VASCULAR BIOLOGY, ATHEROSCLEROSIS, AND ENDOTHELIUM BIOLOGY

Essential Role of IL-12 in Angiogenesis in Type 2 Diabetes

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Recently, IL-12 emerged as a critical player in type 2 diabetes complications. We previously reported that ischemia-induced angiogenesis is compromised in type 2 diabetic mice. In this study, we determined that IL-12 disruption rescued angiogenesis and arteriogenesis in type 2 diabetic mice. To induce type 2 diabetes, wild-type (WT), p40IL-12/−/− (p40−/−), and p35IL-12/−/− (p35−/−) mice were fed a high-fat diet (HFD) for 12 weeks. Body weight, glucose test tolerance, and insulin test tolerance were assessed. After 12 weeks of an HFD, the femoral artery was ligated and blood flow recovery was measured every week for 4 weeks. WT, p40−/−, and p35−/− mice fed an HFD became obese after 12 weeks and exhibit glucose intolerance and insulin resistance. Blood flow recovery was fully restored in 2 to 3 weeks after femoral artery ligation in all groups of mice fed a normal diet. However, after 12 weeks of an HFD, blood flow recovery was compromised in WT mice, whereas it was fully recovered in p40−/− and p35−/− mice. The mechanism of blood flow recovery involves an increase in capillary/arteriole density, endothelial nitric oxide synthase/Akt/vascular endothelial growth factor receptor 2 signaling, and a reduction in oxidative stress and inflammation. The disruption of IL-12 promotes angiogenesis and increases blood flow recovery in obese type 2 diabetic mice by an endothelial nitric oxide synthase/Akt/vascular endothelial growth factor receptor 2/oxidative stress—flammation—dependent mechanism. (Am J Pathol 2017, 187: 2590–2601; http://dx.doi.org/10.1016/j.ajpath.2017.07.021)

Diabetes mellitus affects >347 million individuals worldwide, leading to >4.6 million deaths each year, and its prevalence substantially continues to increase globally.

Cardiovascular dysfunctions are the primary chronic complications, which cause tremendous morbidity, disability, and mortality in patients with type 2 diabetes. Approximately 80% of deaths in patients with diabetes are closely related to vascular complications. Diabetes affects the function and the structure of the small and large blood vessels. The development of new vessels in ischemic organs is blunted in diabetic patients, which contributes to delayed wound healing, increased risk of rejection of transplanted organs, exacerbated peripheral limb ischemia (foot ulceration and amputation), and even cardiac mortality. Moreover, the mechanisms underlying diabetes-impaired angiogenesis are not completely understood.

We previously reported that angiogenesis is blunted in type 2 diabetic mice under chronic ischemia in the hind limb because of inflammation. Recently, inflammation has emerged as a central contributor to the progression of insulin resistance and the pathogenesis of type 2 diabetes and its vascular complications. IL-12 is a heterodimeric cytokine composed of two disulfide-linked protein subunits, p35 and p40, produced by dendritic cells, macrophages, and natural killer cells. IL-12 has been shown to increase in type 2 diabetes and has been involved in the pathogenesis of atherosclerosis, macrovascular complications, and diabetic retinopathy. However, little is known about whether IL-12 plays a role in the process of ischemia-induced angiogenesis and arteriogenesis in type 2 diabetes.

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Accordingly, using genetically deficient p40\(^{-/-}\) and p35\(^{-/-}\) mice, we examined whether IL-12 is involved in ischemia-induced neovascularization in the setting of type 2 diabetes. We used a well-established mouse model of neovascularization with unilateral hind-limb chronic ischemia and a high-fat diet (HFD) to induce type 2 diabetes.\(^2,15\)

Our aim was to determine the role and mechanism of IL-12 in ischemia-induced neovascularization in mice fed an HFD. We hypothesized that the disruption of IL-12 is an important mechanism that triggers angiogenesis, which protects tissues against chronic ischemia in type 2 diabetes.

**Materials and Methods**

**Animal Model and Hind-Limb Ischemia Surgery**

C57BL/6J males [wild type (WT)], p40\(^{-/-}\), and p35\(^{-/-}\) on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, ME). To induce diabetes, mice were fed an HFD, 60% high fat [TD.06414 Adjusted Calories Diet 60/Fat; ENVIGO (Teklad, Madison, Wisconsin)] for 12 weeks.\(^2,15\) After 12 weeks, hind-limb ischemia was performed.

Hind-limb ischemia was induced in all mice, as previously reported,\(^5-7\) by ligating the left common femoral artery proximal to the origin of the profunda femoris artery. Mice were anesthetized with 1% to 3% of isoflurane, and a skin incision was made at the left hind limb of the mice. The common femoral vein and nerve were dissected and separated from the femoral artery. The femoral artery was ligated, and then the incision was sutured. These studies were performed according to the principles of the NIH Guide for the Care and Use of Laboratory Animals\(^16\) and were approved by the Eastern Virginia Medical School (Norfolk, VA) Institutional Animal Care and Use Committee.

**Body Weight and Fasting Blood Glucose**

Body weight and fasting blood glucose level were measured once a week for 12 weeks.

**Glucose Tolerance Test and Insulin Tolerance Test**

The glucose tolerance test and insulin tolerance test were performed once a month for 3 months.

**Glucose Tolerance Test**

Mice were fasted overnight. Blood samples were obtained from the tail vein using an Ultra Touch glucometer (True test; Trivida Health Inc, Fort Lauderdale, FL). Mice then received an i.p. injection of 2 g/kg glucose. Then, tail vein lancing (using a small sterile needle) was used to draw blood (approximately 2 μL) to determine blood glucose levels. Blood glucose levels were measured with an Ultra Touch glucometer at 0, 10, 20, 30, 60, 90, and 120 minutes after glucose injection.

**Insulin Tolerance Test**

Mice were fasted for 6 hours and then injected with 1 U/kg i.p. of insulin. Then, blood glucose was measured at 15, 30, 45, and 60 minutes after insulin injection.

**Laser Doppler Measurement of Hind-Limb Blood Flow**

Each mouse was warmed to a core temperature of 37°C. Then, hind-limb blood flow measurements were performed over the region of interest before surgery, immediately after surgery, and serially over the 4-week period with laser Doppler perfusion imaging (Moor Instruments, Wilmington, DE).\(^5-7\)

**In Vivo Treatment with IL-12**

One week before femoral ligation, p40\(^{-/-}\) mice fed an HFD were given injections of recombinant murine IL-12 (i.p., 25 ng, three times per week) for a period of 4 weeks. IL-12 was purchased from Peprotech (Rocky Hill, NJ).

**IL-12 and Migration of Vascular Smooth Muscle Cells**

Vascular smooth muscle cells were cultured in the complete growth medium (Dulbecco’s modified Eagle’s medium) to confluence in a humidified incubator containing 5% CO\(_2\) at 37°C. Confluent cell monolayers were incubated for 24 hours with the starvation media (low-glucose Dulbecco’s modified Eagle’s medium without fetal bovine serum) before the experiments. Then, they were scraped with a 200-μL standard pipette tip to generate scratch wounds and washed three times with phosphate-buffered saline to remove any loosely attached cells. The wounded monolayers were then incubated for 24 hours after treatment with and without 50 ng of IL-12. Images of injured areas at 0 and 24 hours were taken at \(\times 10\) magnification.

**Enzyme-Linked Immunosorbent Assay and Kits**

All of the assays and kits were used according to the manufacturer’s recommendations. IL-12, IL-10, IL-6, and monocyte chemoattractant protein (MCP)-1 enzyme-linked immunosorbent assay kits were purchased from BioLegend (San Diego, CA). Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were purchased from R&D Systems (Minneapolis, MN). The thiobarbituric acid reactive substances assay kit was purchased from Cayman Chemical (Ann Arbor, MI).

**Western Blot Analysis**

Hind-limb tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA) containing a cocktail of protease and phosphatase inhibitors. Equal amounts of protein were loaded into polyacrylamide-SDS gels (Bio-Rad Laboratories Inc., Des Plaines, IL) and transferred onto nitrocellulose
membranes (Bio-Rad Laboratories Inc.). The blots were blocked with 5% bovine serum albumin for 1 hour and probed with primary antibodies for target proteins overnight at 4°C. Immunoblots were next probed with the fluorophore-labeled secondary antibodies (LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature. Final protein expression was detected using the Odyssey imaging system (LI-COR Biosciences) and quantified using ImageJ software version 1.51 (NIH, Bethesda, MD; http://imagej.nih.gov/ij). Antibodies for phosphorylated endothelial nitric oxide synthase (eNOS) (serine 1177), phosphorylated VEGF receptor (VEGFR) 2, total Akt, phosphorylated Akt, total STAT4, NADPH oxidase isofrom (Nox)2, and Nox4 were purchased from Cell Signaling Technology (Danvers, MA). Antibodies for phosphorylated STAT4, total Akt, total STAT3, total JNK, total PKC, total PI3K, total Erk, phosphorylated Akt, total STAT4, NADPH oxidase were measured in lysates of ischemic muscles using a lucigenin chemiluminescence assay. Briefly, ischemic muscles for hind-limb muscles from all groups. The RNA was then reverse transcribed using NEB M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA) and subjected to real-time quantitative PCR using the TaqMan and Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Real-time quantitative PCR was performed in triplicate using the following TaqMan assays (Thermo Fisher Scientific): Nox2 (Mm01287743_m1), Nox4 (Mm00479246_m1), tumor necrosis factor (TNF)-α (Mm00432585_m1), p40 (Mm00434174_m1), p35 (Mm.PT.5813818295), IL-10 (Mm.PT.58 13531087), interferon (IFN)-γ (Mm.PT.5841769240), arginase-1 (Mm.PT.588651372), Mannose receptor C type 1 (Mm.PT.5831835913), C-type lectin domain containing 10A (Clec10a; Mm.PT.5831835913), and chemokine (C-C motif) ligand (Mm.PT.5842151692). A two-step reaction protocol was used with an initial denaturation of 1 minute at 95°C, followed by 40 cycles of 95°C for 15 seconds, then 60°C for 45 seconds, using the Real-Time Thermal Cycler CFX96 Optics Module (Bio-Rad Laboratories Inc.). Final normalized gene expression was calculated using α-actin mRNA as an endogenous control.

Statistical Analysis

Results are expressed as means ± SEM. One- and two-way analysis of variance were used when appropriate. Comparisons between groups were performed with t-tests when the analysis of variance test was statistically significant. P < 0.05 was considered significant.

Results

In WT, p40−/−, and p35−/− mice fed with an HFD for 12 weeks, a similar increase in the body weight and blood glucose levels (Figure 1, A and B) was observed. The glucose tolerance was similar between WT and p40−/− mice fed a chow diet (Figure 1, C and E). However, glucose tolerance for a chow diet was higher within the first 60 minutes in p35−/− mice compared with WT and p40−/− mice (Figure 1, C, E, and G). The insulin tolerance was similar in all mice fed a chow diet (Figure 1, D, F, and H). When these mice were fed an HFD for 12 weeks, the glucose test tolerance was compromised in WT and p40−/− mice but exacerbated in the p35−/− mice (Figure 1, C, E, and G). However, the insulin test tolerance was reduced in all groups of mice (Figure 1, D, F, and H). These results showed that HFD induced insulin resistance in WT, p40−/−, and p35−/− mice. Our data suggest that p35 could be involved in the etiology of obesity and type 2 diabetes induced by HFD feeding.

To determine the in vivo contribution of IL-12 on angiogenesis, femoral artery ligation was performed in WT, p40−/−, and p35−/− mice fed a chow diet and an HFD. Laser Doppler perfusion imaging was performed to measure blood flow before and immediately after femoral artery...
Data are expressed as means ± SEM (A–H). *P < 0.05 versus WT fed a normal diet; †P < 0.05 versus p40−/− fed a normal diet; ‡P < 0.05 versus p35−/− fed a normal diet.

Figure 2C illustrates representative blood flow in all mice fed either a chow diet or an HFD before and after femoral artery ligation. These data indicate that the deletion of IL-12 protects the hind-limb muscle from chronic ischemia by promoting angiogenesis/arteriogenesis and, therefore, blood flow perfusion. To demonstrate that the stimulation of angiogenesis observed in mice lacking IL-12 and fed an HFD was because of the IL-12 deficiency, we tested the effect of administrating exogenous IL-12 (25 ng per mouse) to p40−/− mice fed an HFD 1 week before femoral artery ligation and 1, 2, and 3 weeks after. Blood flow recovery was impaired in p40−/− mice receiving IL-12 after femoral artery ligation compared with untreated p40−/− mice fed an HFD (Figure 2, B and C). Indeed, the administration of IL-12 to p40−/− mice blunted the promotion of angiogenesis. Together, these findings support the critical role of IL-12 in impaired angiogenesis in type 2 diabetes.

The blood flow data were supported by the blood vessel density measurement assessed by immunostaining in hind-limb muscle. The capillary density determined by CD31 (a specific marker for the endothelial cell) staining was significantly reduced in WT mice fed an HFD compared with all other groups of mice (Figure 2, A and B). For a normal chow diet, total blood flow recovery was faster in p35−/− mice (2 weeks) compared with WT and p40−/− mice (3 weeks) (Figure 2A).

Blood flow recovery was significantly compromised in WT mice fed an HFD compared with WT fed a chow diet. The recovery reached only 50% within 4 weeks after the femoral artery ligation (Figure 2B), whereas it reached 100% in p40−/− and p35−/− mice fed an HFD within 2 to 3 weeks (Figure 2B).

VEGF and bFGF are factors known to play a major role in angiogenesis. The tissue level of VEGF and bFGF was measured in the ischemic hind limb of all mice. VEGF and bFGF levels were significantly increased in WT mice fed an HFD compared with WT, p40−/−, and p35−/− mice fed a Chow diet (Figure 2, G and H). Interestingly, VEGF and bFGF levels were reduced in the ischemic limb of p40−/− and p35−/− mice fed an HFD (Figure 2, G and H).

To demonstrate the antiangiogenic effect of IL-12, we performed an in vitro cell injury and migration assay. We found that exogenous IL-12 significantly blunted the migration of smooth muscle cells (Figure 2, I and J). Together, these results established the following: the excess in IL-12 in type 2 diabetic mice blunts angiogenesis and arteriogenesis in response to chronic ischemia; and the disruption of IL-12 triggers the angiogenesis and arteriogenesis events in response to chronic ischemia.

Figure 3, A and B, illustrate the increase in p35 and p40 mRNA levels in the hind-limb muscle from WT mice fed an HFD compared with WT mice fed a chow diet. These results indicate that IL-12 (p40 and p35) was up-regulated in type 2 diabetes.
It is well documented that macrophages are the key cell type in orchestrating the angiogenic and healing responses in ischemic tissues. 20 On the basis of the activation route, macrophages can generally be divided into two major populations: M1 proinflammatory macrophages and M2 anti-inflammatory/proangiogenic macrophages. To determine the status of M1/M2 macrophages in our model of HFD-induced diabetes, we analyzed the expression of genes reflecting...
proinflammatory and anti-inflammatory macrophages in ischemic limbs of WT, p40, and p35 mice. INF-γ and TNF-α, which are downstream signaling to IL-12, were increased in WT mice fed an HFD and reduced in p40 and p35 mice fed an HFD (Figure 3, C and D), suggesting a role of IL-12 in proinflammatory cytokine regulation. Expressions of anti-inflammatory factors and markers for M2 macrophages, arginase-1, IL-10, Clec10a, and MRC1 were all increased in the ischemic limb of p40 and p35 mice fed either a chow diet or an HFD compared with WT mice fed an HFD or a chow diet (Figure 3, E–H).

Moreover, we assessed the levels of IL-12, IL-10, IL-6, and MCP-1 cytokines at the protein levels by enzyme-linked immunosorbent assay. IL-12 was only detected in the ischemic limb of WT mice (Figure 4A). Consistent with previous studies, our results confirmed that p40 and p35 mice were unable to make the bioactive IL-12 cytokine and also showed that an HFD induced an up-regulation of IL-12 in the ischemic limb of WT mice. The IL-10 cytokine was decreased in the ischemic limb of WT mice fed an HFD compared with WT mice fed a chow diet but prevented in p40 and p35 mice (Figure 4B). Moreover, an HFD induced an increase of the IL-6 level in the ischemic limb of WT mice; this was blunted in p40 and p35 mice (Figure 4C).

Interestingly, an HFD increased the level of MCP-1 in the ischemic limb of WT, p40, and p35 mice. Unlike IL-6, MCP-1 level was not affected by p40 or p35 deletion (Figure 4D).

Together, our data showed that IL-12 deficiency up-regulated the expression of anti-inflammatory cytokines and reduced the expression of proinflammatory cytokines in the ischemic limb of type 2 diabetic mice.

Using p40 and p35 antibodies, immunohistochemical analysis confirmed our real-time quantitative RT-PCR data showing the up-regulation of p40 and p35 mRNA levels in the ischemic limb of WT mice fed an HFD (Figure 5, A, D, F, and G).

We also demonstrated that the number of F4/80-positive cells (a marker for macrophages) was significantly higher in WT mice fed an HFD compared with WT mice fed a chow diet (Figure 5, B and I). However, in the p40 or p35 mice, the number of macrophages was low and was not increased by an HFD (Figure 5, E, H, and I). Moreover, the double immunostaining between F4/80 (macrophage marker) and p35 or between F4/80 and p40 revealed that the...
source of IL-12 was mainly from the macrophages (Figure 5C).

We previously reported that oxidative stress was increased in type 2 diabetic mice.19 In the present study, we found an increase of oxidative stress, as assessed by thio- barbituric acid reactive substances malonedialdehyde levels and NADPH oxidase activity, in ischemic hind limb muscle from WT mice fed an HFD compared with WT mice fed a chow diet; oxidative stress was blunted in p40−/− and p35−/− mice fed an HFD (Figure 6, A and B).

Recent evidence demonstrated that NADPH oxidases NOX2 and NOX4 were increased by an HFD.23 Accordingly, real-time quantitative RT-PCR and Western blot analysis showed that Nox2 and Nox4 levels were up-regulated in hind-limb muscles from WT mice fed an HFD but not in p40−/− and p35−/− mice fed an HFD (Figure 6, C–F). We could not detect Nox1 expression in ischemic limb muscle from all mice. These data indicate that IL-12 disruption promotes angiogenesis and arteriogenesis in response to chronic ischemia, more likely through the
inhibition of oxidative stress via the down-regulation of Nox2 and Nox4 expression.

It is well documented that IL-12 acts via the STAT4 signaling pathway. Signal transduction through the IL-12 receptor induces tyrosine phosphorylation of the Janus family kinases TYK2 and JAK2, which, in turn, phosphorylate STAT4. Our data show that phosphorylated STAT4 was increased in the hind-limb muscle from WT mice fed an HFD (Figure 7, A and B). However, the increase in phosphorylated STAT4 was blunted in p40−/− and p35−/− mice fed an HFD (Figure 7, A and B). We did not observe any changes in total STAT4 and JAK2, which, in turn, phosphorylate STAT4. Our data suggest that an HFD was able to activate IL-12 signaling via STAT4 phosphorylation and was prevented by p40 or p35 deletion.

The angiogenic factors (phosphorylated eNOS, Akt, and VEGFR2) were significantly reduced in ischemic hind-limb muscle from WT mice fed an HFD (Figure 7, D–J) but normalized in p40−/− and p35−/− mice fed an HFD (Figure 7, D–J), suggesting that IL-12 promotes angiogenesis by regulating proangiogenic factors.

Discussion

The present study provided evidence that the disruption of IL-12 promotes angiogenesis, which rescues tissue blood flow perfusion in obese type 2 diabetic mice. We also demonstrated that the deletion of IL-12 stimulates neovascularization events through a mechanism that involves a decrease in oxidative stress and an increase in the angiogenic factors (eNOS, Akt, and VEGFR2). The beneficial effects of lacking IL-12 were associated with reduced inflammation, increased M2 macrophages, and reduced oxidative stress in the ischemic limb of type 2 diabetic mice, suggesting that inflammation and oxidative stress account for the impaired ischemia-induced neovascularization in type 2 diabetes (Figure 8). These results are consistent with our previous finding indicating that angiogenesis in response to hind-limb ischemia was abrogated during diabetes. Despite a huge number of positive animal studies on angiogenesis, the clinical trials on therapeutic angiogenesis are not conclusive. With a disease as complex as diabetes, many factors are likely to be involved in angiogenesis defect. In the present study, we used a preclinical relevant murine model of type 2 diabetic obese mice induced by an HFD to study the in vivo contribution of IL-12 on neovascularization. The HFD mice model is a robust and efficient model for impaired glucose tolerance and progressive insulin resistance and type 2 diabetes.

In agreement with previous studies, we showed that p40−/− and p35−/− mice were unable to produce a biological active of IL-12, the heterodimeric cytokine IL-12 (70 kDa) composed of p35 (35 kDa) and p40 (40 kDa) (Figure 4 A), and IL-12 production is enhanced in obese type 2 diabetic patients and animal models. We found an increase of IL-12 in the hind-limb muscle of our diabetic mice (fed an HFD for 12 weeks) compared with nondiabetic mice (fed a chow diet). WT, p40IL-12−/−, and p35IL-12−/− mice fed an HFD for 12 weeks exhibit a gain in body weight, impaired glucose test tolerance, and insulin resistance. These data suggest that IL-12 is unlikely to be involved in the development and etiologies of obesity and type 2 diabetes. However, p35−/− mice fed an HFD appear to be more sensitive to glucose test tolerance than p40 or WT mice. Further investigations are needed to explore the possible link between p35 and insulin resistance and type 2 diabetes development. In fact, a recent study reported an association without cause-effect between lipid abnormalities and p40 levels in obese patients. Moreover, IL-12 secretion and production are enhanced under hyperglycemia and diabetes conditions, suggesting that augmented IL-12 level and release are the consequences of obesity and type 2 diabetes, rather than a cause.

The role of IL-12 in angiogenesis and arteriogenesis is still unknown, especially in obesity associated with type 2
diabetes. This study is the first evidence to show the in vivo contribution of IL-12 in impaired neovascularization in the ischemic hind limb of type 2 diabetic mice. The disruption of IL-12 in type 2 diabetic mice was able to stimulate angiogenesis efficiently and rescue blood flow perfusion in mice with type 2 diabetes and insulin resistance.

For a chow diet, IL-12 disruption did not significantly affect the underlying mechanism of angiogenesis and arteriogenesis in our model of hind-limb ischemia. Nevertheless, IL-12 was reported to inhibit bFGF-induced neovascularization in a s.c. Matrigel. Laser Doppler perfusion measurement is sensitive to temperature changes, and a high temperature could lead to high values in blood flow perfusion assessment.

During the past decade, it became apparent that inflammation is a key feature of obesity and type 2 diabetes. In fact, obesity, insulin resistance, and type 2 diabetes are closely associated with chronic inflammation, characterized...

Figure 7 Effect of p40 and p35 deficiency on IL-12 signaling, survival pathway, and proangiogenic factors in ischemic muscle limb. Representative immunoblots and densitometric analysis of the expression of phosphorylated STAT4 (P-STAT4; PY693; A and B), total STAT4 (T-STAT4; A and C), phosphorylated Akt (P-Akt; serine 473; D and E), total Akt (T-Akt; D and F), phosphorylated endothelial nitric oxide synthase (eNOS) (P-eNOS; serine 1177; G and H), total e-NOS (T-eNOS; G and H), and phosphorylated vascular endothelial growth factor receptor 2 (VEGFR2; tyrosine 1175; I and J). β-Actin was used as loading control. Densitometric measurements were performed to evaluate the fold increase in protein expression or activity. Data are expressed as means ± SEM. n = 6 (A–J). *P < 0.05 versus all groups. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Figure 8 Summary diagram that illustrates potential mechanisms by which IL-12 impairs ischemia-induced angiogenesis in type 2 diabetes via oxidative stress, inflammation, and angiogenic factors. eNOS, endothelial nitric oxide synthase; VEGF, vascular endothelial growth factor.
by abnormal cytokine production and activation of inflammatory signaling pathways.\textsuperscript{31–34}

Blood flow measurement and immunohistochemistry analyses indicate that blood flow recovery was strongly associated with an increase in capillary and arteriole density in the ischemic limb of p40\textsuperscript{−/−} and p35\textsuperscript{−/−} mice fed an HFD. These results highlight the protective role of IL-12 disruption in neovascularization in obese type 2 diabetic mice.

The mechanism by which IL-12 disruption protects the angiogenesis and arteriogenesis could be through the reduction of inflammation (reduction of macrophage infiltration, reduction of proinflammatory cytokines, and increase of anti-inflammatory cytokines). Macrophages have a remarkable plasticity to effectively respond to environmental changes. They can modify their phenotypes M1 or M2 to exert different functions. Both subtypes of macrophages are required during neovascularization; the release of chemokfactors, such as MCP-1 by M1, plays an important role in arteriogenesis.\textsuperscript{35}

In the present study, TNF-α, IFN-γ, and IL-6, potent inducers of inflammation, were highly up-regulated in ischemic limbs from WT mice fed an HFD compared with WT mice fed a chow diet; they were significantly reduced in p40\textsuperscript{−/−} and p35\textsuperscript{−/−} mice fed an HFD. MCP-1, another proinflammatory cytokine, was up-regulated in the ischemic limb of all mice fed an HFD and was not affected by the deletion of p40 or p35. The blockade of MCP-1 activity was able to impair blood flow recovery after hind-limb ischemia in control mice.\textsuperscript{36} Further studies are needed to explore the role of MCP-1 in ischemia-induced neovascularization in type 2 diabetes.

Macrophages, major sources of proinflammatory factors, were also increased by an HFD in WT mice and drastically inhibited in p40\textsuperscript{−/−} and p35\textsuperscript{−/−} mice.

In line with our results, a clear link was established between proinflammatory TNF-α, obesity, and inflammation.\textsuperscript{37} Moreover, TNF-α has been shown to be involved in ischemia-induced neovascularization\textsuperscript{38,39} and could account for the beneficial effect of lacking IL-12 on ischemia-induced neovascularization in diabetes. Furthermore, IFN-γ could also mediate the inhibitory effect of IL-12 on angiogenesis observed in our diet-induced type 2 diabetic mice. The mRNA expression of IFN-γ was up-regulated in the ischemic limb of type 2 diabetic mice, whereas it was down-regulated in the mice lacking p40 or p35 compared with wild type. In fact, IFN-γ has been shown to be a mediator of angiogenesis inhibition by IL-12 using a Matrigel angiogenesis system.\textsuperscript{29,40}

IFN-γ biases M1 polarization,\textsuperscript{41,42} which could explain the inhibitory effect of IL-12 on angiogenesis in type 2 diabetes. Further investigations are needed to determine the effect of the IL-12/IFN-γ/M1 macrophage polarization axis on angiogenesis and arteriogenesis in diabetes.

Indeed, M2 macrophages are known to express angiogenic factors, such as VEGF, insulin-like growth factor-1, and hepatocyte growth factor, and promote wound repair and neovascularization.\textsuperscript{43} Our results show that M2 macrophage markers, ARG1, IL-10, Clec10a, and MRC1, were all up-regulated in the ischemic limb of p40\textsuperscript{−/−} and p35\textsuperscript{−/−} mice compared with WT mice fed an HFD. These results suggest the potential involvement of M2 macrophages in ischemia-induced neovascularization in type 2 diabetic mice.

Recently, oxidative stress has emerged as a feature of obesity and is a major factor in the development of insulin resistance in obesity\textsuperscript{44}; it has been shown to play a major role in diabetic vascular complications.\textsuperscript{19} Indeed, inhibiting oxidative stress in diabetic mice may account for the beneficial role of deleting IL-12 in ischemia-induced neovascularization in type 2 diabetes. The exact mechanism by which IL-12 regulates oxidative stress is yet to be determined. Lacking IL-12 regulates oxidative stress is yet to be determined. Lacking IL-12 protects type 2 diabetic mice against oxidative stress.

The induction of neovascularization is a consequence of a balance between proangiogenic and antiangiogenic factors. In line with previous studies,\textsuperscript{5,7,45} we demonstrated that the VEGF level was increased and eNOS and VEGF signaling (VEGFR2 phosphorylation) were reduced in the ischemic limb of dietary-induced type 2 diabetes. Indeed, an increase of soluble and membrane-bound VEGFR1 may limit the ability of VEGF to bind to VEGFR2, resulting in decreased angiogenesis.\textsuperscript{45}

Moreover, bFGF was also increased in the ischemic limb of WT mice fed an HFD compared with WT mice fed a chow diet. It is well known that bFGF is a potent angiogenic factor that requires the activation of the VEGF system.\textsuperscript{46} Interestingly, the advanced glycation end product has been shown to be present in type 2 diabetes and associated with vascular complications.\textsuperscript{47} Moreover, bFGF has been shown to be modified by the advanced glycation end product in vitro and in vivo was able to reduce its angiogenic properties,\textsuperscript{48,49} which, in turn, can inhibit VEGF-dependent signaling.

We also found that p40 and p35 deficiency enhanced eNOS and VEGF signaling. Moreover, both VEGF and bFGF levels were reduced in p40\textsuperscript{−/−} and p35\textsuperscript{−/−} mice fed an HFD. These results suggest that the eNOS pathway, VEGF expression, bFGF expression, and VEGF signaling are regulated by IL-12. Further studies are needed to delineate the mechanism linking IL-12 to eNOS, VEGF, and bFGF signaling.

In conclusion, we provided new insights into the essential role of IL-12 in ischemia-induced neovascularization. The disruption of IL-12 in obese type 2 diabetic mice promotes the induction of angiogenesis and arteriogenesis and, therefore, blood flow perfusion in response to chronic ischemia. The protective mechanism of IL-12 disruption in obesity and type 2 diabetes involved the following: i) the reduction in oxidative stress and IL-12 downstream signaling (STAT4 phosphorylation), ii) the increase in angiogenic factor activity (eNOS, Akt, and VEGFR2), iii) the reduction of inflammation and proinflammatory...
cytokines, and iv) the increase of anti-inflammatory cytokines and M2 macrophage markers (Figure 8).

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