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Over-expression of catalase in myeloid cells causes impaired post-ischemic neovascularization

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Abstract

Objective—Myeloid lineage cells (MLCs) such as macrophages are known to play a key role in post-ischemic neovascularization. However, the role of MLC-derived reactive oxygen species (ROS) in this process and the chemical identity of the ROS remain unknown.

Methods and Results—Transgenic mice with MLC-specific over-expression of catalase (TgCat-MLC mice) were created on a C57BL/6 background. Macrophage catalase activity was increased 3.4-fold compared to wild-type mice. After femoral artery ligation, LASER Doppler perfusion imaging revealed impaired perfusion recovery in TgCat-MLC mice. This was associated with fewer collateral vessels, as assessed by micro CT angiography, and decreased capillary density. Impaired functional recovery of the ischemic limb was also evidenced by a 50% reduction in spontaneous running activity. The deficient neovascularization was associated with a blunted inflammatory response, characterized by decreased macrophage infiltration of ischemic tissues, and lower mRNA levels of inflammatory markers such as tumor necrosis factor-α, osteopontin, and matrix metalloproteinase-9. In vitro macrophage migration was impaired in TgCat-MLC mice, suggesting a role for H₂O₂ in regulating the ability of macrophages to infiltrate ischemic tissues.

Conclusions—MLC-derived H₂O₂ plays a key role in promoting neovascularization in response to ischemia and is a necessary factor for the development of ischemia-induced inflammation.

Keywords

limb ischemia; reactive oxygen species; angiogenesis; hydrogen peroxide; macrophages

Neovascularization is an important repair mechanism to preserve tissue integrity after ischemia, and it is a key determinant of the final outcome of vascular occlusive diseases such as coronary or peripheral vascular atherosclerosis¹, ². The term describes a complex process that includes the sprouting of new capillary beds (angiogenesis), the transformation of small arterioles into large conductance arteries (collateral vessel formation, also named arteriogenesis), and the in-situ differentiation of endothelial progenitors into mature endothelial cells (vasculogenesis)³. In the setting of acute ischemia, it is thought that the
largest contributor to blood flow recovery is provided by arteriogenesis in the form of collateral arteries. Whereas hypoxia is the major driving force in angiogenesis, arteriogenesis seems to be more dependent on mechanical forces and inflammation. Previous studies have shown that myeloid lineage cells (MLCs), in particular macrophages, play a central role in orchestrating the inflammatory response that promotes collateral vessel growth. Several factors released by macrophages, such as reactive oxygen species (ROS), tumor necrosis factor alpha (TNF-α), osteopontin (OPN), and matrix metalloproteinases (MMPs), among others, modulate post-ischemic neovascularization.

ROS such as superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) are key regulators of physiological and pathophysiological responses in the vasculature. In excess amounts, ROS are mediators of cellular injury. In contrast, at physiological levels, ROS participate in strictly regulated redox signaling pathways, modulating cell migration, proliferation, and matrix remodeling, all of which are necessary events for neovascularization. In addition, the proangiogenic effects of vascular growth factors such as vascular endothelial growth factor (VEGF) and angiopoietin-1 appear to be mediated at least in part by the production of ROS. Potential enzymatic sources of ROS in the vascular wall are diverse and include mitochondria, xanthine oxidase, uncoupled nitric oxide synthase, and the NADPH oxidase isoforms present in a variety of cell types. In vivo studies using knockout mice for the gp91phox subunit of NADPH oxidase show altered neovascularization after hind limb ischemia, thus highlighting the importance of this particular source of ROS.

Despite the strong evidence for the role of ROS as major mediators of the angiogenic response to ischemia, the specific ROS involved remains unclear. Compared to O$_2^-$, H$_2$O$_2$ is uncharged, can diffuse freely across lipid membranes, and is longer-lived, which makes it a good candidate for redox signaling in the vasculature. In this study we demonstrate that H$_2$O$_2$ derived from inflammatory cells is an important mediator of post-ischemic neovascularization. Using a transgenic mouse model whereby levels of H$_2$O$_2$ in MLCs are decreased by the tissue-specific over-expression of catalase, we show impaired collateral vessel formation and functional recovery of the hind limb after ischemia. This was associated with an impaired migratory activity of catalase-overexpressing macrophages and an overall reduced inflammatory response in the ischemic tissues.

Methods

Details are provided in an expanded Methods section in the online Data Supplement, available at http://atvb.ahajournals.org.

Generation and Characterization of Transgenic Mice with Specific Over-expression of Human Catalase in MLCs

To develop mice with MLC-specific over-expression of catalase (Tg$^{Cat-MLC}$ mice), we used a Cre/LoxP system similar to that described previously, modified to have the cre-recombinase cDNA under the control of the MLC-specific promoter Lysz. Due to the genetic design used, cells other than MLCs are expected to show green fluorescent protein (GFP) fluorescence, while selective expression of cre-recombinase in MLCs will lead to excision of the GFP gene and loss of fluorescence in this cell population (for details, please see the online methods supplement and supplemental Figure I). All mice used in this study were on a C57BL/6 background. Control mice used for all experiments, referred from now on as wild type (WT) mice, were littermates of the transgenic mice. Only male mice between 8 and 10 weeks were used. All procedures were approved by the Emory University Institutional Animal Care and Use Committee and were in compliance with the standards for
the care and use of laboratory animals of the Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD.

The expression of human catalase and other antioxidant enzymes was assessed by western blot of thioglycollate-induced peritoneal macrophages. Catalase activity in peritoneal macrophages was measured as previously described\textsuperscript{24}. The specificity of the cre-mediated recombination in MLCs was assessed by immunocytochemistry of peritoneal cells and immunohistochemistry of lung tissues as detailed in the online supplement.

**Mouse Model of Unilateral Hind limb Ischemia and Assessment of Neovascularization**

Ligation and excision of the left superficial femoral artery, LASER Doppler perfusion imaging (LDPI) and micro-CT imaging were performed as previously described\textsuperscript{9}. Capillary density, expressed as number of capillaries per muscle fiber, was assessed by immunostaining of gastrocnemius muscle against CD31 and β-dystroglycan. Functional recovery of the ischemic limb was assessed by monitoring of spontaneous activity in cages equipped with a running wheel.

**Quantification of Mobilized Endothelial Progenitor Cells (EPCs) and EPC Culture Assay**

EPCs were quantified from bone marrow or peripheral blood mononuclear cells (MNCs) as Sca-1\textsuperscript{+}/Flk-1\textsuperscript{+} cells by flow cytometry. EPC culture assay was performed as previously described\textsuperscript{25, 26}.

**Assessment of Inflammation in the Ischemic Hind Limb**

At post-operative day 5, paraffin sections of gastrocnemius muscle were obtained and processed for conventional hematoxylin and eosin staining or immunostaining against the macrophages-specific marker MAC-3 or against an anti-neutrophil antibody. Levels of CD68, OPN, TNF-α, MMP-9, VEGF and SDF-1 mRNA in gastrocnemius muscle were measured by quantitative real-time PCR (qRT-PCR) and normalized to ribosomal 18S RNA content.

**Macrophage Migration Assay**

Macrophage migration in response to MCP-1 was assessed using a modified Boyden chamber assay as previously described\textsuperscript{9}.

**Measurement of H\textsubscript{2}O\textsubscript{2} Production**

H\textsubscript{2}O\textsubscript{2} production from gastrocnemius muscle or peritoneal macrophages was measured by Amplex Red assay (Invitrogen, Carlsbad, California) as per the manufacturer’s instructions.

**Statistical Analysis**

Data are presented as mean ± SEM. When applicable, values were compared by Student t test or ANOVA with Bonferroni post-hoc analysis. \( p < 0.05 \) was considered to be statistically significant.

**Results**

**Phenotypic Characterization of Transgenic Mice**

The expression of human catalase in MLCs of Tg\textsuperscript{Cat-MLC} mice was evaluated by western blot analysis of isolated peritoneal macrophages using an antibody that preferentially identifies the human enzyme\textsuperscript{24}. Figure 1A demonstrates the over-expression of human catalase in Tg\textsuperscript{Cat-MLC} macrophages. Other antioxidant enzyme systems were not altered...
compared to littermate WT controls (Figure 1A). To confirm that the over-expression of catalase resulted in an increase in enzymatic activity, we assayed for catalase activity in peritoneal macrophages. As shown in Figure 1B, Tg\textsuperscript{Cat-MLC} macrophages exhibited a 3.4-fold increase in catalase activity compared to WT macrophages (1169.4 ± 241.2 vs 343.1 ± 63.4 mU/mg of protein, respectively, p < 0.05).

To verify that the excision of the GFP cDNA by cre-recombinase (which allows for the expression of the human catalase gene) was confined to MLCs, we performed immunocytochemistry on thioglycollate-induced peritoneal cells of Tg\textsuperscript{Cat-MLC} mice (Figure 1C), as well as immunostaining of lung sections (Figure 1D). After 72 hours of thioglycollate injection, most peritoneal leukocytes are macrophages\textsuperscript{27}. Figure 1C shows that peritoneal cells stained for the macrophage specific antigen MAC-3 did not show GFP fluorescence, while cells with intrinsic GFP fluorescence were not stained for MAC-3. This absence of co-localization between GFP fluorescence and macrophage markers was also confirmed in confocal images of lung sections where, unlike the alveolar epithelium, resident alveolar macrophages lacked GFP (Figure 1D). These data demonstrate that human catalase expression is indeed confined to MLCs and not present in other cell types.

Baseline characteristics before ischemia, including muscle capillary density (supplemental Figure II) and expression of angiogenic factors such as VEGF and SDF-1 (supplemental Figure III), were not different between genotypes. There was no obvious effect of MLC-specific over-expression of catalase on number of total circulating leukocytes or percentages of granulocytes, monocytes and lymphocytes (supplemental Figure IV). Similarly, no differences in litter size, body weight, blood pressure, susceptibility to infections, or other physical descriptors were found (data not shown).

**LASER Doppler Perfusion Imaging**

LDPI measurements were done from day 2 through day 28 after femoral artery ligation and excision. The acquired images illustrated a significant lag in recovery in Tg\textsuperscript{Cat-MLC} mice (Figure 2A). Quantitative perfusion measurements confirmed this discrepancy (Figure 2B). WT mice showed a steady recovery in perfusion, reaching a maximum perfusion ratio of 52 ± 5% at day 28 relative to the non-ischemic leg. In contrast, Tg\textsuperscript{Cat-MLC} mice showed a blunted curve, which diverged from the WT curve after day 7 and reached a maximum perfusion ratio of only 37 ± 2% at day 28 (Figure 2B, p < 0.05). The difference in perfusion between WT and Tg\textsuperscript{Cat-MLC} mice achieved statistical significance at day 14 and was maintained at the subsequent time points.

**Micro-CT Imaging of Collateral Vessels**

Micro-CT angiography was employed to quantitatively assess vascular anatomy. Qualitative observation of the 3-D image reconstructions of the vasculature performed 28 days after hind limb ischemia showed reduced collateral vessel formation in Tg\textsuperscript{Cat-MLC} mice relative to WT (Figure 3A). Quantitative analysis of the obtained images using 3-D histomorphometric software revealed an approximate 30% reduction in the vascular volume to tissue volume ratio and a 15% reduction in vascular density in Tg\textsuperscript{Cat-MLC} mice compared to WT (Figure 3B).

**Capillary Density Analysis**

Capillary density was analyzed by immunohistochemistry using antibodies against CD31 and β-dystroglycan to detect endothelial cells and skeletal muscle fibers, respectively (Figure 3C). At post-operative day 28, gastrocnemius muscle from Tg\textsuperscript{Cat-MLC} mice showed decreased capillary to muscle fiber ratios compared to WT (1.81 ± 0.04 vs 2.44 ± 0.16 capillaries/muscle fiber, p < 0.05, Figure 3D).
Motor Activity Monitoring

As a physiological measure of functional ischemic limb recovery, spontaneous running distance on an activity wheel was measured on postoperative days 28 through 35. Despite the hind limb ischemia, WT mice exhibited a very active profile, running more than 40 kilometers over the course of a week (Figure 3E). In contrast, TgCat-MLC mice displayed significantly decreased motor activity, as shown by a 50% reduction in total distance run relative to WT animals (21.9 ± 6.9 vs 44.1 ± 3.9 Km, p < 0.02, Figure 3E).

EPC Mobilization and Differentiation in Response to Ischemia

To investigate whether a deficiency in EPC mobilization could contribute to the impaired neovascularization in TgCat-MLC mice, we quantified EPCs by flow cytometry. Three days after hind limb ischemia, we found no differences in the number of circulating EPCs in peripheral blood or in bone marrow (supplemental Figure V). We next analyzed the differentiation potential of peripheral blood and bone marrow MNCs into EPCs in culture, as assessed by the uptake of acLDL and the binding of BS-1 lectin. Despite the impaired neovascularization in TgCat-MLC mice, there was a trend towards an increase in the number of cultured EPCs compared to WT in peripheral blood (4.8 ± 1.0 vs 2.5 ± 0.5 cells per mm², respectively, p = 0.21, n = 3 per genotype) and bone marrow (167.2 ± 8.3 vs 113.6 ± 16.5 cells per mm², respectively, p = 0.16, n = 3 per genotype).

Inflammatory Response to Ischemia

The degree of collateral vessel formation after ischemia is directly related to the inflammatory response in the ischemic tissues. To assess whether the impaired neovascularization seen in TgCat-MLC mice was associated with different levels in inflammatory cell infiltration, we performed histological analysis of the ischemic limbs. Hematoxylin and eosin staining of ischemic distal limbs from WT mice revealed an intense infiltration of inflammatory cells surrounding areas of necrotic muscle fibers (Figure 4A). Immunostaining identified macrophages as the dominant cell type (Figure 4A insert). In contrast, TgCat-MLC mice exhibited significantly lower levels of inflammatory infiltration as compared to WT (Figure 4A). Computer-assisted quantification of the low magnification hematoxylin and eosin images revealed a ≈ 50% decrease in the infiltration of inflammatory cells in TgCat-MLC mice (Figure 4B). At the same time point (post-operative day 5), the presence of neutrophils in ischemic tissues was minimal (supplemental Figure VI).

To further assess if the difference in inflammatory cell infiltration was associated with differential expression of inflammatory markers within the two genotypes, we performed qRT-PCR analyses of muscle tissues. As shown in Figure 4C, mRNA levels of the macrophage-specific marker CD68 was significantly lower in ischemic limbs of TgCat-MLC mice compared to WT. This is consistent with the reduced number of macrophages seen on immunohistochemistry. We also investigated the expression of other inflammatory genes known to be involved in post-ischemic neovascularization, including TNF-α, OPN, and MMP-9. The up-regulation of these factors in response to ischemia was markedly blunted in TgCat-MLC mice compared to WT (Figure 4C), such that expression was not significantly different from the non-ischemic, control limb. A similar trend was seen for ICAM-1 expression, although it did not reach statistical significance (data not shown). There was no difference in VEGF mRNA or protein expression, or in SDF-1 mRNA levels, between ischemic limbs of WT and TgCat-MLC mice (supplemental Figure VII).

Levels of H₂O₂ in gastrocnemius muscle were increased in the ischemic leg compared to the non-ischemic leg in both genotypes (Figure 4D). However, there were no significant differences in H₂O₂ production between WT and TgCat-MLC ischemic muscle (Figure 4D). In contrast, and as expected by their increased catalase activity, production of H₂O₂ was
lower in isolated $\text{Tg}^{\text{Cat-MLC}}$ macrophages both at baseline and after stimulation with LPS (Figure 4E).

**Macrophage Migration**

Many studies have demonstrated that $\text{H}_2\text{O}_2$ plays an important role in regulating cell migration in different cell types including vascular smooth muscle (VSMCs) and endothelial cells (ECs)\textsuperscript{13, 30}. We therefore sought to determine whether catalase over-expression by macrophages led to changes in their migratory function. For this purpose we performed an in vitro macrophage migration assay in a modified Boyden chamber using MCP-1 as the chemotactic factor. Although stimulation with MCP-1 induced macrophage migration in both genotypes compared to media alone, $\text{Tg}^{\text{Cat-MLC}}$ cells displayed a significantly impaired migratory response compared to WT (Figure 5).

**Discussion**

The major finding in our study is that $\text{H}_2\text{O}_2$ derived from cells of myeloid origin plays a key role in post-ischemic neovascularization. Transgenic mice with tissue-specific over-expression of catalase in MLCs had impaired blood flow recovery, collateral vessel development, capillary density, and functional capacity of the hind limb after acute ischemia. This was associated with a blunted inflammatory response in the ischemic tissues, characterized by lower levels of inflammatory markers and reduced macrophage infiltration.

Despite the mounting evidence for the involvement of ROS as major regulators of neovascularization, the role of each specific ROS species, as well as the major cellular sources responsible for their effects, remains unclear. Several animal studies on post-ischemic neovascularization have focused on modulating $\text{O}_2^{\cdot -}$ levels by genetic manipulation of Nox enzymes or superoxide dismutase isoforms\textsuperscript{23, 31, 32}. Such changes in $\text{O}_2^{\cdot -}$ are expected to be accompanied by changes in its dismutation product, $\text{H}_2\text{O}_2$. The role of $\text{H}_2\text{O}_2$ in angiogenesis has been studied in vitro, where it has been shown to induce the formation of tube-like structures by ECs\textsuperscript{33} and promote the proliferation and migration of ECs and VSMCs\textsuperscript{13-16}. However, confirmation of the angiogenic potential of $\text{H}_2\text{O}_2$ in vivo is lacking.

Our study provides novel evidence for the role of MLC-derived $\text{H}_2\text{O}_2$ in promoting neovascularization in vivo, as shown by the impaired perfusion recovery in $\text{Tg}^{\text{Cat-MLC}}$ mice. The deficit in perfusion seen on LDPI analyses was explained in part by a reduction in the number of collateral vessels formed, as assessed by micro-CT. These results are in agreement with a previous report where, in an inverse model, catalase deficient mice form larger arterioles in response to angiopoietin-1\textsuperscript{17}. Of note, while the LDPI perfusion ratios between the ischemic and non-ischemic legs of WT mice were below 1 at 28 days, the ratios for vascular volume obtained by micro-CT were higher than 1. This suggests the existence of some degree of “ineffective” collateral vessel formation, consisting of non-connecting vessels that provide abnormal perfusion to tissues.

In addition to the effects on collateral vessels, overproduction of catalase by MLCs resulted in decreased capillary density after ischemia. The absence of differences in capillary density in the non-ischemic muscle, along with the LDPI data showing differences in perfusion only at later time points (after day 7), is consistent with an impairment in angiogenesis, as opposed to differences in preexisting collaterals\textsuperscript{34}.

Although $\text{O}_2^{\cdot -}$ has been implicated in the mobilization of EPCs after hind limb ischemia\textsuperscript{35}, we found no differences between genotypes in the number of EPCs in peripheral blood and bone marrow after surgery, suggesting that this process is not $\text{H}_2\text{O}_2$-sensitive. Surprisingly,
there was a trend towards an increase in the number of EPCs cultured from Tg\textsuperscript{Cat-MLC} MNCs compared to WT. In the setting of overall impaired neovascularization, this may represent a compensatory mechanism to overcome the deficiencies in angiogenesis and collateral vessel formation.

Finally, and most importantly, the impaired neovascularization in Tg\textsuperscript{Cat-MLC} mice correlated with dramatic differences in functional recovery of the ischemic limb, as evidenced by a 50% reduction in spontaneous motor activity compared to their littermate controls.

The role of inflammation in promoting post-ischemic neovascularization, in particular collateral vessel formation, has been well documented by numerous groups.\textsuperscript{3} Macrophages appear to play a central role in driving new vessel formation. They secrete vascular growth factors, cytokines, MMPs, and ROS, all of which promote proliferation and migration of vascular cells. Indeed, macrophage infiltration of ischemic tissues appears to be a necessary event for collateral vessel development,\textsuperscript{7} and it may be related to their ability to burrow channels for vessel growth through the action of proteolytic enzymes.\textsuperscript{10} Our results clearly show a significant reduction in the inflammatory response mounted by macrophages in Tg\textsuperscript{Cat-MLC} mice, as evidenced by the immunohistochemistry analyses, as well as quantification of CD68 mRNA in ischemic tissues. This correlated with decreased expression of inflammatory markers such as OPN, TNF-\textgreek{a}, and MMP-9; factors that have been implicated in the formation and growth of collateral arteries.\textsuperscript{9, 11} In contrast, expression of VEGF and SDF-1 after ischemia were similar in both genotypes. Thus, these factors did not seem to account for the observed differences in the angiogenic response.

Since H\textsubscript{2}O\textsubscript{2} regulates cell migration in various cell types,\textsuperscript{13} we hypothesized that macrophages from Tg\textsuperscript{Cat-MLC} mice may have an inherent deficiency in their migratory function. Results from our in vitro migration assays are consistent with this hypothesis, suggesting that the reduced number of infiltrating macrophages in Tg\textsuperscript{Cat-MLC} mice is a consequence of their impaired ability to migrate in response to an ischemic insult.

Although MLCs from Tg\textsuperscript{Cat-MLC} mice clearly produced less H\textsubscript{2}O\textsubscript{2} (Figure 4E), the total amount of H\textsubscript{2}O\textsubscript{2} released by the ischemic muscle was not significantly different than WT (Figure 4D). Other cellular sources, such as ECs, VSMCs, or skeletal muscle fibers, may produce enough H\textsubscript{2}O\textsubscript{2} in response to ischemia to hinder any differences arising specifically from MLCs. The absence of differences in global H\textsubscript{2}O\textsubscript{2} levels in ischemic tissues raises the possibility that H\textsubscript{2}O\textsubscript{2} does not have a direct role in neovascularization, but acts indirectly, perhaps by impeding the access of macrophages to the area of ischemia. However, it is also possible that H\textsubscript{2}O\textsubscript{2} directly regulates new vessel formation, but its actions depend on its specific localization at the site of macrophage infiltration. Supporting this latter notion, studies have shown that the actions of H\textsubscript{2}O\textsubscript{2} can be localized not only as a paracrine vascular signal,\textsuperscript{36} but also within different intracellular compartments.\textsuperscript{37}

A limitation of our study is posed by the relative lack of cellular specificity of the lysozyme promoter. Although we found differences in macrophage-mediated inflammation in our transgenic mice, it is important to point out that some activity of the promoter is expected in other inflammatory cells of myeloid origin such as neutrophils.\textsuperscript{38} These cells are known to infiltrate tissues very early and transiently after ischemia,\textsuperscript{39} but their role on post-ischemic neovascularization is less clear, with studies showing diverse effects.\textsuperscript{40, 41} In our model of hind limb ischemia most neutrophils were absent by post-operative day 5, which is consistent with previous reports.\textsuperscript{39} At this same time point macrophage infiltration was intense, and differences in the expression of inflammatory markers between genotypes were significant. These findings demonstrate that H\textsubscript{2}O\textsubscript{2} regulates macrophage-mediated inflammatory events that are relevant to the formation of functional collateral vessels.
Although less likely, the data do not rule out a role for neutrophil-derived H$_2$O$_2$ in post-ischemic neovascularization.

Finally, studies show that different concentrations of ROS have disparate effects on angiogenesis. For instance, in young, healthy mice, Nox2 deficiency impairs post-ischemic neovascularization$^{23}$, whereas, in older mice exposed to tobacco smoke, the same enzyme deficiency augments post-ischemic angiogenesis$^{32}$. This suggests that while some level of ROS is necessary for neovascularization, an excess is detrimental. Indeed, the existence of an optimal “oxidative window” was also demonstrated by Reed et al. in the coronary circulation$^{32}$. Thus, generalizing our results to conditions of increased oxidative burden require further studies.

In summary, in this study we identify H$_2$O$_2$ as one of the key mediators secreted by MLCs which regulates post-ischemic neovascularization. The mechanisms by which MLC-derived H$_2$O$_2$ stimulates collateral vessel formation involve the facilitation of macrophage infiltration in ischemic tissues and mounting of an inflammatory response. Targeting MLC-derived H$_2$O$_2$ could lead to improved therapies for regulating neovascularization.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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**References**


Figure 1. MLC-specific catalase over-expression in TgCat-MLC mice
(A) Western blot analyses of peritoneal macrophages show over-expression of human catalase in TgCat-MLC mice. Levels of superoxide dismutase (SOD)-1, SOD-2, and glutathione peroxidase (GPx) 1/2 protein were not different from WT mice. Shown are representative blots of three independent experiments. (B) Macrophage catalase activity was up-regulated 3.4 fold in TgCat-MLC mice compared to WT mice. *p < 0.05. (C) Immunocytochemistry of peritoneal cells from TgCat-MLC mice shows absence of GFP fluoresce (green) in macrophages (red) but not in other cells types. Nuclei are stained with DAPI (blue). (D) Merged confocal images of lung sections stained for GFP (green), alveolar macrophages (red) and DAPI (blue). GFP fluorescence can be seen in the alveolar...
epithelium of TgCat-MLC but not in WT mice. The absence of co-localization between red and green (yellow) indicates selective recombination of GFP in macrophages.
Figure 2. LDPI analysis
(A) Representative LDPI images demonstrate the time course of ischemic limb reperfusion in Tg\textsuperscript{Cat-MLC} and WT mice. (B) Quantitative analyses are presented as perfusion ratios (ischemic/non-ischemic leg), up to post-operative day 28. A significant lag in perfusion recovery in Tg\textsuperscript{Cat-MLC} mice was evident at day 14 and maintained through day 28 (n = 14 to 16 per genotype). *p < 0.05 vs WT.
Figure 3. Analysis of collateral vessel formation, capillary density, and spontaneous motor activity

(A) Representative Micro-CT angiographies of ischemic limbs from WT and TgCat-MLC mice obtained at post-operative day 28. (B) Quantitative analysis showed decreased vascular volume / tissue volume ratios as well vascular density in TgCat-MLC mice (n = 8 per genotype). Data are expressed as fold change compared to the non-ischemic leg. *p < 0.05 vs WT. (C) Representative photomicrographs of ischemic gastrocnemius muscles stained with antibodies against CD31 or β-dystroglycan, allowing the detection of capillaries or muscle fibers, respectively, 28 days after surgery. Scale bar = 20 μm. (D) Quantification of capillary density in ischemic gastrocnemius muscle of treated mice using anti-CD 31
antibody and reported to the number of muscle fibers (n = 3 per genotype). *p < 0.05 (E)
Spontaneous motor activity recorded over a 7 day period and expressed as total distance run, in meters. Monitoring began at post-operative day 28 (n = 6 per genotype). *p < 0.02.
Figure 4. Inflammatory cell infiltration and expression of inflammatory markers in ischemic tissues
(A) Representative photomicrographs of ischemic distal limbs at post-operative day 5, stained with hematoxylin and eosin. Inserts show corresponding immunostaining for macrophages (red). Green color represents autofluorescence from muscle fibers. Insert scale bar = 100 μm. (B) Quantitative assessment of the basophilic areas of inflammatory cell infiltration from hematoxylin and eosin images, expressed as percentage of total area (n = 5 per genotype). *p < 0.04. (C) mRNA levels of the corresponding inflammatory markers were measured in ischemic and non-ischemic gastrocnemius muscle at post-operative days 5 and normalized to 18S rRNA (n = 6 per genotype). Data are expressed as fold increase.
compared to non-ischemic legs of WT mice. *p < 0.05 vs WT non-ischemic, **p < 0.05 vs TgCat-MLC non-ischemic, §p < 0.05 vs WT ischemic. (D) H$_2$O$_2$ production from ischemic and non-ischemic gastrocnemius muscle was assessed by Amplex Red assay at post-operative day 5 (n = 3 per genotype). *p < 0.02 vs WT non-ischemic, **p < 0.02 vs TgCat-MLC non-ischemic. (E) H$_2$O$_2$ production by peritoneal macrophages was assessed by Amplex Red assay at baseline (Ctl) or after stimulation with 10 μg/mL of LPS (LPS). *p < 0.05 vs WT Ctl, **p < 0.05 vs WT + LPS.
Figure 5. Macrophage migratory activity
Migration of isolated peritoneal macrophages in response to DMEM media alone (Ctl) or media containing 0.5 \( \mu \text{g/mL} \) MCP-1. *\( p < 0.01 \) vs WT Ctl, **\( p < 0.01 \) vs Tg\textsuperscript{Cat-MLC} Ctl, §\( p < 0.02 \) vs WT + MCP-1.