Shock wave treatment after hindlimb ischemia results in increased perfusion and M2 macrophage presence

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List of abbreviations

CTR … control

DAPI … 4',6-diamidino-2-phenylindole

IL6 … interleukin 6

IL13 … interleukin 13

IFNy … interferon gamma

LDPI … Laser Doppler perfusion imaging

LPS … lipopolysaccheride

MCP-1 … monocyte chemotactic protein 1

PBS … phosphate buffered saline

RT-PCR … reverse transcription polymerase chain-reaction

SW … shock waves

SWT … shock wave therapy

TGFb … Transforming growth factor beta

TNFa … Tumor necrosis factor alpha

Author contributions

C.T., J.H., P.P. designed experiments and wrote manuscript

D.L., M.G. performed animal experiments

A.U., C.T. performed analyses

M.G. revised manuscript

ABSTRACT:

Background:

Shock wave therapy (SWT) has been shown to induce angiogenesis in ischemic muscle. However, the mechanism of action remains unknown. Macrophages are crucial for angiogenic responses after ischemic injury. The M2 macrophage subset enables tissue repair and induces angiogenesis. We hypothesized that the angiogenic effects of SWT are at least partly caused by enhanced macrophage recruitment.

Methods:

C57BL/6 mice were subjected to hind limb ischemia with subsequent SWT or sham treatment. Muscles were analyzed via immunofluorescence staining, RT-PCR and western blot. Gene expression and proteins involved in macrophage recruitment was analyzed. Tissue sections were stained for macrophages including subsets, capillaries and arterioles. Laser Doppler perfusion imaging was performed to assess functional outcome.

Results:

Treated muscles showed increased expression of the pivotal macrophage recruiting factor monocyte chemotactic protein 1 (MCP-1). Higher levels of macrophage marker CD14 were found. Increased numbers of macrophages after SWT could be confirmed in immunofluorescence stainings. The expression of the M2 polarization promoting chemokine IL-13 was significantly elevated in the treatment group. We found elevated mRNA expression of the M2 scavenger receptor CD163 after SWT. Immunofluorescence stainings confirmed increased numbers of M2 macrophages

after treatment. SWT resulted in higher number of capillaries and arterioles. Assessment of functional outcome revealed significantly improved limb perfusion in treated animals.

Conclusion:

SWT causes increased macrophage recruitment and enhanced polarization towards reparative M2 macrophages in ischemic muscle resulting in angiogenesis and improved limb perfusion. SWT represents a promising new treatment option for the treatment of ischemic heart disease.

Key words: shock wave therapy, ischemic heart disease, macrophages, angiogenesis

Short Title: SWT induces angiogenesis ischemic muscle via macrophage recruitment

1. INTRODUCTION

Heart failure from ischemic origin still represents a major socio-economic health burden in western countries (Mozaffarian et al., 2015). It is associated with significant impairment of prognosis and quality of life for affected patients. Ischemia is concomitant with the loss of viable myocardium resulting in remodelling of the heart and the formation of functionally impaired scar tissue(Jessup and Brozena, 2003). Available treatments aim to reduce risk factors, alleviate symptoms and/or to beneficially influence cardiac remodeling (Dickstein et al., 2008). However, there is no regenerative treatment available for affected patients. Experimental approaches mainly focus on (stem) cell therapies (Laflamme and Murry, 2011). Although favourable results have been reported, none of them have reached broad clinical use due to distinct limitations like possible development of teratomas, immune response upon injection, cross-species contamination with animal serum due to cultivation, limited expansion potential in vitro, lack of homing to the site of injury, limited ability for differentiation and ethical controversies depending on the cell source. Selection and harvesting as well as the way of administration of a feasible cell type remains challenging (Cunningham et al., 2012; Karp and Leng Teo, 2009).

Shock waves are a specific kind of sound-wave waves which have been used for kidney stone lithotripsy for decades (Chaussy et al., 1980). They have been shown to induce tissue regeneration when used with lower energies than for lithotripsy. They have been successfully used for the treatment of numerous pathologies including bone non-unions and chronic wounds (Elster et al., 2010; Gerdesmeyer et al., 2003; Ottomann et al., 2012; Schaden et al., 2007; Wang et al., 2009). Recently, SWT was reported to reduce infarction size and improve ventricular function in animal models of ischemic heart failure (Nishida et al., 2004). First clinical

applications resulted in a relieve of angina symptoms in patients suffering from coronary heart disease. Thereby, induction of angiogenesis at the boarder zone of the infarcted area and subsequent contractile improvement of hibernating myocardium were described. Although numerous effects including increased VEGF and NO production, enhanced progenitor cell recruitment and induction of endothelial cell proliferation have been described, the underlying mechanisms remain largely unknown (Aicher et al., 2006; Holfeld et al., 2014b; Tepekoylu et al., 2013; Weihs et al., 2014).

After an infarction myocardium is biologically highly active. Inflammatory processes orchestrate the remodelling process and determine the quality of infarction healing. Thereby, macrophages play a crucial role. Two subsets of macrophages have been described. Pro-inflammatory M1 macrophages can be activated by IFN-y, TNF-a or LPS. They are known to induce tissue destruction by the production of pro-inflammatory cytokines like TNF-a and IL6 and the production of reactive oxygen species. This subset of macrophages is increased in chronic inflammatory diseases like atherosclerosis (Gordon, 2003; Nahrendorf et al., 2010).

In contrast, M2 macrophages can be activated by factors like IL13 or TGF-b. This macrophage subset is needed for reparative processes. It contributes to angiogenesis and tissue regeneration by secretion of pivotal angiogenic growth factors, production of anti-inflammatory cytokines and transport of prosurvival factors and reparative enzymes. M2 macrophages can by identified by their scavenger receptor CD 163 (Gordon, 2003; Nahrendorf et al., 2010).

In this project we hypothesized that SWT promotes recruitment of macrophages and enhances the polarization towards M2 macrophages. The hypothesis was tested in a mouse model of hind limb ischemia, which represents a commonly used experimental model for the evaluation of novel angiogenic therapies in the field of ischemic heart disease (Cao et al., 2003; Theurl et al., 2010). Limb perfusion as well as angiogenesis was analyzed to investigate regeneration of the muscle

2.MATERIALS AND METHODS

2.1.Experimental Setup

Male, 12-14 weeks old C57/BL6 mice (Charles River, Sulzfeld, Germany) weighing 25–30 g were randomly divided into 4 groups (CTR 72h, SWT 72h, CTR 28d, SWT 28d, n = 6). Sample size was n=6 for each timepoint and analysis. Treatment animals underwent femoral artery excision and subsequent SWT. Control animals (CTR) were left untreated. The treatment was applied immediately after hind limb ischemia surgery while the animals were still anesthetized. The animals were sacrificed 72 h and 28 days after therapy. Gastrocnemius muscle was harvested 72h 28d after therapy and RT-PCR and immunofluorescence stainings were performed. Laser Doppler perfusion was measured before treatment, and 1 week, 2 weeks, 3 weeks and 28d after treatment. The timepoints were chosen due to observed shock wave effects in previous projects (Holfeld et al., 2014a). The experimental setup is depicted in **supplemental Figure 1**.

2.2. Animal experiments

The experiments were approved by the institutional animal care and use committee at Innsbruck Medical University and by the Austrian ministry of science. The investigation conformed to the ‘‘Guide for the Care and Use of Laboratory Animals’’ published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996; available from: www.nap.edu/catalog/5140.html).

2.3. Hindlimb ischemia model

The model was performed as described previously (Holfeld et al., 2014a). Animals were anesthetized by an intraperitoneal injection of ketamine hydrochloride (Graeub, Switzerland; 80 mg/kg body weight) and xylazine hydrochloride (aniMedica, Germany; 5 mg/ kg body weight). The left femoral artery was ligated and excised between the inguinal ligament and proximal to the branching into saphenous and popliteal artery using 7-0 polypropylene sutures (Ethicon, USA). The entire gastrocnemius muscle was harvested and processed for further analyses.

2.4. Shock wave treatment

Shock wave therapy was performed as published previously (Tepekoylu et al., 2013). Briefly, the animals received therapy to the area above the adductor muscles after hind limb surgery and skin closure, still under anesthesia. Common ultrasound gel was used for coupling. The commercially available Orthogold 180 with applicator CG050-P (TRT LLC, Tissue Regeneration Technologies, Woodstock, GA, USA) served as shock wave device. The diameter of the applicator’s membrane is 4.5 centimeters. 300 impulses were delivered to the ischemic area with an energy flux density of 0,1 mJ/mm^2 at a frequency of 5 Hz. The rationale for the treatment parameters is our experience from previous studies (Holfeld et al., 2014a; Holfeld et

al., 2014b; Tepekoylu et al., 2013). At these energy levels, no adverse effects have been observed.

2.5. RT-PCR

For all RT-PCR analysis, 6 animals per group were used. The entire gastrocnemius muscle was harvested in all the groups. Muscles were homogenized using a Mixer Mill MM440 device (Retsch, Haan, Germany) as described previously (Nef et al., 2009). Total RNA was isolated from homogenized muscle samples using TRI Reagent (Sigma-Aldrich, USA) according to the manufacturer's protocol. Then cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad laboratories, USA). Real-time polymerase chain reaction (PCR) was performed using the StepOnePlus Real-Time PCR with SYBR green reagents (Applied Biosystems, USA) and the following oligonucleotides as described previously(Paulus et al., 2006): primer sequences: b-actin forward CTCTCCCTCAATCCTG, reverse CTCCTCAGGGGCCACACGCA; MCP-1 forward GCAGAGAGCCAGACGGGAGGAA, reverse CCAACACGTGGATGCTCCAGCC; CD14 forward TGCAGGCGCTCCGAGTTGTG, reverse TGATGGAGCTCCGGCGGTA, CD163 forward ACGTGTGGGGCTCCGTCTGT, reverse GGACCCCAGGCTCCGAGTGT, m-IL-13 forward GCAGCAGCTTGAGCACATTT, reverse GCAGACAGGAGTGTTGCTCT. After a denaturation step at 95 °C for 10 min, the cycling started. Annealing was performed at 60 °C for 10 s, followed by a synthesis step at 72 °C for 25 s. SYBR Green fluorescence was detected at 78 °C. After 40 cycles, the experiment was finished by running a melting curve with an augmentation of 0.3 to 95 °C followed by fluorescence detection at the end of each augmentation step. The melting curve was

used to determine the specificity of the primer pairs. Results were calculated using the $2^{-\Delta\Delta C_T}$ method as described previously (Schmittgen and Livak, 2008). Results are shown as fold change compared to the control group.

2.6. Immunofluorescence staining

For all histological analyses, six animals per group were analyzed. Staining was performed as described previously (Holfeld et al., 2014a). Briefly, muscle samples were fixed in 4% formaldehyde and subsequently embedded in paraffin. Prior to the staining procedure, heat-mediated antigen retrieval was performed in sodium-citrate buffer (10 mM sodium-citrate, 0,05%Tween 20, pH 6,0) followed by fixation in methanol for 10 min at 4°C. After blocking for 30 min. with 2% BSA in PBS, samples were incubated with monoclonal rat anti-CD31 (nova, Hamburg, Germany), rabbit polyclonal anti-alphha smooth muscle actin, rat anti-F4/80 (both Abcam, Cambridge, UK), and rabbit anti CD163 (Bioss Antibodies, Woburn, MA) antibodies (Abcam, Cambridge, UK) over night at 4uC. Alexa Fluor 568 goat anti-rat IgG as well as Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies, Carlsbad, Ca) served as secondary antibodies. DAPI (Life Technologies, Carlsbad, Ca) was used for nuclear counterstaining. For macrophage quantification, the total number of F4/80 positive cells was related to the total number of nuclei per high power field. To determine the percentage of M2 macrophages, the number of CD163 positive cells was related to the number of F4/80 cells per high power field. Images were analyzed using AxioVision Rel.4.8 software (Carl Zeiss, Oberkochen, Germany). Positive cells were counted using ImageJ (NIH, Bethesda, MA). Analyses were performed by a single blinded researcher. Five slides per sample were investigated. Three random areas per slide were photographed and analyzed.

2.7. Laser Doppler Perfusion Imaging (LDPI)

Blood flow measurements were performed before treatment and weekly after treatment by a laser Doppler perfusion image analyzer (Moor Instruments, USA) as previously reported (Holfeld et al., 2014a). To minimize data variables attributable to ambient light and temperature mice were kept on a heating plate at 37ºC for approximately 10 minutes before measurement in a darkened room. Blood perfusion is expressed as the laser Doppler perfusion image index representing the ratio of left (operated, ischemic leg) to right (not operated, non ischemic leg) limb blood flow as described previously (Holfeld et al., 2014a). A ratio of 1 prior to surgery indicated equal blood perfusion in both legs.

2.8. Western Blotting

Cells were homogenized and protein isolated. SDS-gels were loaded with 20µg protein. Proteins were immunodetected on Hybond C supermembrane (Amersham Pharmacia Biotech, Amersham, UK) with PageRuler marker (Thermo Scientific, Waltham, MA) as a standard. The blots were probed with rat anti F4/80 antibody (abcam, Cambridge, UK) or rabbit anti CD163 antibody (Bioss Antibodies, Woburn, MA). Detection was then performed by incubating the membranes with the corresponding secondary anti-rabbit or anti-rat biotinylated antibody (Dako, Santa Clara, CA) and development was performed using ECL Western blotting detection reagent (Amersham Pharmacia Biotech, Amersham, UK). Beta-tubulin (Sigma, St.Louis, MO) served as loading control.

2.9. Statistical Analysis

All results are expressed as mean +/- SEM (standard error of the mean). Statistical comparisons between 2 groups were performed by student's t-test using GraphPad PRISM 6 for Mac OS X (GraphPad Software Inc., La Jolla, CA, USA). Continuous variables were either compared with analysis of variance (Bonferroni) after testing for normality of distribution or the Mann–Whitney test. P-values <.05 were considered statistically significant.

3. RESULTS

3.1. Enhanced macrophage recruitment and M2 polarization in shock wave treated ischemic muscle

First, we aimed to investigate whether SWT results in increased macrophage recruitment. Therefore, mRNA expression of the chemoattractant monocyte chemotactic protein 1 (MCP-1) was measured in the treated muscles with and without SWT. Indeed, we found increased MCP-1 levels after treatment (gene expression in fold change: CTR 1.0±0.1371 vs. SWT 2.122±0.2939, p=0.0016) **(Fig.1A)**. Next, we aimed to analyze whether we could find increased numbers of macrophages in the treated tissue. Expression of the macrophage marker CD14 was measured. mRNA levels of CD14 were significantly upregulated after SWT (gene expression in fold change: CTR 1.0±0.3361 vs. SWT 3.59±0.6351, p=0.0012) **(Fig. 1B)**.

M2 polarization promoting chemokine IL-13 was measured. We found significant increase of IL-13 mRNA expression after treatment (gene expression in fold change: CTR 1.0±0.1581 vs. SWT 5.963±2.015, p=0.014) **(Fig. 1C)**.

Expression levels of the M2 macrophage specific scavenger receptor CD 163 was analyzed. CD 163 levels were significantly increased after SWT, showing indeed increased presence of M2 macrophages after treatment (gene expression in fold change: CTR 1.0±0.1545 vs. SWT 4.25±0.8838, p=0.0008) **(Fig.1D)**.

Subsequently, western blotting of treated muscles was performed to analyze protein expression after SWT. Increased protein levels of the macrophage marker F4/80 and the M2 macrophage marker CD 163 were found after SWT **(Fig. 1E).**

3.2. SWT results in higher numbers of M2 macrophages

In order to confirm the RT-PCR findings, we performed immunofluorescence staining of the treated muscles. Sections were stained for F4/80, a commonly accepted marker for macrophage identification in tissue sections. We found significantly elevated numbers of F4/80 positive cells 72h (percentage of positive cells per HPF: CTR 30±2vs SWT 43±5, p=0.0265) and 28d after SWT (percentage of positive cells per HPF: CTR 8±3 vs. SWT 26± 4, p=0.0072) **(Fig. 2A)**. In a next step we aimed to investigate whether we could find higher numbers of M2 macrophages in treated muscles. We therefore stained tissue sections for F4/80 and CD163 and quantified the amount of cells that were positive for both markers. The amount of CD163 positive macrophages was significantly higher in treated animals indicating enhanced M2 macrophage presence 72h (percentage of F4/80 / CD163 positive cells: CTR 62±3 vs. SWT 84±4, p=0.0001) and 28d after SWT (percentage of F4/80 / CD163 positive cells: CTR 28±12 vs. SWT 71±11, p=0.016) **(Fig. 2B)**.

3.3. Increased numbers of capillaries and arterioles after SWT

Next, we quantified capillaries and arterioles after SWT. CD31 staining was used as endothelial marker. SW treated muscles showed higher numbers of capillaries compared to untreated controls (number of capillaries per HPF: CTR 8.18±1.9 vs. SWT 16.25±2.09, p=0.009) **(Fig 3A).** Additional a-SMA (alpha smooth muscle actin) staining was performed for the identification of arterioles. Significantly higher numbers of arterioles could be found after SWT (number of arterioles per HPF: CTR 1.11±0.26 vs. SWT 3.78±0.52, p=0.0003) **(Fig. 3B)**.

3.4. Improved limb perfusion after SWT

Finally, we aimed to assess whether angiogenesis after SWT results in an improved clinical outcome. Hindlimb ischemia surgery was performed. Limb perfusion showed no difference between the groups before treatment (laser Doppler perfusion image index: CTR 0.2785±0.026 vs. SWT 0.3261±0.0147, p=0.19). Perfusion was measured weekly until 4 weeks after treatment. No significant difference between the groups could be observed 2 weeks (laser Doppler perfusion image index: CTR 0.6706±0.092 vs. SWT 0.6993±0.01, p=0.84) and 3 weeks (laser Doppler perfusion image index: CTR 0.6071±0.084 vs. SWT 0.7164±0.12, p=0.48) after treatment. However, limb perfusion was significantly improved in treated animals compared to untreated control animals 1 week (laser Doppler perfusion image index: CTR 0.4399±0.072 vs. SWT 0.7391±0.08, p=0.025) and 4 weeks after treatment (CTR 0.5977 ± 0.12 vs. SWT 0.9683 ± 0.038, p=0.014) **(Fig. 4)**.

4. DISCUSSION

Heart failure due to ischemic heart disease represents a serious condition with limited therapeutic options. Currently, available strategies mainly focus on alleviating symptoms rather than regeneration of functionally impaired tissue (Jessup and Brozena, 2003) . There is a strong need for novel regenerative approaches for this disease.

Shock waves are developing as a regenerative tool for various pathologies. They have proven useful in the regeneration of tissue after ischemic injury (Mittermayr et al., 2011; Nishida et al., 2004; Weihs et al., 2014). Thereby, the induction of angiogenesis seems to be crucial for its regenerative properties. It is not well understood how SWT promotes angiogenesis, albeit several angiogenic effects have been described: increased VEGF production with subsequent VEGFR2 phosphorylation (Holfeld et al., 2014a), ERK activation (Wang et al., 2004), higher NO levels (Yan et al., 2008), ATP release and cell proliferation (Weihs et al., 2014), endothelial progenitor cell recruitment (Aicher et al., 2006; Tepekoylu et al., 2013). However, the role of macrophages has not been investigated yet.

Macrophages play a pivotal role in the remodelling process after infarction, especially in tissue repair and the induction of angiogenesis. This project aimed to elucidate whether macrophages are involved in SW induced angiogenesis. We tested our hypothesis in a model of hind limb ischemia by femoral artery excision in mice. Ischemic muscles were treated with SWT immediately after surgery.

RT-PCR showed increased levels of MCP-1, a major chemokine for macrophages. MCP-1 is known to recruit monocytes and enable migration towards the site of injury (Dipietro et al., 2001). Indeed, significantly higher expression of the macrophage marker CD14 indicated increased macrophage presence in treated

tissue. In parallel, we found higher numbers of F4/80 positive cells in immunofluorescence stainings after SWT.

In a next step we were interested in whether SWT not only causes macrophage recruitment, but also influences polarization of macrophages. Macrophages are divided into two subsets, namely M1 and M2 macrophages. The pro-inflammatory M1 macrophages can cause tissue damage via production of reactive oxygen species, whereas M2 macrophages are crucial for angiogenesis and tissue repair (Gordon, 2003). M2 macrophages promote healing in ischemic myocardium via extracellular matrix remodeling, release of anti-inflammatory cytokines, production of angiogenic growth factors resulting in angiogenesis (Nahrendorf et al., 2010).

mRNA levels of M2 polarization promoting chemokine IL-13 were increased after treatment. Expression of the M2 specific scavenger receptor CD 163 was upregulated after SWT. We analyzed tissue sections for F4/80 and CD 163 and found higher numbers of M2 macrophages in SW treated ischemic muscles. Thus, we could show for the first time that SWT results in (1) increased macrophage recruitment to ischemic muscle and (2) enhanced macrophage polarization towards regenerative M2 macrophages. A well-orchestrated macrophage response is crucial for adequate repair of ischemic muscle. M1 macrophages are responsible for the cleavage of necrotic tissue by phagocytosis and proteolytic activity. Thus, extracellular matrix is degraded and molecules released by dying cells are removed. This is crucial to prepare the injured tissue for angiogenesis and regeneration by subsequently activated M2 macrophages (Nahrendorf et al., 2007). M1 macrophages recruited by SWT might be responsible for phagocytosis of necrotic

tissue and thus prepare ischemic muscle for regenerative processes. However, their exact role remains unclear.

To investigate whether SWT resulted in angiogenesis, we quantified capillaries and arterioles in stained tissue sections. We confirmed higher numbers of both in the treatment group indicating enhanced angiogenesis after SWT.

Finally, we were interested whether the higher number of vessels had resulted in functional improvement, namely perfusion of the ischemic limbs. We therefore measured perfusion 28 days after therapy and found improved blood flow after SWT.

Summarizing, the recruitment of macrophages and enhancing their polarization towards a reparative M2 subset might be a crucial mechanism in SW mediated angiogenesis. Further studies are needed to investigate how the mechanical stimulus is translated into the release of chemokines for macrophage recruitment. Angiogenesis due to SWT could become a promising tool for the regeneration of ischemic muscle. Shock waves have been used in clinical routine for decades and no severe adverse effects have been reported so far. A translation of its use for the treatment of ischemic limb as well as ischemic heart disease therefore seems reasonable.

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7. CONFLICTS OF INTEREST

None. The authors have declared that no competing interests exist. The sponsors of this study had no role in study design, data collection, analysis, and decision to publish or prepare the manuscript.

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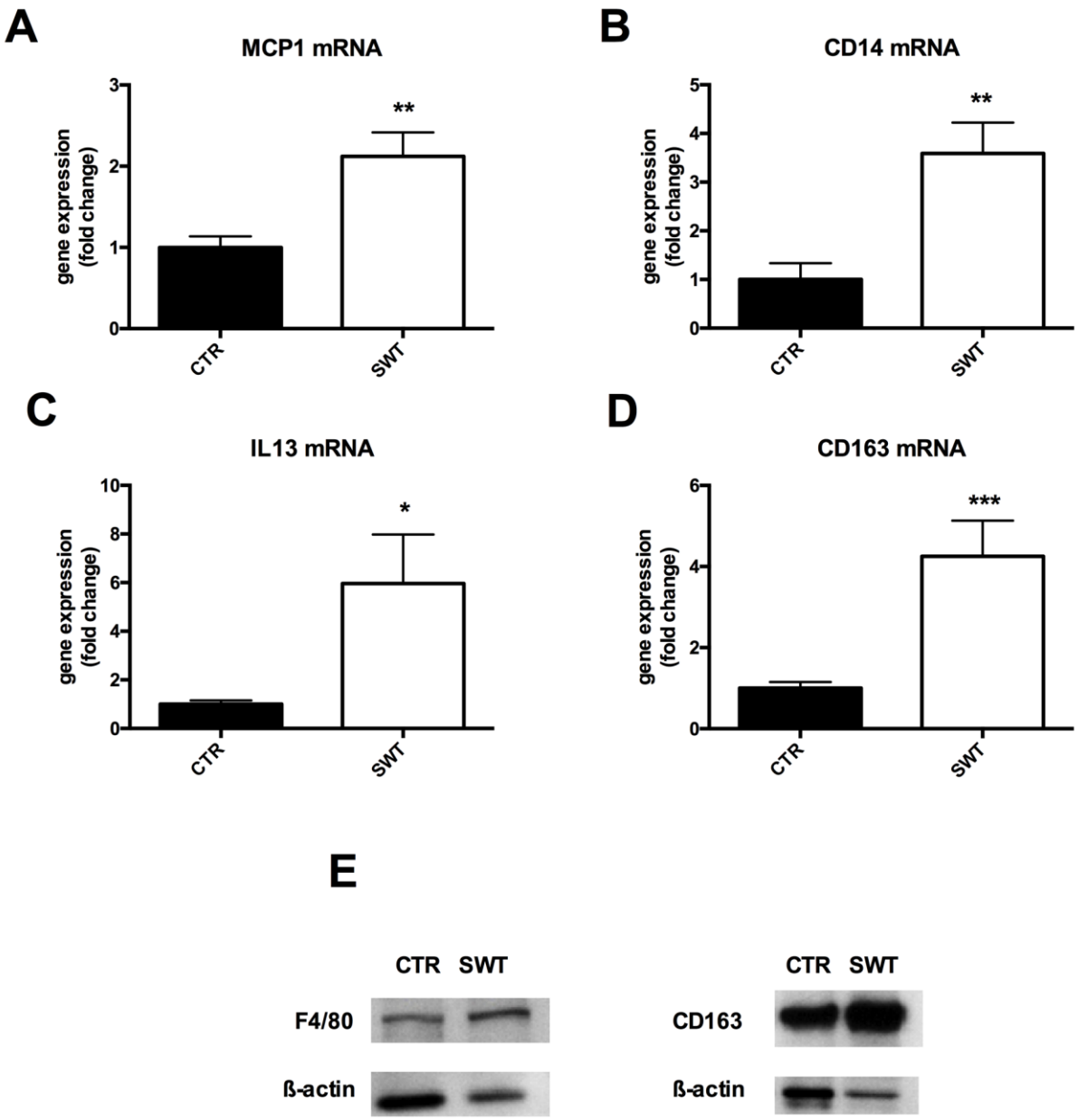

Figure 1: Enhanced macrophage recruitment and M2 polarization in shock wave treated ischemic muscle

Ischemic muscle was treated with SWT and after 72h analyzed for the expression of genes and proteins involved in macrophage recruitment via RT-PCR. Results are shown as fold change.

A Treated ischemic muscles showed increased expression of the pivotal recruiting factor monocyte chemotactic protein 1 (MCP-1) after SWT compared to untreated controls 72h after treatment (n=6, **p<0.01).

B In parallel we found a significant increase of the macrophage marker CD14 in treated muscles hinting for increased macrophage presence after SWT (n=6, **p<0.01).

C The expression of the M2 polarization promoting chemokine IL-13 was significantly increased in the SWT group (n=6, *p<0.05).

D Increased levels of the M2 scavenger receptor CD163 could be found after SWT compared to untreated controls (n=6, ***p<0.001).

E Western blotting of treated muscle showed increased protein expression of the macrophage marker F4/80 and the M2 macrophage marker CD 163. Beta-tubulin was used as loading control.

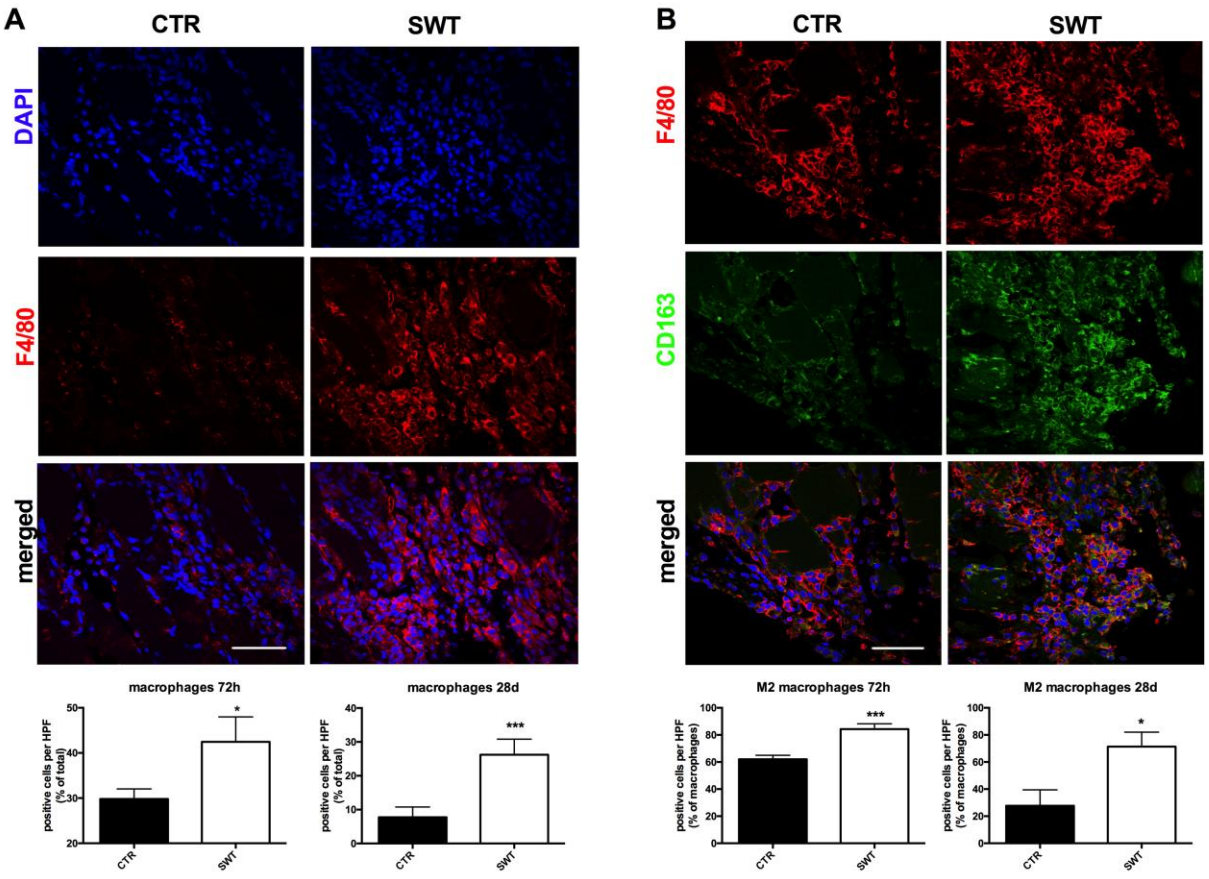

Figure 2: SWT results in higher numbers of M2 macrophages

Ischemic muscles were treated with SWT. Subsequently immunofluorescence staining for the macrophage marker F4/80 and the M2 macrophage marker CD163 was performed**.**

A Representative views of immunofluorescence stainings for DAPI (cell nuclei) and F4/80 (macrophages) 72h after SWT. Tissue sections were stained for macrophage infiltration 72h and 28 days after treatment. Macrophages were identified using F4/80 as a marker. Total cell number was divided by positive cells. Results are shown as percentage of positive cells per HPF. We found significantly elevated numbers of macrophages 72h and 28d after SWT (n=6, *p<0.05,***p<0.001). Scale bar: 50µm.

B Representative views of immunofluorescence stainings for DAPI (cell nuclei), F4/80 (macrophages) and CD 163 (M2 macrophages) 72h after SWT. In a next step we aimed to investigate whether we could find higher numbers of M2 macrophages

in treated muscles. We therefore stained tissue sections for F4/80 and CD163 72h and 28 days after treatment and quantified the amount of cells that were positive for both markers. The amount of F4/80 and CD163 positive cells was significantly higher in treated animals 72h and 28d after SWT indicating enhanced M2 macrophage presence after SWT (n=6, *$p<0.05$). Scale bar: 50µm.

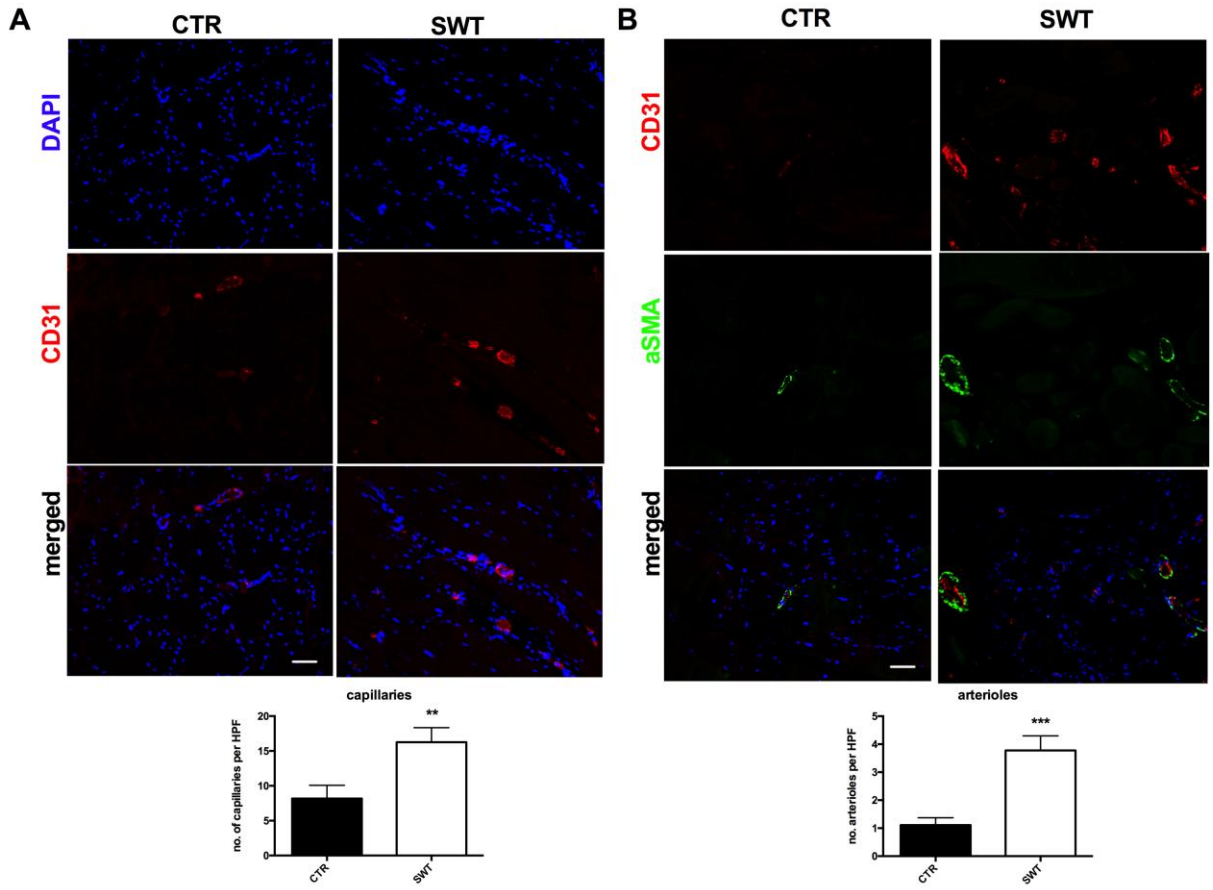

Figure 3: Increased numbers of capillaries and arterioles after SWT

Treated muscle was analyzed for capillaries (CD31) and arterioles (CD31 and aSMA) after SWT.

A Representative views of immunofluorescence stainings for DAPI (cell nuclei) and CD31 (endothelial cells). To analyze whether SWT resulted in angiogenesis, capillaries in tissue sections were quantified 28 days after treatment. We found significantly increased numbers of capillaries after SWT compared to untreated controls (n=6, **p<0.01). Scale bar: 50µm.

B Representative views of immunofluorescence stainings for DAPI (cell nuclei), CD31 (endothelial cells) and a-SMA (smooth muscle cells). SWT resulted in higher numbers of arterioles per HPF (n=6, ***p<0.001). Scale bar: 50µm.

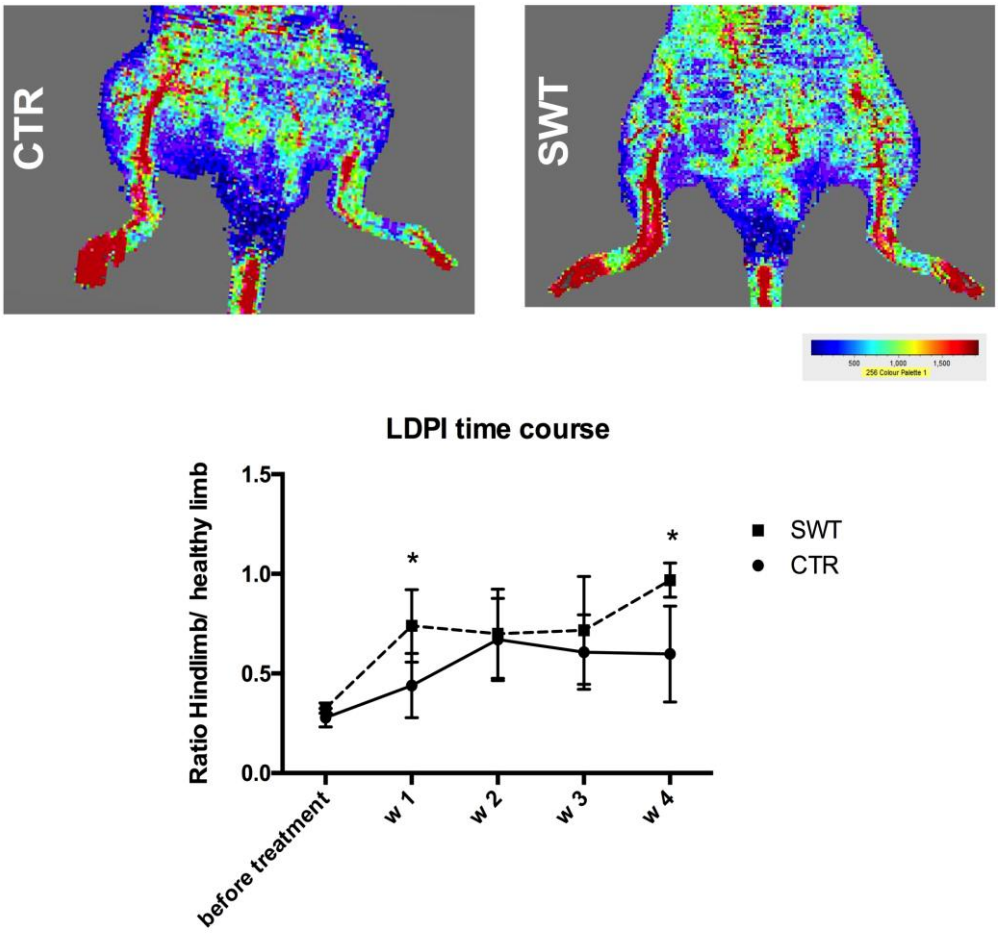

Figure 4: Improved limb perfusion after SWT

Limb perfusion was measured before treatement and weekly after treatment using LDPI.

Mice were subjected to hind limb ischemia by femoral artery excision (left leg). Successful surgery was confirmed by Laser Doppler perfusion imaging. Immediately after surgery, animals received a single SWT to the ischemic muscle. Limb perfusion was assessed before treatment, 1 week, 2 weeks, 3 weeks and 28 days after treatment and showed a significant improvement after SWT 1 week and 28d after treatment. Representative perfusion images of a treated animal and a control animal 28d after treatment are displayed. Quantification of measurements is expressed as ratio of left leg (operated, ischemic) to right leg (non-operated, non-ischemic) (n=6, *$p<0.05$).