vs 37.79 ± 4.60 pg/mL, $p = 0.001$; Figure 3).

Discussion

The IL-21 receptor was initially discovered in immune cells, where it was shown to regulate lymphoid and myeloid cell proliferation and differentiation.[11] In this study, we showed that IL-21R expression is up-regulated in endothelial cells from calf muscle biopsies in PAD patients. These data are consistent with our results from the distal muscle in mice with HLI and endothelial cells cultured under hypoxic conditions.[9]

In a pre-clinical model of PAD, we showed that blocking IL-21R using two complimentary methods reduced perfusion recovery and the extent of angiogenesis following HLI.[9] Blocking IL-21R activation by using either IL-21 or IL-21R-specific antibodies is being assessed in human studies to limit transplant rejection. As PAD may co-exist in patients requiring organ transplantation, it is important to assess whether loss of function of this pathway could have detrimental effects for patients with PAD.

In this report, we not only found the receptor to be up-regulated in PAD patients versus control calf muscle biopsies, but we found the increased IL-21R expression to be predominantly in the endothelium from the most symptomatic leg, which parallels what we found in mice with HLI.[9] Interestingly, the IL-21 ligand level is higher in blood from PAD patients when compared to healthy controls. Given the fact that loss of IL-21 ligand or receptor results in impaired perfusion in mice,[9] it is certainly plausible that increased endothelial cell IL-21R expression in ischemic muscle and elevated blood IL-21 ligand levels in PAD patients may be at least partially adaptive. The presence of IL-21R in ischemic muscle may establish systemic IL-21 as a potential strategy to treat PAD.

It has been shown that IL-21R functions mainly through STAT, which is a group of transcription factors.[16] On activation, STAT becomes phosphorylated on tyrosine residue and forms homodimers that translocate to the cell nucleus, where they modulate the transcription of target genes.[16] STAT1 activation has been reported to induce endothelial apoptosis and inhibit proliferation.[17] A recent study in a cancer model showed IL-21R is expressed in mouse endothelial cells and IL-21 ligand-mediated receptor activation has ‘angiostatic effects’ through STAT1 pathway activation in endothelial cells.[18] 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 recovery from these diseases.[19] In previous work, we found that IL-21R expression is up-regulated in response to ischemia in both HLI mouse models and cultured human endothelial cells, and under these conditions, activation of the IL-21R by IL-21 increases angiogenesis through activation of the STAT3 pathway.[9] Exactly how IL-21R expression has divergent effects on endothelial cells based on the presence or absence of hypoxia remains unknown. Nevertheless, we did find evidence for STAT3 activation (p-STAT3) in the endothelium of IL-21R+/CD31+ cells, suggesting

that this pathway may be activated in humans with PAD. Whether increased ligand-mediated IL-21R signaling would have beneficial effects in PAD patients may be worth further investigation.

Limitations

Owing to a lack of an antibody suitable for use in western blots, we quantified IL-21R levels by immunofluorescence. Sample numbers were relatively small but all values had reasonable levels of significance. Finally, not knowing the stability of IL-21 in plasma, the samples used to determine plasma IL-21 levels were obtained from a different group of patients than those used for the muscle biopsies. Despite these limitations our study provides human data supporting up-regulation of IL-21R expression in the endothelial cells from distal limb muscle of patients with PAD. The differential expression of this receptor in PAD may serve as a future therapeutic target.

Conclusion

In summary, we have demonstrated increased IL-21R expression in endothelial cells from gastrocnemius muscle biopsies and elevated circulating IL-21 levels in PAD patients. Taken together with our previous study of mice with HLI, these data suggest IL-21 ligand-receptor function could serve as a pathway to protect and treat PAD.

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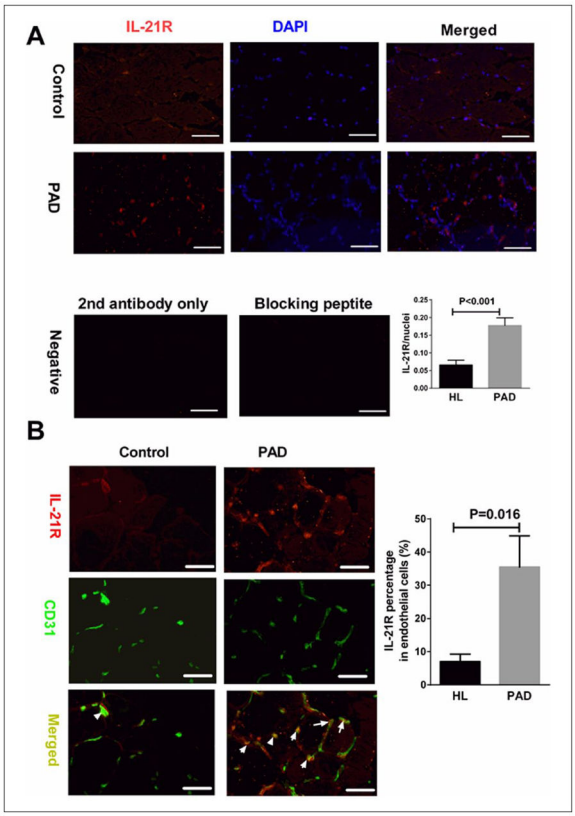

Figure 1.

Interleukin-21 receptor (IL-21R) levels were significantly higher in the ischemic limb from patients with peripheral artery disease (PAD) compared to healthy controls. (A) Ischemic limbs of PAD patients showed a higher ratio of IL-21R positive cells/nucleus (0.18 ± 0.02, n = 9) compared to non-ischemic limbs (0.06 ± 0.01, n = 7, p<0.001). (B) Immunofluorescence of muscle specimens from PAD patients and healthy controls: IL-21R (red), CD31 (green, endothelial cell marker) and merged (yellow). IL-21R staining was weaker and the ratio of IL-21R+ cells was lower in the non-ischemic muscle. A quantitative analysis shows that among the endothelial cells (CD31+), the percentage of IL-21R+ cells are significantly higher in the ischemic muscle from PAD patients compared to controls. PAD indicates ischemic muscle specimens from PAD patients; control indicates non-ischemic muscle from healthy controls. Scar bar = 50 μm.

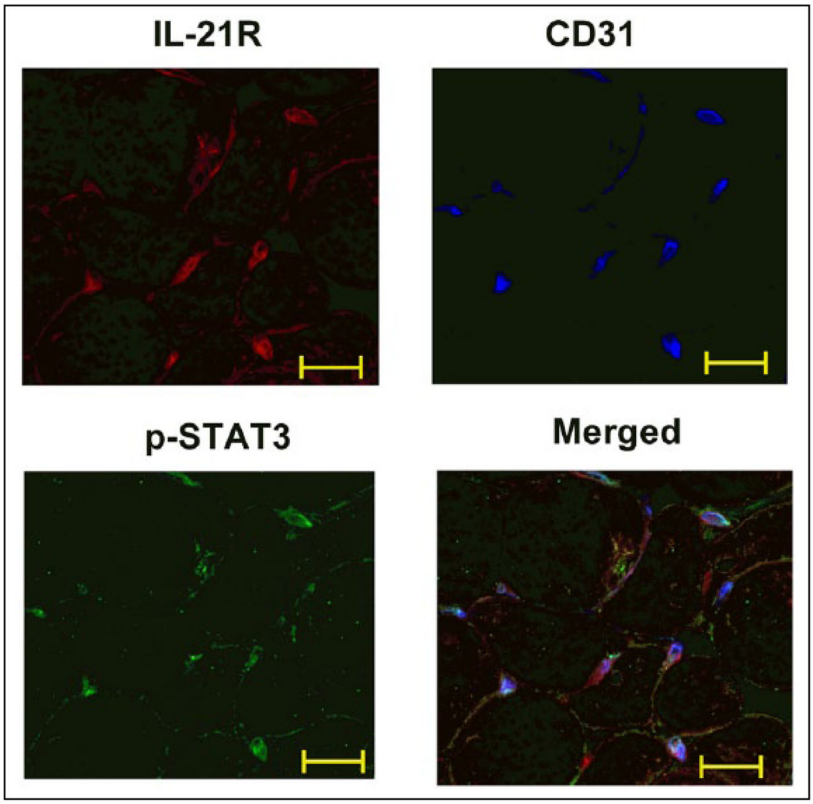

Figure 2.

Immunofluorescence of phosphorylated STAT3 (p-STAT3, green), interleukin-21 receptor (IL-21R) (red), and CD31 (blue) in the ischemic muscle from patients with peripheral artery disease (PAD). Phosphorylated STAT3 is detected in IL-21R+ endothelial cells in human PAD muscle specimen. Scar bar = 50 μm.

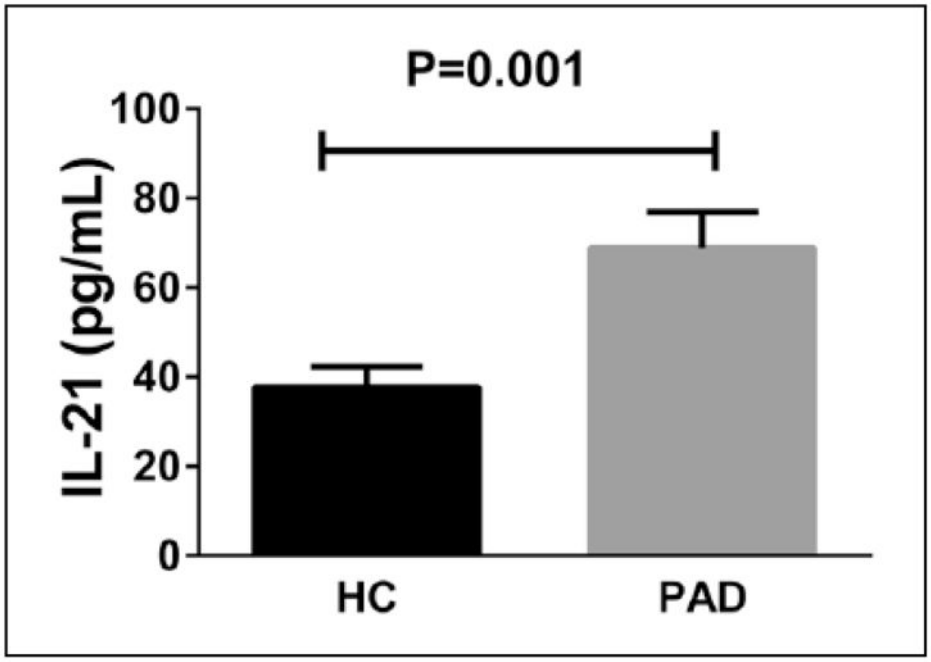

Figure 3.

Interleukin-21 (IL-21) ligand level is significantly higher in the blood from patients with peripheral artery disease (PAD) compared to healthy controls (HC) (p=0.001, n=19–20/group). The IL-21 level was measured by ELISA using capture and detecting antibodies.

Table 1

Participant characteristics (mean ± SEM).

Patient information	Control (*n*=20)	PAD (*n*=20)
ABI	1.06 ± 0.028	0.63 ± 0.05 **
Gender (F/M)	12-Aug	11-Sep
Age (year)	67.1 ± 2.4	66.2 ± 2.1
Diabetes Mellitus (%)	40	40
Hypertension (%)	100	90

** $p<0.01$.