Functional tissue restoration; Therapeutic effects of chemokine overexpression

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Abstract
When a tissue is injured, for example by acute or chronic ischemia, the hypoxic cells will release damage-associated molecular patterns (DAMPs) and initiate a healing process in which leukocytes are highly involved in re-establishing the vasculature. The maturing vasculature, post injury, can be assessed by measuring perfusion response to external stimuli. In this study we have examined if angiogenesis can be enhanced and through that result in better functional perfusion, if angiogenesis is stimulated by an ischemic injury and if overexpression of any of the chemokines CCL2 or CXCL12 further enhances the angiogenic process. The results showed that those ischemic hindlimbs expressing pCXCL12 but not pCCL2 day 7 and 21 after the ischemia induction had a tendency of improved heat-induced hyperremia response, which correlated with a substantial infiltration of macrophages where pCXCL12 seems to polarize the macrophages to the phenotype associated with tissue remodelling. Thereby does the muscle overexpression of pCXCL12 serve as a possible future strategy to enhance restoration of functional tissue perfusion in ischemic limbs in humans.

Sammanfattning
När en vävnad skadas, exempelvis vid en bestående ischemi, kommer cellerna i den hypoxiska miljön att frisätta damage-associated molecular patterns (DAMPs) och initiera en läkningsprocess med kärlnybildning, angiogenes. Genom att inducera en ökning av lokalt blodflöde via en extern värmekälla i ben och fot, värmeinducerad hyperemi, kan möjligheten att öka det ökade blodflödet, den funktionella perfusionen, mätas. I denna studie har vi undersökt om en ökad angiogenes och därmed en bättre funktionell perfusion kan stimuleras vid en ischemisk skada genom administrering av plasmider kodandes för kemokinerna CCL2 eller CXCL12 som lokalt ökar koncentrationerna av dessa kemokiner i muskel. Denna studie visar att de ischemiska benen hos möss transfekterade med pCXCL12 dag 7 och 21 efter ischemisk induktion är bättre på att höja blodflödet ut i extremiteterna vid värmeinduktion. Detta korrelerar med infiltrationen av makrofager där pCXCL12 verkar polarisera makrofagerna till den fenotyp som associeras med våvnadsmodifering. Dessa resultat indikerar att överexpression av pCXCL12 kan vara en möjlig strategi för återställande av funktionella vävnadsperfusion i ischemiska extremiteter hos människor.
Introduction

Oxygen is essential to all eukaryotic organisms and insufficient oxygen supply to tissues and organs severely impair organ and cell function\(^1\). The human body have mechanisms to continuously maintain and, if needed, restore the tissue oxygen homeostasis, by utilizing tools like parts of the nervous system\(^2\), hormonal regulation\(^3\), modification of vascular tone\(^4\) and tissue remodelling\(^5\). Chronic or long-term insufficient oxygen supply may however overcome all the attempts of the body to compensate for the local shortage of oxygen and as a result several pathological states can occur depending on the affected site, for example renal failure\(^6\) or entire limb dysfunction\(^7\).

Tissue perfusion

The cardiovascular system consists of many components: the three circulatory systems (pulmonary, systemic and coronary circulation), blood, arteries, arterioles, capillaries, venules and veins. The task of the system, which covers a great part of the internal body surface, is foremost to supply the cells with oxygen and nutrients as well as remove the metabolic waste. The metabolic exchange occurs at the capillary level in the tissue where the exchange occurs through diffusion. In the alveoli of the lung, CO\(_2\) diffuse into the air and the oxygen from the air diffuse into the blood stream and attaches to the haemoglobin of the red blood cells and will be transported out to the tissue, maintaining physiological oxygen tension \([pO_2]\)\(^8\).

The heart, by mechanical force, creates a systemic pressure in the arteries and arterioles. The body also possesses many tools to influence the blood pressure. The renin-angiotensin system will control the pressure by control of the fluid volume in the circulation together with vasodilators that are released by the tissue for example CO\(_2\) and lactate\(^9\). The arteries, nut not the veins, are surrounded by the tunica media with contractile smooth muscle cells that offers vascular dynamics, stability and elasticity. The inner vascular wall, tunica intima, consists of one-layer endothelial cells and the outer vascular wall, tunica externa, consists of connective tissue and a basement membrane which is shared with chaperoning cells such as different pericytes. The continuous capillaries on the other hand only consists of one layer of endothelial cells and due to the thin vessel wall it is possible for oxygen and CO\(_2\) to diffuse through and form equilibrium with the surrounding tissue\(^8\). Oxygen is transported bound to haemoglobin in red blood cells, erythrocytes which comprises of 39-49% and 37-44% of the cellular component of blood in men and women respectively\(^8,10\).

To protect the tissue, the blood also contains leukocytes. These cells are described as the host defenders against foreign substances\(^11\), but new research also think that these cells have many regulatory functions in remodelling of tissue\(^12,13\).

Different mechanism of angiogenesis

If the vascular system is interrupted, new capillaries needs to be formed. This process is known as angiogenesis. There are two types of angiogenesis, sprouting and intussusceptive. The intussusceptive angiogenesis (IA) is newly discovered and the underlying mechanisms are not yet clear but the process seems to be faster and less energy dependent compared to sprouting angiogenesis\(^14\). The formation of the new capillaries starts with the endothelial cells purse the diameter of the vessel. With the help of invading fibroblasts and pericytes the two pipes can be separated into new capillaries\(^15\).

The sprouting angiogenesis on the other hand has been thoroughly investigated\(^14\) and the mechanisms by which the new capillaries are produced are now mechanistically obtained\(^16\).
First the basement membrane (BM) and the extra cellular matrix (ECM) are degraded. Then the endothelial cells proliferate to be able to migrate into the paths of degraded ECM and they can form the new sprouts. At last the new sprouts couple with existing capillaries and the new vessels are formed.

To make room for new capillaries in the tissue, the enzyme matrix metalloproteinase-9 (MMP-9) is produced to degrade components in the ECM. This facilitates the movements of the angiogenic cells, such as endothelial cells, that will form the new capillaries. The chemokine stromal-derived factor-1 (CXCL12), or SDF-1 as it also is called, has been shown to enhance the MMP-levels in bone-marrow derived cells so migration of the cells easier can be achieved. When the new endothelial tubs have formed, pericytes together with a basement membrane containing the collagens and proteoglycans stabilize the tube.

**Leukocytes in angiogenesis**

When the homeostasis in tissue is disturbed ex. During hypoxia in an otherwise sterile muscle or a open wound in tissue, local activation of the endothelium can be observed. Many factors are released from the tissue to attract leukocytes. Some of the known factors are chemokines, damage-associated molecular patterns (DAMPs) and damages associated signals such as high mobility group box 1 (HMGB1).

When a tissue is wounded, local signals induced by the trauma is released into the surrounding tissue. Theses signals, for example histamine, tumour necrotic factor (TNF) and interleukin-1 (IL-1) activate nearby endothelial cells. The endothelial cells will by this signal themselves produce vasodilators such as nitric oxide (NO). It act as a relaxer to the surrounding arteriolar smooth muscles and increase blood flow to the wounded site. Circulating leukocytes become activated and attracted by gradients of cytokines from the wounded tissue, and the leukocyte recruitment cascade is thereby initiated. This cascade starts by rolling leukocytes on the endothelial cells by binding to selectins, where after they adhere to the vessel wall followed by intravascular crawling of the leukocytes to optimal spots where they transmigrate out to tissue. In the tissue the leukocytes continue migrating towards the chemokine gradient. At the afflicted site, the neutrophils eliminate invading microbes by phagocytosis and the release of reactive oxygen species (ROS). The classical activation of macrophages to the M1-type is mediated by IFN-γ, and results in anti-microbial effector cells ready to eliminate a foreign pathogen. Monocytes in the circulation are recruited with the CCR2 to the site of infection. Their ligand is the monocyte chemo-attractant protein-1 (MCP-1, also called CCL2). At the site of infection the monocytes will differentiate to macrophages.

Many activating factors have been discovered and the different factors seems to lead to different functions of the macrophage. Currently macrophages can be divided into at least two groups, M1- and M2-type. The M2 seems to contain further different subtypes- M2a, M2b and M2c, which may have different effector roles.

**Tissue hypoxia**

If tissue is continuously deprived of its normal blood supply, the tissue becomes hypoxic and the cells starts to release DAMPs (Table 1) which alerts the surrounding tissue as well as components of the circulatory system.
Hypoxia stimulates the sprouting form of angiogenesis and if the [pO₂] gets too low in the tissue, the cells react to the low tension and the ubiquitination of the hypoxic-inducible factor (HIF) is reduced 33, 34. There are two different sub-types of this factor, HIF-1alpha and HIF-2alpha 35, 36. The HIF-1alpha is a transcriptional factor for many components in the angiogenic process 36. One is CXCL12, which act as a chemokine and ligand for the CXCR4 receptor, this chemokine is thought to be the primary signal in the recruitment of leukocytes and other bone-marrow derived cells to the hypoxic site 37. Earlier studies show that an enhanced angiogenesis can be observed following treatment of hindlimb ischemia models with plasmids containing the HIF-alpha gene administrated to the ischemic limb38.

Aim and Hypothesis
The process on which the leukocytes are recruited and their involvement in tissue remodelling during hypoxia is not yet fully charted. Bits of the chart have during the last years been explored and the functions are examined but they are far from understood 39.

In this study we aim at stimulating the sprouting angiogenesis in a mouse model of hindlimb ischemia by overexpression of the chemokines CCL2 or CXCL12, both suggested to recruit and retain pro-angiogenic leukocytes. We hypothesize that proangiogenic leukocytes play a role in both angiogenesis and vascular functionality which were assessed by measurements of the vascular response to limb heat challenge.

Materials and Methods

Animals
Male C57B1/6 mice (25-30 g, Taconic) were used. The mice had free access to pelleted food and tap water during the study. All experiments were approved by the Regional Animal Ethics Committee in Uppsala, Sweden.

Induction of hindlimb ischemia
The mouse was anesthetized with isoflurane Forene (Abbott Scandinavia AB), in a glass beaker. The mouse was then allowed spontaneous inhalation of 3% isoflurane during the process. The mouse was kept warm with a warming pad set on 37°C. The fur was removed using a razor and hair removal cream (Veet). An incision over the femoralis vasculature of 5-10 mm was done in the skin on the right leg. The a. femoralis was localized and separated from the v. femoralis and n. femoralis. To avoid an extent of immune response through vessel damage after the operation, the invasion was done with blunt forceps. Surgical knots with a ligation thread were done on the artery, two proximally and one distally and the artery was cut in between (Figure 3A). The wound was closed with 3 to 4 stitches (Ethilon polyamide 6, 4-0, Onemed). The animals were monitored post operation and normal body temperature was ensured by keeping the cage close to an infrared lamp, Infracare (Philips).

Plasmid transfection
Three different plasmids were used in the study (Figure 1A,B): control plasmid encoding only reporter sequence –copGFP-T2A-Luc2- but no chemokine referred to as pCTR, plasmid encoding reporter sequence and the chemokine CCL2, -CCL2-P2A–copGFP-T2A-Luc2- referred to as pCCL2 and plasmid encoding reporter sequence and the chemokine CXCL12, -CXCL12-P2A–copGFP-T2A-Luc2- referred to as pCXCL12. Immediate after induction of
hindlimb ischemia and before closing the wound, plasmid (40 μg in 200 μl PBS) was injected in the right gastrocnemius and the quadriceps muscle.

**Non-invasive limb hyperaemia measurements**

Measurements of total blood perfusion of the ischemic and healthy leg were done with Laser Doppler Flowmetry (PeriFlux System 5000, Perimed) with dual lasers, 635 nm and 780 nm and fibre separation were 0.25 resp. 0.5 mm connected to a PowerLab 4/30 (ADInstruments). Basal perfusion and heat-induced hyperemia in ischemic limbs were measured day 7, 14, 21 and 28 post ischemia induction. Also the contralateral healthy leg was measured as reference. The mouse was briefly anesthetized with isoflurane (Abbott) and put on a heating pad, 37°C. A mask with a continuous flow of isoflurane (2-3%) and medical gas was covering the head of the mouse for the entire experiment. The foot pad skin temperature was continuously measured with an isolated thermometer (ADInstruments). Two heating baths were heated up to 22°C resp. 70°C. The baths were both connected to a small tubing system surrounding the mouse limb giving local temperatures of 22°C resp. 34°C. A baseline of 20 minutes was recorded while the water bath on 22°C was flowing in the tube. The hyperaemia was then recorded for 30 minutes when the limb was exposed to the heat (34°C). The software LabChart 7 Pro (ADInstruments) was used to register signals and analysing data.

**Non-invasive foot pad measurements**

The paws of the anesthetized mouse were put parallel on a black, taped plastic tube in which water of different temperatures could flow. The scanner, PeriCam PSI System (Perimed) was focused to 10 cm +/- 1 mm on the paws. During baseline, 22°C water streamed through the plastic tube. Baseline was recorded for 5 minutes or until a steady perfusion was measured. After baseline registration, a change to 70°C water was made. The perfusion during hyperaemia was recorded for 10 minutes. The regions of interest (ROI) were marked and analysed on the healthy and the ischemic footpads using the software program PIMSoft (Perimed). During awakening, the mouse was kept warm using a heating source, Infracare (Philips).

**Ischemic Scoring**

The mice were visually assessed before femoral artery ligation and before each measurement on the days 7, 14, 21 and 28. The Modified Ischemia Score designed by Westvik et al., which is specified in Table 2 was used to determine the ischemia-related appearance and necrosis of the limbs.

**Protein concentration**

From each of the groups on day 1, 3 and 7 post ischemia induction and plasmid delivery, serum was collected. Serum was also collected from an untreated control group, without a femoral artery ligation. The two kit Mouse CXCL12/SDF-1α (R&D Systems) and Mouse/Rat CCL2/JE/MCP-1 Immunoassay (R&D Systems) were used to determine protein concentrations in serum. These kit were followed and were then analysed with the wavelength 450 nm with correction wavelength 540 nm.

**Muscle isolation**

Prior to euthanization at the last day of experiments, 50 μl SBA-lectin Alexa Fluor-488 or 549 (Invitrogen) was injected in the jugular vein and was allowed to circulate for more than 5 min.
After euthanization both gastrocnemius muscles were isolated and the soleus muscle were removed. The muscles were snap frozen in liquid nitrogen in NEG 50 (Thermo Scientific).

**Immunohistochemistry**

The isolated gastrocnemius muscles with injected fluorescence from the healthy and ischemic limbs were sectioned in 7nm thick sections in -22°C at Superfrost plus glass slides (ThermoScientific, Menzel-Gläser). All the following steps were performed in the dark to protect the injected antibody-fluorescence. The glass slides were air dried for 30 minutes and then fixated in ice-cold methanol for 10 minutes. The samples were then permeabilized in 0.5% Triton X (Sigma Aldrich) solution for 15 minutes. To block the background 300µl Background Sniper (Biocare Medical) was added to each glass slide for 45 minutes. Then the samples were incubated with antibodies targeting vasculature and macrophages. All antibodies were conjugated to Alexa Fluor monoclonal antibody-kit 488 or 555 (Invitrogen). The anti-mouse-CD31-mAb PECAM-1 (clone 390, eBioscience) was used to visualize vasculature. To visualize all the macrophages, anti-mouse-F4/80-mAb (clone BM8, eBioscience) and Macrophage Mannose Receptor (MMR) (clone 310301, R&D Systems) antibodies were used. For each glass slide, 300 µl solutions were made with 1µl antibody, 5% fetal calf serum, FCS, (Sigma Aldrich) in PBS. The incubation lasted for several hours in 4°C. The glass slides were washed in PBS with 0.05% Tween 20 (Prolabo) in 5 minutes 3 times. The nuclei were stained with Hoechst 33342 (Invitrogen) diluted to 1:10000 in 10 minutes and then washed in PBS with 0.05% Tween 20 in 5 minutes 3 times. The slides were mounted with Fluromount-G (SouthernBiotech) and a cover slip (Menzel-Gläser).

**Image acquisition and quantification**

To image the staining, a laser scanning confocal microscope (Nikon C1 on a TE2000-U base with the objective Plan APO VC 20x/0.75) were used. The images were analysed in EZ-C1 software (Nikon) and Image J (NIH). To analyse the capillaries a grid of 350x350 was placed over the picture and cells on the intersections were counted, to determine the density of capillaries. The macrophages were analysed in EZ-C1 software (Nikon) and cells that stained positive for both F4/80- and MMR-antibodies were considered as M2. The cells only positive for F4/80-antibody were considered as M1.

**Statistics**

The values are expressed as mean ± SEM. Student’s two-tailed unpaired t-test was used analysing two groups and One Way ANOVA was used analysing more than two groups. p<0.05 were considered significant.

**Results**

**Transfected gastrocnemius muscle produce CCL2 and CXCL12**

In previous studies, the expression of the delivered plasmid was determined. A peak of plasmid expression in the gastrocnemius muscle occured on day 2 and the expression could be observed for proximally 30 days (Figure 1 C, D). To examine if the expression of delivered chemokine leaked into the serum, collected serum from ischemic mice on day 1, 3 and 7 and untreated control mice were analysed with immunoassays. The mice transfected with pCCL2 had significant higher chemokine concentration in serum on day 7 after ischemic induction than pCTR-transfected group (Figure 2A). The pCXCL12 treated group had at day 3 higher
serum levels of the chemokine than the untreated group and at day 7 higher CXCL12-concentrations than both the untreated control and the pCTR treated group (Figure 2B).

**Functional limb perfusion**
We further wanted to investigate if the effect of pCCL2 or pCXCL12 transfection could enhance the functional limb perfusion after the ischemic limb induction. Measurements of delta blood perfusion were done on day 7, 14, 21 and 28 with the Laser Doppler setup (Figure 3B and C). At day 7 the delta perfusion was significant higher in the pCXCL12 treated mice than the pCTR treated (Figure 3D). At day 21, the pCXCL12 group had a higher functional limb perfusion than both the pCTR and the pCCL2 treated mice. A loss of subjects after day 21 resulted in few observations on day 28. Measurements of blood pressure throughout the limb heat-induced hyperemia protocol showed stable blood pressure (86.70 mmHg) in one mouse.

**Functional foot pad perfusion**
With the Laser Speckle Contrast Analysis we could not observe any significant changes between the groups (Figure 4). A tendency that the pCXCL12 treated mice had improved functional perfusion in their foot pad may be observed (Figure 4B).

**Ischemia scoring**
As the legs and paws of the mice did not restore their functional perfusion and none of the mice recovered from the ischemia induction after 28 days, a visual assessment of their limbs were grading the severity of the ischemia (Figure 5). Both the pCCL2 and the pCXCL12 treated mice showed to have a tendency to maintain their functional scoring longer than the pCTR group.

**Capillary density in ischemic muscle**
With the immunohistochemistry of the muscles the amount of capillaries were examined. The capillary density, number of capillaries per mm², of the gastrocnemius muscle had increased in the pCXCL12 treated group day 7 post ischemia induction compared to both the pCTR treated and the healthy muscle (Figure 6B). The amount of perfused capillaries (Figure 6A) did not differ between the groups. When compared the amount perfused and the density of the capillaries a difference between the groups was not seen (Figure 6C).

**Macrophage population in ischemic muscle**
To identify which type of macrophages recruited to the site of ischemia, the immunohistochemical analysis was used, which showed that the M1 macrophages were more abundant at 7 days in the muscle than after 28 days (Figure 7A). It also showed that there were a significant higher amount of the type M2 macrophages in the pCXCL12 treated muscles at day 7 (Figure 7B). Very few macrophages were found in the pCCL2 treated mice (Figure 7A), but of these the majority was of the M2 phenotype (Figure 7C).

**Discussion**
The regeneration process of injured tissue caused by local consistent ischemia is not fully explored though the mechanisms of the sprouting angiogenesis have been extensively studied. The function of the complex population of leukocytes has recently taken a new turn when remodelling and regulating macrophages were discovered. The M2 macrophages act to
repair wounds and construct new capillaries through promoting angiogenesis while the M1 macrophages are shown to be responsible for the phagocytosis and killing of bacteria\textsuperscript{41}. In this study we used transfected ischemic hindlimbs to investigate the angiogenic process by measuring functional blood perfusion, and the macrophage population was counted and specified by their surface proteins to be either of M1- or M2-type.

Plasmid overexpression, apart from being a safe alternative to introduce genes\textsuperscript{42}, is also an approach that gives a transient effect. We found that the transient effect lasted for a period of approximately 30 days when 40 µg DNA was administered as a single injection. In this system, a local area containing high concentrations of the overexpressed chemokine was engineered. However, there was evidence that the local bioengineered site was not perfect as the chemokines could be found in the circulation after 7 days. However, the concentrations were within the normal range of healthy mice. Further studies could investigate the kinetics of the overexpression more in detail with for example repeated delivery of plasmid as well as correlate leukocyte recruitment and vascular function to tissue chemokine concentrations.

The higher limb functional perfusion in the pCXCL12 treated group seen on day 7 and 21 indicates that the overexpression of CXCL12 affects the limb vasculature. Strikingly this effect most certainly was potent enough to extend its effect all the way to the footpad as a tendency to increase functional perfusion was seen in the pCXCL12 group compared to the pCTR group. Interestingly, we found that some of the ischemic foot pads of the pCXCL12 treated ischemic hindlimbs had a higher baseline perfusion than the healthy contralateral foot pad suggesting tissue remodelling (Figure 4A).

Seen in the results from the Modified Ischemia Score, none of the groups recovered from the ischemic limb induction and a high rate of auto amputation was seen after day 21. This is interesting since reports of others\textsuperscript{33, 44} suggest a normalisation of baseline perfusion in the ischemic limb after day 21 and they use the normalization of basal perfusion in the footpad as recovery from the femoral artery ligation. We believe that a protocol extending 28 days using the hindlimb ischemia model might inappropriate for the aim of this study.

We suggest a shortening of the protocol to 21 days, and believe that the most important events deciding the outcome take place during the first 7 days post ischemia induction and plasmid delivery. In this study we found that 7 days after ischemia induction there were a higher capillary density in the pCXCL12 treated group compared to the pCTR group. At 28 days post ischemia induction there were no differences in capillary density in the animals that were investigated. In a therapeutic perspective, higher vascular density in early stages may improve the outcome of ischemia why the tissue may benefit from CXCL12 plasmid delivery early after ischemia induction, for example after an iliac stenosis.

The observed high recruitment of macrophages and especially the M2-type to the ischemic site could correlate to the improved capillary density at day 7. However we do not know if the macrophage recruitment directly affects the capillary density or the perfusion of the newly formed capillaries. It has been reported that the macrophages are important for the repair of damaged tissue by inducing angiogenesis. Thus, they need to be stimulated to be recruited and to act pro-angiogenic\textsuperscript{45}. As leukocytes affects the recruitment of other leukocytes to the afflicted site\textsuperscript{37} it would be interesting to investigate the early recruitment of neutrophils in these hindlimbs since neutrophils have been proven to be essential for induction of angiogenesis\textsuperscript{46}. 
Poor limb perfusion is the clinical problem that the findings of this project may adhere to. Impaired limb perfusion is common and can have many different causes, amongst the most common is as a result of long-term hyperglycemia in type 2 diabetes mellitus patients\(^4^7\) or for example atherosclerosis\(^4^8\), and both diabetes and hypertension are risk factors associated with CLI\(^4^8\). The diagnosis for early stages of impaired limb perfusion is called peripheral arterial diseases (PAD) which can over time develop into in the much more severe chronic lower limb ischemia (CLI)\(^4^9,^5^0\) defined as limb pain even at rest. The perfusion in the lower limb is reduced in CLI and the more distal parts then become less perfused with blood and hypoxia arises. Ulcers and even necrosis in the tissue can occur from the ischemia and they are hard to treat since the tissues is much deprived of its blood\(^4^9\).

There are many ongoing clinical trials addressing both PAD and CLI patients. However many of them fail due to side effects\(^5^1\), insufficient delivery of therapeutic agent\(^5^1,^5^2\) or the choice of therapeutic agent\(^5^3\), where VEGF-A have been the most studied for a long time\(^5^4,^5^5\).

Based on this study, we believe that overexpression of CXCL12 may be an interesting strategy to improve PAD since it does not affect the vasculature directly such as VEGF-A but rather asserts a wider therapeutic effect recruiting proangiogenic macrophages with the capacity to further secrete proangiogenic factors and even mechanically participate in the tissue remodelling.

**Acknowledgements**

I would like to thank and express my gratitude to my supervisor Associate Professor Mia Phillipson and PhD Student Evelina Vågesjö at the Department of Medical Cell Biology for the ten weeks of constant learning, with the guidance in performing experiments and writing reports. Thank you!
References


20. Stratman AN, Malotte KM, Mahan RD, Davis MJ, Davis GE. Pericyte recruitment during vasculogenic tube assembly stimulates endothelial basement


40. Westvik TS, Fitzgerald TN, Muto A, Maloney SP, Pimiento JM, Fancher TT, *et al.* Limb ischemia after iliac ligation in aged mice stimulates angiogenesis


Figure Legends

Table 1. Necrotic DAMPs and their receptors. Illustrated by Kaczmarek et al. are different DAMPs released from necrotic cells and their receptors. Seen are the HMGB1 with different receptors such as Toll Like Receptor 2 (TLR2).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Adaptor</th>
<th>PAMPs</th>
<th>DAMPs</th>
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<tbody>
<tr>
<td>TLR2</td>
<td>Myd88</td>
<td>Lipopolysaccharide</td>
<td>HMGB1, HSPs</td>
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<td>TLR3 (endosomal)</td>
<td>TRIF</td>
<td>Viral, bacterial DNA</td>
<td>Ribo- and -nucleoproteins, mRNA</td>
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<td>Myd88/TRIF</td>
<td>LPS</td>
<td>HMGB1, HMG1, HSPs, Hyaluronan Biglycan</td>
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<td>Myd88</td>
<td>Diacyl lipoprotein</td>
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<td>Myd88</td>
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<td>ssDNA HMGB1</td>
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RNA or DNA sensors
- RIG-I
- MAVS
- dsRNA
- dsDNA

DAMPs or alarmins-specific receptors
- CD14/CD40/CD91: TLR4 cofactor/TRAF6
- CD24: Siglec
- RAGE: Myd88
- DNGR-1: Myd88
- P2Y P2X: ATP
- IL1R1: Myd88
- ST2L/IL1RAcP: Myd88

Table 2. Modified Ischemia Score. The score of the necrosis in the ischemic limbs were assessed by the grade developed by Westvik et al. Ranging from 7, with no necrosis to 0 where auto amputation was a criteria.

<table>
<thead>
<tr>
<th>Modified Ischemia Score</th>
<th>Tissue grade</th>
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<tr>
<td>0</td>
<td>auto amputation</td>
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<tr>
<td>1</td>
<td>leg necrosis</td>
</tr>
<tr>
<td>2</td>
<td>foot necrosis</td>
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<tr>
<td>3</td>
<td>two or more toe discoloration</td>
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<td>5</td>
<td>two or more nail discoloration</td>
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<tr>
<td>6</td>
<td>one nail discoloration</td>
</tr>
<tr>
<td>7</td>
<td>no necrosis</td>
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Figure 1. **Muscle transfection with plasmids.** Map of the plasmid backbone and the three different inserts used for intramuscular expression (A, B). Non-invasive imaging of plasmid luciferase expression in hindlimb of an anesthetized mouse using IVIS bioimaging (C). The plasmid expression was measured over time by means of light intensity corresponding to amount of luciferase in the external abdominal oblique muscle due to its superficial location (D). The substrate, D-Luciferin was given in excess.
Figure 2. Secretion of plasmid encoded chemokines. Circulatory levels of the chemokine CCL2 in unaffected mice and mice with ischemic hindlimbs 1, 3 and 7 days post femoral artery ligation (from left to right, n=5, n=3, n=3, n=3, n=8 and n=10) (A). Circulatory levels of the chemokine CXCL12 in unaffected mice and mice with ischemic hindlimbs 1, 3 and 7 days post femoral artery ligation (from left to right, n=4, n=3, n=3, n=3, n=7 and n=6) (B). * Indicates difference (p<0.05) determined by Student’s t-test.
**Figure 3. Functional limb perfusion measurements.** Complete femoral artery ligation on right limb of an anesthetized mice (A). Illustration of research protocol (B). Representative image of Laser Doppler flowmetry measurements with a baseline of 20 minutes and heat-induced hyperemia of 30 minutes (C). Delta perfusion, baseline to hyperaemia, measured with the Laser Doppler in ischemic hindlimbs over time of mice transfected with pCTR, pCCL2 and pCXCL12 and delta perfusion of the contralateral untransfected healthy limb (left to right, healthy n=3, 3, 3, day 7 n=2, 3, 3, day 14 n=3, 3, 3, day 21 n=2, 3, 2, day 28 n=1, 2, 2) (D). * Indicates difference (p<0.05) determined by One-way ANOVA test, Bonferroni post hoc test.
**Figure 4. Functional foot pad perfusion measurements.** Representative images showing footpad perfusion of baseline (left) and heat-induced hyperremia (right) acquired by Laser Speckle Contrast Analysis (A) and quantifications of delta perfusion, baseline to hyperremia, in healthy and ischemic footpads (left to right, healthy n=3, 3, 3, day 7 n=3, 3, 3, day 14 n=3, 3, 3, day 21 n=2, 3, 2, day 28 n=1, 1, 1) (B).
Figure 5. Modified ischemia score\textsuperscript{40} over time demonstrating ischemia-related effects in the foot pad (healthy n=9, pCTR n=3, pCCL2 n=3, pCXCL12 n=3).
Figure 6. Capillary density in ischemic gastrocnemius. The perfused capillary density per mm$^2$ in the ischemic muscle at day 7 and (A). The total amount of capillaries per mm$^2$ at day 7 and 28 (B). Percentage of perfused capillaries in the ischemic muscle at day 7 and 28 (C) (left to right, healthy n=10, day 7 n=3, 1, 2, day 28 n=1, 2, 2) * Indicates difference (p<0.05) determined by One-way ANOVA test, Bonferroni post hoc test.
Figure 7. Macrophage population in the ischemic gastrocnemius. Density of type 1 macrophages in the ischemic muscle compared to the mean of the contralateral healthy limbs (left to right, healthy n=5, day 7 n=5, 4, 3, day 28 n=1, 3, 2) (A). Density of the type 2 macrophages 7 and 28 days post ischemia induction (left to right, healthy n=5, day 7 n=5, 4, 3, day 28 n=1, 3, 2) (B). Percentage of type 2 macrophages in the ischemic muscle, 7 and 28 days post ischemia induction (left to right, healthy n=5, day 7 n=5, 5, 3, day 28 n=1, 3, 2) (C). * Indicates difference (p<0.05) determined by One-way ANOVA test, Bonferroni post hoc test.