From the clinic and polyclinic of Cardiac Surgery
Director: Prof. Dr. med. Gustav Steinhoff
At Rostock University

Applying ischemia and reperfusion in the cremaster muscle model to identify the role of Toll-like receptor 2 and 4 and their intracellular pathways on stem cell homing

Dissertation

To acquire the academic degree of
Doctor of Medicine (Dr. med.)
at the faculty of Medicine
at Rostock University

presented by

Saifullah Abubaker
from Erbil

Date of Submission: 21.02.2020
Date of Defense: 17.11.2020

Dean: Prof. Dr. med. univ. Emil C. Reisinger

https://doi.org/10.18453/rosdok_id00002889

1. Reviewer: Prof. Dr. med. Gustav Steinhoff (clinic and polyclinic for Cardiac surgery, University Heart Center Rostock)

2. Reviewer: Prof. Dr. med. Brigitte Vollmar (Institute for Experimental Surgery, University of Rostock)

3. Reviewer: Prof. Dr. med. Oliver Hakenberg (clinic and polyclinic for Urology, University of Rostock)

List of abbreviations

°C	Grad Celsius
CCD	Charge-coupled Device
CD	Cluster of Designation
cDNA	Complementary DNA
CFDA-SE	Carboxyfluorescein Diacetate-Succinimidyl Ester
CLR	C-type Lectin Receptor
CXCR4	C-X-C Chemokine Receptor Type 4
DAMP	Danger Associated Molecular Pattern
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
FasL	Fas ligand
Fig	Figure
g	Gramm
G	Gravitational constant
HMGB-1	High Mobility Group Protein B1
ICAM-1	Intercellular Adhesion Molecule 1
IR	Ischemia-Reperfusion
IRF	Interferon Regulatory Factor
IVM	Intravital Microscopy
kg	Kilogram
ko	Knockout
LFA-1	Lymphocyte Function-Associated Antigen 1
MACS	Magnetic Cell Separation
MAPK	Mitogen-Activated Protein Kinase
mg	Milligram
µl	Microliter
µm	Micrometer
min	Minute
ml	Milliliter
mm	Millimeter
MSC	Mesenchymal Stem Cell
MyD88	myeloid differentiation primary response protein
NaCL	Natrium chloride

NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-Like Receptor
NO	Nitric Oxide
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate buffer Saline
PCR	Polymerase Chain Reaction
PERFECT	Intramyocardial Trans**P**lantation of Bon**E** Ma**R**row Stem Cells **F**or Improv**E**ment of Post-Infarct Myo**C**ardial Regenera**T**ion in Addition to CABG Surgery
PRR	Pattern Recognition Receptor
RIG	Retinoic acid-Inducible Gene
RLR	RIG-I-Like Receptor
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDF-1	Stromal Derived Factor-1
SDS	Sodium Dodecyl Sulfate
TIRAP	TIR domain-containing Adaptor Protein
TLR	Toll-Like Receptor
TNFR1	Tumor Necrosis Factor Receptor 1
TNFR2	Tumor Necrosis Factor Receptor 2
TNF-α	Tumor Necrosis Factor-αlpha
TNF-β	Tumor Necrosis Factor- βeta
TRAIL	TNF-Related Apoptosis Inducing Ligand
TRAM	TRIF-Related Adaptor Molecule
TRIF	TIR-domain-containing adaptor inducing Interferon β
VEGF–VEGFR	Vascular Endothelial Growth Factor- Vascular Endothelial Growth Factor Receptor
VLA-4	Very Late Antigen-4
WT	Wild Type

Figure legends:

Fig. 1: Image showing devices and tools for femoral artery cannulation and cremaster muscle preparation.

Fig. 2: Image showing the femoral neurovascular bundle in a mouse. From medial to lateral: femoral vein, femoral artery, and femoral nerve.

Fig. 3: Representative example of left femoral artery cannulation in different magnifications.

Fig. 4: Representative example of the mouse cremaster muscle tissue following microsurgical preparation in different magnifications.

Fig. 5: Representative example of the mouse following femoral artery cannulation via the femoral catheter and microsurgical preparation of cremaster muscle before intravital microscopy.

Fig. 6: Representative shot of intravital microscopy showing cremasteric microvasculature, namely arteriole and venule immediately after injection of fluorescently stained stem cells (picture captured under 20x Olympus LUC Plan FLN- water immersion objective).

Fig. 7: Quantitative analysis of c-kit$^+$ cells showing rolling in control groups.

Fig. 8: Quantitative analysis of c-kit$^+$ cells showing rolling in control groups and TLR knockout groups

Fig. 9: Quantitative analysis of c-kit$^+$ cells showing rolling in experimental groups.

Fig. 10: Representative picture of intravital microscopy showing rolling stem cell in postcapillary venule of the cremaster muscle (picture captured under 20x Olympus LUC Plan FLN- water immersion objective).

Fig. 11: Quantitative analysis of c-kit$^+$ cells showing firm adhesion in control groups.

Fig. 12: Quantitative analysis of c-kit$^+$ cells showing firm adhesion in wildtype mice and TLR knockout groups.

Fig. 13: Quantitative analysis of c-kit$^+$ cells showing firm adhesion in experimental groups.

Fig. 14: Representative Picture of Intravital microscopy exhibiting adherent stem cell in postcapillary venule of the cremaster muscle (picture captured under 20x Olympus LUC Plan FLN- water immersion objective).

Fig. 15: Quantitative real-time PCR analysis for TNF-α gene expression in wild type mice and TLR knockout groups.

Fig. 16: Quantitative real-time PCR analysis for TNF-α gene expression in experimental groups.

Contents

1. Introduction

1.1 The Heart and Stem cell

The idea that the adult heart to be unable to regenerate has been revised through studies that have demonstrated the existence of de novo myogenesis in the heart with different range of potential (Laflamme & Murry, 2011). This new view opened a remarkable therapeutic option for treating the injured heart. Cardiac regeneration might be through either dormant inhabitant cells or migrated from bone marrow in case of recruitment via injured myocardium or transplanted. The issue surrounds with controversy, to which extent the stem cells are able to regain the function of the heart, whether the stem cells directly differentiate to contractile myocardium and replacing dead cells or through other mechanisms support regeneration like paracrine effect and angiogenesis (Lyngbæk, Schneider, Hansen, & Sheikh, 2007).

Stem cells are defined as cells able of self-proliferation as well as differentiation to functionally active cells (Weissman, 2000).
According to the root of development stem cells can be categorized into two groups:
1. Embryonic stem cells which are originated from the inner cell mass of early embryos with a capacity of formation of mesoderm, endoderm, and ectoderm.
2. Adult stem cells which are distributed in diverse tissues and has the ability to give rise to at least one sort of functionally developed offspring. Adult stem cells were originally supposed to possess restricted differentiation range, but the studies exhibited the divergence of differentiation into endodermal, mesodermal and ectodermal ancestry cells, such as induced pluripotent stem cell that can be formed from different somatic cells. Stem cell can be classified according to the spectrum of differentiation into Totipotent, Pluripotent, Multipotent, and Unipotent. Totipotent stem cell carries the broadest spectrum of cellular production like fertilized oocyte, which gives rise to all three germ layers, mesoderm, endoderm, and ectoderm, in addition to the future placenta for maintenance of the embryo. Pluripotent stem cells like inner cell mass of blastocysts (days 4-14 after fertilization) are capable of

formation of mesoderm, endoderm, and ectoderm without extra-embryonic tissues. Multipotent stem cells have the capacity to produce all cell sorts in one specific lineage. Unipotent stem cells can give rise to just one cell lineage (Sobhani et al., 2017).

A prominent type of adult stem cells is bone marrow stem cells (Sobhani et al., 2017). Hematopoietic stem cells are essential in the development of the hematolymphoid system, perpetuation, and reproduction (Weissman, 2000).
Preclinical studies showed that Bone marrow stem cells are capable of producing cells other than blood cells and have competency to differentiate into different types of cells such as skin (Kataoka et al., 2003), skeletal muscle (Ferrari et al., 1998) blood vessels (Asahara et al., 1999) hepatocytes (Lagasse et al, 2000) and cardiomyocytes (Orlic et al., 2001).

The heart possesses extremely limited adaptive capacity in response to injury (Keith & Bolli, 2015) and self-regeneration appears to poorly compensate myocardial loss after myocardial insult (Zhang, Mignone, & MacLellan, 2015), this might be because the heart possesses minimal number of internal stem cells (Beltrami et al., 2003), despite this it is of profound importance to find out that there is sign of regeneration in the human heart (Bergmann et al., 2009).
Based on findings in preclinical studies, therapeutic stem cell application in many clinical trials has been established with promising results (Hare et al., 2012; Chugh et al., 2012).
Various routes for stem cell transplantation have been described, such as delivery in the coronary artery (Janssens et al., 2006) or direct injection in the myocardium as in our institution in the PERFECT clinical trial (Donndorf, Kaminski, Tiedemann, Kundt, & Steinhoff, 2012).

The POSEIDON randomized trial reported that Transendocardial injection of allogeneic and autologous MSCs revealed to interface with ventricular remodeling and improved functional ability and quality of life (Hare et al., 2012).
In the SCIPIO Trial utilizing intracoronary infusion of c-kit^{+} cells, the observed improvement in LVEF was around 7.7% after four months and rising to around 13.6 % after one year of the stem cell application. More detailed estimation of regional EF

showed enhancement of local function, which accompanied by shrinkage of infarct size from 30.4 ± 5.1 g at baseline to 23.5 ± 3.7 g at four months and 22.6 ± 5.5 g at 12 months respectively after stem cell infusion. This reduction in infarct size concomitant with apprise in living myocardial tissue + 11.6 g at four months and + 31.5 g at 12 months after Stem cell therapy (Chugh et al., 2012).

1.2 Stem cell homing

The milieu of stem cell implements crucial influence on how effectively this repair system can regenerate the damaged myocardium. Generally, through three ways stem cell therapy can be translated: 1. Autologous transplantation, 2. Allogeneic transplantation, and 3. Enhancement of resident cardiac stem and progenitor cells (Wang, Chen, Houser, & Zeng, 2013).

After implantation, the stem cells are facing in the initial step the challenge of survival. Microenvironmental difficulties like inflammation, lack of blood supply, and or apoptosis might be the causes of death of around 90% of transplanted cells within one week of application. After engraftment, around 1% of cells survive a period of 4 weeks (Zhang, Wang, Li, Duan, & Yang, 2013).

Acute coronary syndrome induces attraction of a number of diverse bone marrow stem cells through raised production of chemoattractants including cytokines, chemokines, kinins, bioactive phospholipids, growth factors, and the complement cascade. The mobilized stem cells comprise endothelial progenitor cells, hematopoietic stem cells, circulating angiogenic cells, pluripotent very small embryonic-like cells and mesenchymal stromal cells. It has been shown that the mobilized CD34+ (known as a marker of endothelial progenitor cells) cells exhibit increased levels of CXCR4, LFA-1 (Lymphocyte Function-Associated Antigen 1), VLA-4 (Very Late Antigen-4) and ICAM-1 (Intercellular Adhesion Molecule 1) receptors, which basically induces homing of these cells in the damaged region. As a result of myocardial damage in acute coronary syndrome, the level of inflammatory mediators increases. Many of these mediators are chemoattractants to promote migration of bone marrow stem cells to the site of injury. Circulating stem and progenitor cells express several receptors for chemokines,

cytokines and growth factors. Different pathways are involved in chemotactic response of stem cells to the increased level of inflammatory mediators at the site of injury (Wojakowski, Landmesser, Bachowski, Jadczyk, & Tendera, 2012). A pivotal factor in retaining of stem cells in the bone marrow is stromal-derived factor-1 (SDF-1, also termed CXCL12) through interaction with its receptor CXCR4 (C-X-C chemokine receptor type 4). This Receptor highly expressed on hematopoietic and other stem cells. Activated SDF-1 at the site of injury is an essential chemoattractant for BM-derived cells exhibiting CXCR4 (Borlongan, 2011).

Numerous other chemotactic routes also involved in stem cell homing such as stem cell factor–CD117 (c-kit factor), VEGF–VEGFR (Vascular Endothelial Growth Factor-Vascular Endothelial Growth Factor Receptor), hepatocyte growth factor–c-met and leukemia inhibitory factor– leukemia inhibitory factor receptor. Furthermore, there are several factors affecting stem cell mobilization, including age, related cardiovascular risk factors like diabetes mellitus, and medication. These factors influence functional potency and the number of stem cells. Distorted Endothelial regeneration in diabetic patients might be as a result of decreased endothelial NO synthase-derived nitric oxide (NO) production. On the other side, some factors enhance stem cell mobilization, such as the use of statin improves stem cell migration and the ability of neovascularization. Additionally, physical exercise induces the release of VEGF, increases stem cell mobilization (CD34+/CD45+ and CD133+/CD45+) and improves the migratory ability of these cells (Wojakowski, Landmesser, Bachowski, Jadczyk, & Tendera, 2012).

Moreover, in stem cell homing, adhesion molecules are found to have a crucial role. For example, P- and E-selectin are crucial for initial tethering and **'Rolling'** of hematopoietic stem cells and progenitor cells along the endothelial wall of blood vessels. As a next step, cellular **'Adhesion'** and transendothelial migration is mediated through Integrin molecule (Sahin & Buitenhuis, 2012).

Under normal conditions, hematopoietic stem and progenitor cells present in circulating blood with circadian change. Stress situation like inflammation is leading to mobilization of more stem cells. It seems that this recruitment is part of the response to danger signaling under the setting of the innate immune system for protection, maintenance as well as regeneration of body tissues and organs. TLR has been

identified as a new player in stem cell trafficking (Ratajczak et al., 2010). For example, Rossini et al. reported that cardiac c-kit^{+} stem cells migration and proliferation are enhanced through HMGB-1 (a TLR ligand) (Rossini et al., 2008).

1.3 Ischemia-reperfusion injury

The ischemia-reperfusion injury occurs in many diseases like in myocardial infarction, cerebral stroke, renal and intestinal ischemia, and also in relation to cardiovascular surgeries (Arumugam et al., 2009). The consequences of ischemia-reperfusion injury may go beyond the affected tissue leading to multiple organ failure (Park, Kim, Brown, D'Agati, & Lee, 2011).

Ischemia is the most common cause of cardiac injury due to interruption of myocardial perfusion either long-lasting, which results in permanent tissue damage or short-lasting. In the latter case, restoration of blood flow leads to another type of injury named "reperfusion injury." The end result of diverse cardiac diseases is an irreversible cardiac failure, and Immune system may play a decisive role in every step of cardiac disease development (Epelman, Liu, & Mann, 2015).

The injury resulting from ischemia-reperfusion enhances an inflammatory response referred as sterile inflammation (Eltzschig & Eckle, 2011), in which the reaction happens without the involvement of any pathogenic factors (Epelman, Liu, & Mann, 2015).

Various changes at cellular level happen as a result of myocardial ischemia-reperfusion: firstly 'no-reflow' phenomenon in which ischemic-reperfused endothelial cells induce leukocyte accumulation via an exhibition of adhesive molecules. Secondly, after reperfusion, the viable myocardium shows temporary impairment in its function because of myocardial hibernation, also known as "stunning." Thirdly, the injured tissue is liable to arrhythmia. Fourthly, the ongoing progression of tissue damage is continuous even after the reestablishment of blood flow. During reperfusion a cascade of adverse effects is taking place, starting with increasing intracellular Ca2+

concentration with the commencement of proapoptotic signalings like cytochrome c or Diablo homolog (Arslan, De Kleijn, & Pasterkamp, 2011).

Different ways of cell death would be induced as a consequence of Ischemia-reperfusion injury, like necrosis and apoptosis. Necrosis leads to rupture of these cells due to swelling of organelles and release of intracellular components which are remarkably enhancing factors for cytokine generation and attraction of inflammatory cells. On the other hand, apoptosis is characterized by internal programming leading to cellular regression with retaining membrane until late in the process (Hotchkiss, Strasser, McDunn, & Swanson, 2009).

As a result of cellular death the released intracellular matrix to extracellular, which are danger-associated molecular patterns (DAMPs) such as HMGB-1 (Hoebe et al., 2003) to neighbor cells and to circulating cells to stimulate Toll-like receptors (TLRs) and consequent production of inflammatory cytokines (Arslan, De Kleijn, & Pasterkamp, 2011).

The induced inflammatory mediators enhance neutrophil infiltration to the affected region leading to endothelial damage by the production of reactive oxygen species (ROS), proteases, cytokines, and lipids. Neutrophil invasion to the site of injury related to aggravation of cardiac damage and prevention of this infiltration seems to have guarding effect. On the other hand, it has been shown that inflammatory cells and cytokines are involved in cardiac repair. It is of profound importance to apply treatment aiming in protecting the heart from damage and enhancing the healing (Timmers et al., 2012).

1.4 Toll-like receptors

Pattern recognition receptors (PRRs) are critical members of the innate immune system in detecting exogenous molecules from infectious agents referred as pathogen-associated molecular patterns (PAMPs) or endogenous danger signals produced from destroyed cells, known as damage-associated molecular pattern

(DAMPs).
Four groups of PRRs have been recognized. These groups comprise Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and NOD-like receptors (NLRs) (Takeuchi, O., & Akira, S., 2010). TLRs are one of the most studied members of PRRs.

Toll gene was first described in 1985 by Christiane Nüsslein-Volhard from the Max Planck Institute in Tübingen. She found that this gene plays a prominent role in early embryonic development of Drosophila. Later, scientists from Hoffmann's laboratory in Strasbourg found that the Toll gene is of profound importance in the immune system of Drosophila. Then, Ruslan Medzhitov and Charles Janeway at Yale University identified that the mammalian possesses Toll-like receptor, which upon activation produces NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) in a similar way to Toll in Drosophila. Uncovering new mechanisms of TLRs engagement in many infectious diseases and inflammatory responses made them a valuable family member of PRRs. TLRs are involved in the pathophysiology of many diseases, including cardiovascular diseases (Hansson & Edfeldt, 2005).

10 TLRs are present in humans. Different types of cells are expressing TLRs, including endothelial cells and cardiomyocytes (Ha, Liu, Kelley, Williams, & Li, 2013).
TLRs are transmembrane proteins (Kawai and Akira, 2010), which exert their effects after stimulation through intracellular adaptors including: MyD88 (myeloid differentiation primary response protein), TIRAP (TIR domain-containing adaptor protein), TRIF (TIR-domain-containing adaptor inducing interferon β), and TRAM (TRIF-related adaptor molecule) (Frantz, Ertl, & Bauersachs, 2007).

TLRs conduct signaling predominantly through two main downstream pathways, MyD88, and TRIF pathways. All TLRs are adopting MyD88-dependent pathway with the exclusion of TLR3, which in turn resulting in excitation of nuclear factor-kappa B (NF-kB) and mitogen-activated protein kinases (MAPKs) to produce inflammatory mediators. Activation of TRIF-dependent Pathway can be done through TLR3- and TLR4-triggering, enhancing interferon regulatory factor (IRF) 3 and NF-kB for promoting type I interferon (interferon α/β) and inflammatory mediators. TIRAP and TRAM are acting as organizing accessories that connect TRIF and MyD88 to TLRs.

TIRAP associates MyD88 to TLR2 and TLR4. TRAM recruits TRIF to TLR4. TLR4 is the only TLR that utilizes all four adaptors (Kawai and Akira, 2010).

Evidence shows that TLRs (Chen et al., 2017) with associated ligands like HMGB-1 (Tsung et al., 2005) and intracellular adaptors (Ye et al., 2016) play a crucial role in mediating inflammatory response after ischemia-reperfusion injury.
Recently has been recognized that TLRs enrolled in stem cell reparative mechanisms.
Limana et al. showed recovery of cardiac performance through the application of TLR-ligand (HMGB-1) in the peri-infarcted left ventricle at four h after ischemia via enhancement of endogenous cardiac c-kit^{+} progenitor cells proliferation and differentiation (Limana et al., 2005).
The exact underlying pathophysiology and way of the intervention of TLRs and associated signaling pathways related to stem cell activity in combination with ischemia-reperfusion injury demand more clarification and detailed studies.

1.5 TNF-α

Myocardial ischemia-reperfusion injury stimulates TLRs to induce several cytokines, which in turn play a crucial role in the regulation of inflammation. One of these critical cytokines is TNF-α (Takeishi & Kubota, 2009). Under normal physiological condition, the cardiomyocyte does not express TNF-α (Chen et al., 2011).
TNF-α was first described in 1975 as an antitumor agent (Carswell et al., 1975). This cytokine beside its common name also referred as endotoxin-induced factor in serum, cachectin, and differentiation-inducing factor. TNF group, to which TNF-α belongs, consists of numerous essential cytokines participating in mechanisms of tumorlysis, apoptosis, and regulation of inflammation. This family comprises TNF-alpha (TNF-α), TNF-beta (TNF-β), CD40 ligand (CD40L), Fas ligand (FasL), TNF-related apoptosis inducing ligand (TRAIL), and LIGHT (Chu, 2013).
TNF-converting enzyme translates membrane-associated TNF-α (mTNF-α) to secreted TNF-a (sTNF-α), which is an active pattern (Tian, Yuan, Li, Gionfriddo, & Huang, 2015).

The induced TNF-α signaling has been showed to be crucial in maintaining homeostasis, though overactivation of TNF-α leads to drawback effects (Cicha & Urschel, 2015).

TNF-α is a well-known cytokine released during a stressful situation. Both destructive and constructive roles of this inflammatory mediator were reported. This bidirectional action may be demonstrated through its different receptors, TNFR1 and TNFR2. The studies showed that TNF-α exerts adverse impact via TNFR1, while affirmative action through TNFR2 stimulation (Jiang & Liao, 2010).

In the setting of therapeutic stem cell application, it has been found that TNF-α plays a vital role in stem cell–endothelial interaction (Kaminski et al., 2008).

2. Aim of the research

Our research aimed to clarify the role of TLR2 and TLR4 and their secondary messengers in stem cell trafficking after stem cell injection using selective knockout mice for TLR2 and TLR4 receptors and associated intracellular pathways of MyD88 and TRIF in a well-established intravital microscopic examination in the cremaster muscle model. In order to provide similar ambient to myocardial injury following ischemia and reperfusion, we applied a sequence of 15 min ischemia followed by 15 min reperfusion in the cremaster muscle of the mouse.

In this study, we wanted to observe the migratory behavior of stem cells in relation to local endothelium in destination tissue suffered from ischemia and reperfusion injury.

3. Material and Methods

3.1 Animals of experiments

The study is carried out according to the animal welfare and permission from the State Office for Agriculture, Food Safety and Fishing in Mecklenburg Vorpommern/Germany under file number LALLF M-V/TSD/7221.3-1.1-111/12.

The experimental animals were supplied from Charles River Company (Sulzfeld, Germany). All animals were male mice. The genetic background of the control group was C57BL/6J. To minimize differences between experimental groups, the knockout mice had the same original background race of the control group. The following knockout mice were investigated:

- Toll-like receptor 4 knockout with strain name B6.B10ScN-*Tlr4*$^{lps\text{-}del}$/JthJ
- Toll-like receptor 2 knockout with strain name B6.129-*Tlr2*^{tm1Kir}/J
- MyD88 knockout with strain name B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J
- TRIF knockout with strain name C57BL/6J-*Ticam1*^{Lps2}/J

To prevent influence of changing the breeding environment and emotional disturbance, the animals were acclimatized at least for three days after reaching the Institute for experimental surgery in Rostock. At the time of operation, the mice were at least eight weeks and weighing 20-25g.

The animals were bred in standardized cages with free access to food and water as well as regular 12-hour day/night rhythm under constant room temperature and humidity.

3.2 Animal groups

The experiments established in six groups, and in each group were six mice included:

1. Group 1 (*Control-WT*, n=6): negative control group comprises wild type male mice of strain C57BL/6J **without ischemia and reperfusion**.

2. Group 2 (*IR-WT*, n=6): positive control group consists of wild type male mice of strain C57BL/6J with the application of a sequence of 15 min **ischemia** followed by 15 min **reperfusion** of cremaster muscle.
3. Group 3 (*IR-TLR2-ko*, n=6): Toll-like receptor 2 knockout male mice (B6.129-*Tlr2*^{tm1Kir}/J) exposed to 15 min **ischemia** and 15 min **reperfusion** of cremaster muscle.
4. Group 4 (*IR-TLR4-ko*, n=6): Toll-like receptor 4 knockout male mice (B6.B10ScN-*Tlr4*$^{lps-del}$/JthJ), in which the cremaster muscle treated with a sequence of 15 min **ischemia** and 15 min **reperfusion**.
5. Group 5 (*IR-MyD88-ko*, n=6): MyD88 knockout male mice (B6.129P2(SJL)-*Myd88*$^{tm1.1Defr}$/J) with 15 min **ischemia** and 15 min **reperfusion.**
6. Group 6 (*IR-TRIF-ko*, n=6): TRIF knockout male mice (C57BL/6J-*Ticam1*^{Lps2}/J) exposed to the same sequence of 15 min **ischemia** followed by 15 min **reperfusion** as in Groups 2,3,4 and 5.

3.3 C-kit$^+$ stem cells isolation

Bone marrow c-kit$^+$ cells were isolated through utilizing magnetic microbeads coated with anti-c-kit monoclonal antibody (CD117 Beads), Miltenyi Biotec MS columns®, and the MiniMacs® cell separation system (Miltenyi Biotec, Bergisch Gladbach, Germany). The donor male mice were originally C57BL/6J, B6.129-Tlr2Tm1kir/j, and C3H/HeJ. To roll out differences in circulating TLRs in order to study the effect of endothelial TLR we used in our experiments universally the donor male mice C57BL/6J.

Checklist:

- Devices:
  - Sterile hood
  - Centrifuge for tubes 50 ml, 15 ml, and 1.5 ml capacity with below settings:
    - 4 °C x 300 G x 10 min
    - 20 °C x 300 G x 10 min
  - Refrigerator 4 °C
  - Water bath

- Culture hood (incubator)
- Light Microscope for cell counting

❖ Tools and Instruments

- Icebox
- 4 ml DMEM in a 15 ml tube
- Skin scissor
- Petri dish
- Pens
- 50ml MACS within 50 ml tube
- Syringe 5 ml
- 26 G needle
- Pipette (10 µl-1000 µl)
- Pipette (0.5 µl -10 µl)
- Pasteur pipette
- Cell Filter (40 µm)
- Boy pipette
- Tube for boy pipette
- Trypan blue
- Paper for cell count
- Slide for cell count
- CD117 Beads
- MACS magnet
- MACS Column x 2
- MACS Filter x 1
- 15 ml tube x2
- Sterile PBS in a water bath
- 1.5 ml tube

Procedure:

Bilateral femur and tibia of the donor mouse were amputated, dissected, and transferred to a sterile hood in 15 ml tube, which contains 4 ml of DMEM. In order to prevent temperature fluctuation, which might affect the cells, the isolation procedure was undertaken on ice. The bones were placed into a petri dish and kept in DMEM. With small scissor the open ends of the bones were cut. Using 26 Gauge needle and 5

ml syringe the bone marrows were flushed out with MACS (Magnetic Cell Separation) buffer. The procedure was repeated until bones appeared white. Then with 1 ml pipette the larger pieces of bone marrow were degraded to be more homogenous, keeping in mind the shearing stress. In the next step, the 40 µm cell filter (blue) was prepared and rinsed with 1 ml MACS. Using a Pasteur pipette to filter the cell suspension through the cell filter into a fresh 50 ml tube. Thereafter the petri dish was rinsed with 5 ml MACS and applied on cell filter. Swirling gently, if no clumps formed and appearance was uniform, the cells were sediment in the centrifuge at 300 x G for 10 min, 4 °C. The supernatant was discarded and resuspended the cells in 1 ml MACS and added 14 ml MACS with boy pipette. At this point the cells were counted taking 4 µl from cells, 6 µl from MACS and adding 10 µl Trypan blue. The whole volume was 20 µl. Then 10 µl from the mixture has been taken for cell count.

The cells were centrifuged again at 300 x G for 10 min at 4 °C. Meanwhile, CD117 Beads Volume was calculated. For every 10^7 mononuclear cells / 1 ml, 20 µl Beads and 80 µl MACS were added to the cell sediment. First, the larger volume was added, then the smaller volume. After homogenization, the cells were placed in the refrigerator at 4 °C for 15 min.

For washing the cells, 5 ml MACS was added and then centrifuged at 300 x G for 10 min at 4 °C. Meanwhile, MACS Isolation was prepared: 30 µm preseparation filter and column in separation magnet on MACS multistand over a fresh 15 ml tube. The pre-separation filter and the column in the separation magnet were rinsed with 0.5 ml MACS in order to be prepared for passing cells smoothly through to avoid cell injury. The supernatant was resuspended in 0.5 ml MACS. Apply cell suspension on filter and column and wait until no more drops come down. Washing was repeated three times with 0.5 ml MACS. The negative fraction passed through the filter and the column without being caught by the magnet. The positive fraction remained in the column.

To optimize the isolation, the column with a positive fraction placed on the second MACS Column and rinsed three times with 0.5 ml MACS.

The second column (which contain positive cell fraction) was removed from multistand, and away from the magnet, the cells were eluted into a fresh 15 ml tube by forcing 1 ml MACS buffer using the provided pusher.

Finally, the isolated cells were counted and centrifuged at 300 x G for 10 min at 14-20 °C. The total numbers were around 0.4×10^6 c-kit$^+$ cells.

3.4 Stem cell staining

In order to visualize the stem cells under intravital microscopy (IVM), the cells were marked with fluorescent dye Carboxyfluorescein Diacetate-Succinimidyl Ester (CFDA-SE) (Thermo Fisher Scientific, USA). Sterile PBS was warmed to 25 °C in a water bath. At this step, working has been done at room temperature. After centrifuging the isolated stem cells, the supernatant was discarded, and resuspension was done within 500 µl of prewarmed PBS. Then 2 µl of CFDA has been added to the solution (CFDA: PBA + cells 2:500). The cells were incubated for 15 min at 37 °C in the incubator. Thereafter they were centrifuged at 300 x G for 10 min at 20 °C. Finally, the cells were resuspended in 300 µl PBS and prepared for the next step of the experiment. The cells were placed in 1.5 ml Eppendorf tube and covered with dark paper to prevent cells from exposure to light.

3.5 Cremaster muscle preparation

The method of opening the cremaster muscle under intravital microscopy is utilized for visualization of cremaster microvasculature to its finest branches for studying the dynamic changes in these small blood vessels (Baez, 1973).

Checklist:

- Storage platform for the mouse
- Two microforceps
- One microscissor
- Skin scissor
- Needle holder
- 6.0 prolene suture
- Clamp
- 2 x 10 cm long plaster threads
- 2 x NaCl in one ml Insulin syringe
- 1 Insulin syringe for anesthesia injection

- 2 ml tube
- 12 x plaster strips
- A femoral catheter (thinned and flushed)
- cold nitrogen within a special container
- Icebox

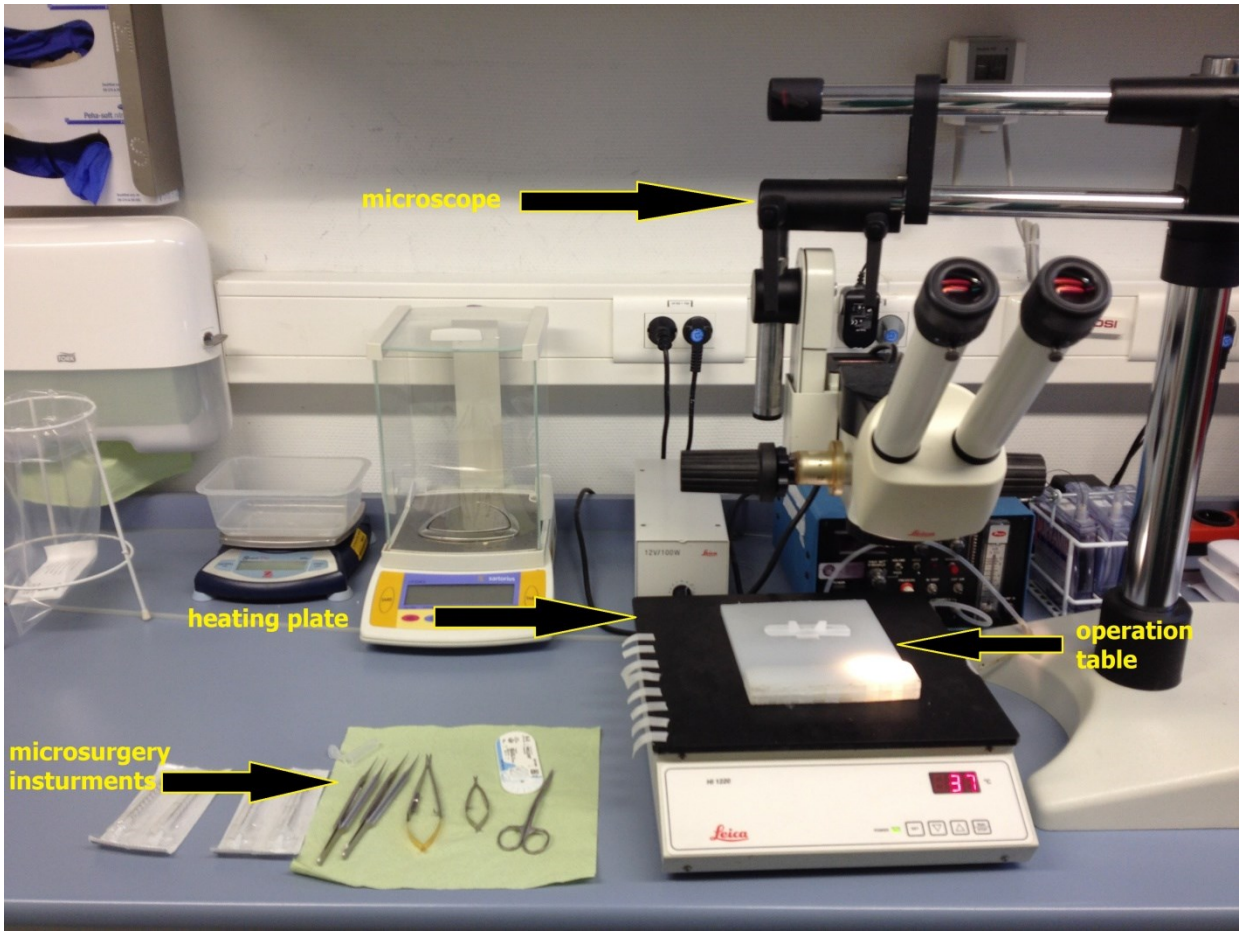


Fig. 1: Image showing devices and tools for femoral artery cannulation and cremaster muscle preparation.

Procedure:

Prior starting the operation, the mouse was anesthetized through intraperitoneal injection of Ketamin (Ketanest) 75mg/kg and Xylazin (Rompun) 25 mg/kg. For exact preparation of the anesthesia, the below formula was used.

Mouse in g (weight) x 10 = in microliter from the solution containing Kitamin and Xylazin

Preparation of the solution of Kitamin and Xylazin:

Ketamin 10 % (100 mg/ml)

150 µl Ketamin + 850 µl NaCl

Xylazin 2 % (20 mg/ml)

250 µl Xylazin + 750 µl NaCl

Both mixed in a 2 ml Tube and according to the weight of the mouse, the required amount was injected. After a few minutes of narcosis preparing the area of operation was undertaken by shaving the left medial thigh, left inguinal region, right testicle and right groin. The hair remnants were removed using moistened Q-tip.

To prevent airway blockage, the tongue was put aside and fixated at the angle of the mouth.

The mouse was placed on stage (operation table), and the limbs were fixated. The mouse and operation table was placed on a heating plate (Leica HI1220) at 37 °C, to avoid hypothermia (Fig. 1). After that under using an operation microscope (Leica M651) with tenfold magnification, an incision at the distal part of the left thigh was done. The superficial connective tissue was dissected and the whole left femoral artery prepared (Fig. 2). The two caudal branches were cauterized. Through attachment to a suture material, the medial and lateral superficial adipose tissues were fixated. The fatty tissue over the neurovascular bundle was removed. Thereafter the femoral nerve accompanying the artery was transected using microscissor. The distal end of the femoral vein and artery were ligated. A knot was formed around the proximal part of the femoral artery and vein without ligation. At this step, the magnification power of microscope was switched to 16 fold. An oblique incision of the femoral artery to about 1/3 to 1/2 of the vessel diameter was done with microscissor, followed by subsequent dilatation of the opening with the tip of the scissor. The prepared catheter (Portex non-sterile Polythene Tubing 0.28mm ID, SIMS Portex, UK) was cautiously introduced to the femoral artery (Fig. 3). Correct positioning of the catheter was checked through blood aspiration and slow injection. Then the proximal knot was slightly tightened to secure position of the catheter.

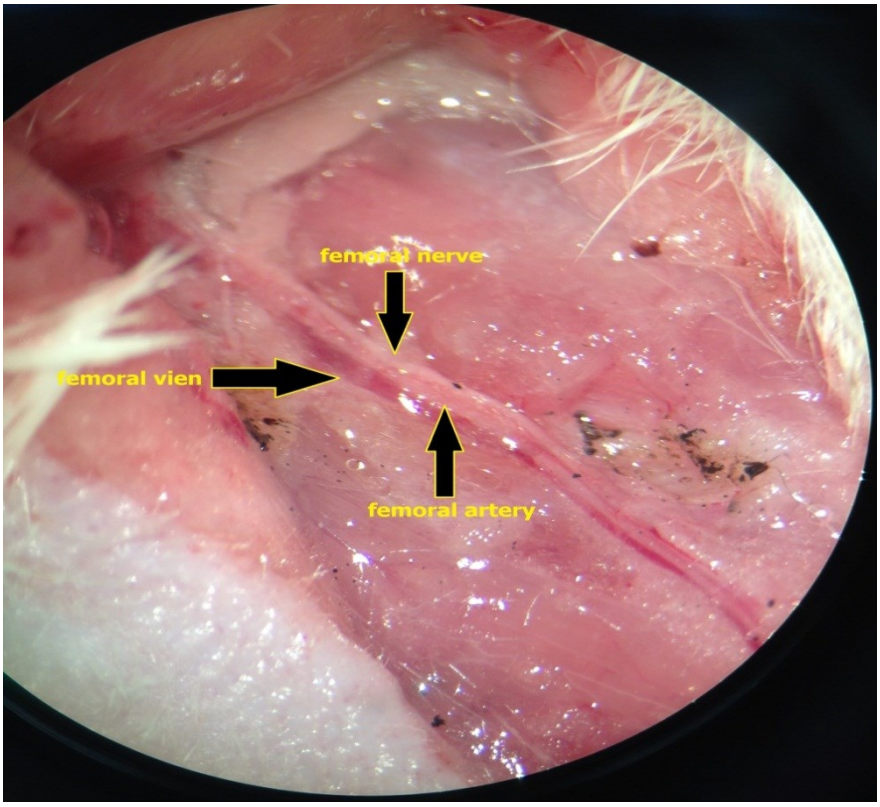
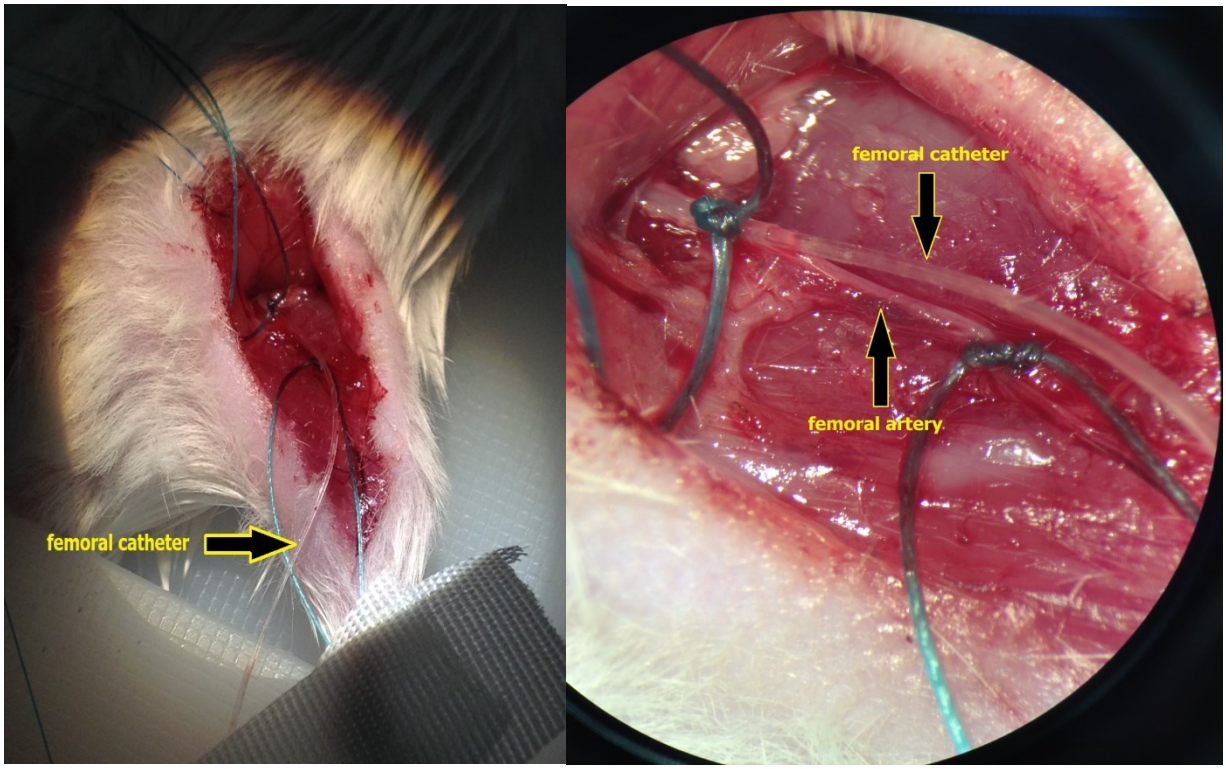


Fig. 2: Image showing the femoral neurovascular bundle in a mouse. From medial to lateral: femoral vein, femoral artery, and femoral nerve.



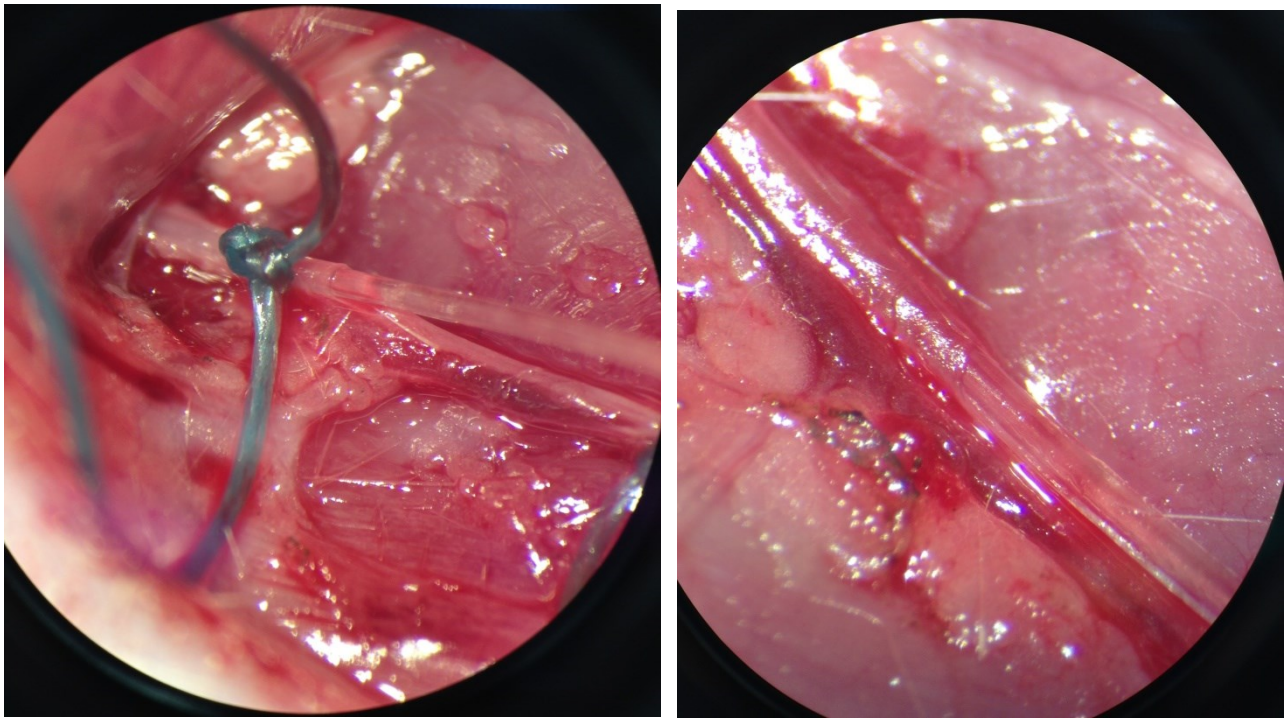


Fig. 3: Representative example of left femoral artery cannulation in different magnifications.

The next step is the preparation of the cremaster muscle. After switching again to tenfold magnification power, a skin incision was made at caudal portion of the right testicle. The subdermal adipose tissue was carefully removed. The tip of cremaster muscle was fixated using 6-0 Prolen (Ethicon, Somerville, New Jersey, USA). The rest of surrounding connective tissue was removed and muscle exposed proximally as much as possible. An incision was done on the anterior surface of the muscular sac and then proximally and distally extended. Respecting the underlying structures, the testicular sac was separated from the cremaster muscle. As a next step, the cremaster muscle was opened, and the ends were fixated using prolen 6-0 suture material (Fig. 4 and Fig. 5). It is necessary to keep the cremaster muscle during the whole procedure moist, and the catheter should be washed regularly to avoid clot formation within it. In case of ischemia-reperfusion, an atraumatic microvascular neurosurgical clamp Aesculap FT 250 T (Aesculap, Braun Melsungen AG, Melsungen, Germany) was applied to the base of cremaster muscle (proximal vascular bundle) leading to establish ischemia for 15 min followed by reperfusion for 15 min.

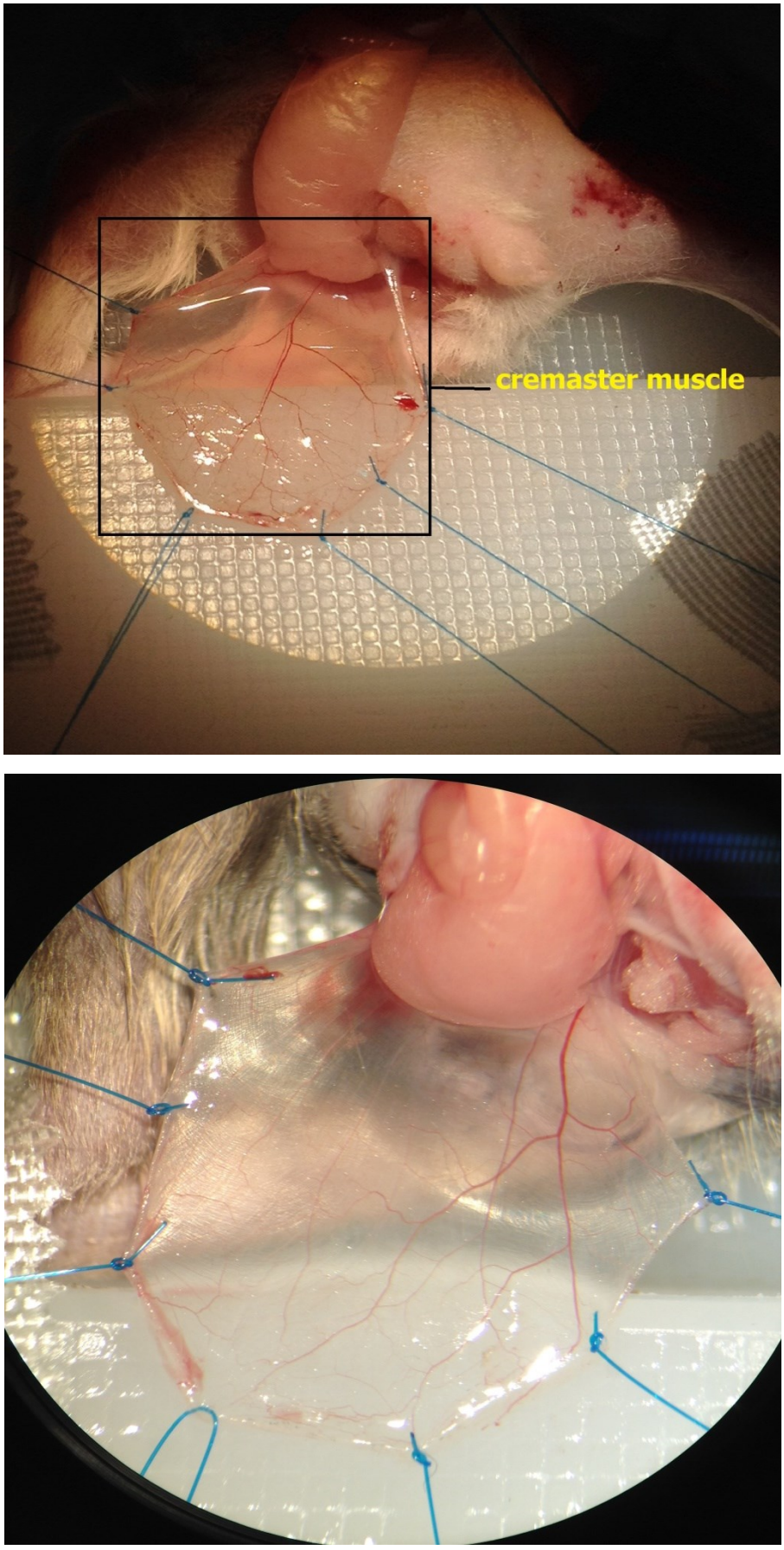


Fig. 4: Representative example of the mouse cremaster muscle tissue following microsurgical preparation in different magnifications.

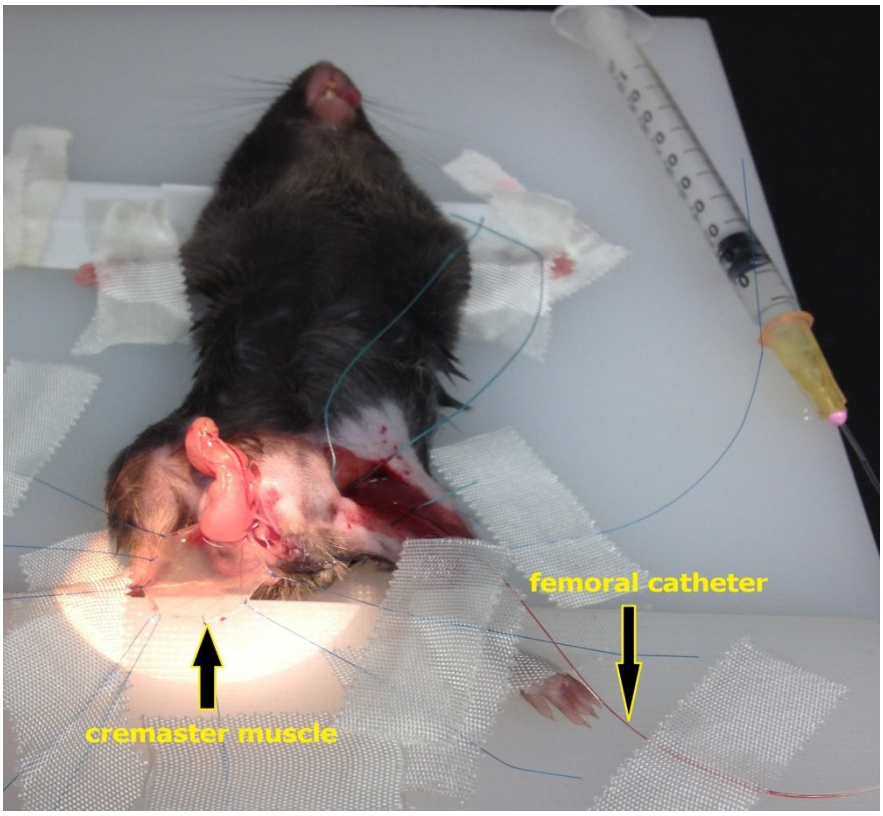


Fig. 5: Representative example of the mouse following femoral artery cannulation via the femoral catheter and microsurgical preparation of cremaster muscle before intravital microscopy.

3.6 Intravital Fluorescence Microscopy (IVM)

After preparation of cremaster muscle, the mouse was transferred under intravital microscopy (IVM).

Procedure:

Intravital microscopy was performed using an Axio-tech fluorescence microscope (Carl Zeiss, Jena, Germany), modified for epi-illumination and connected to a charge-coupled device (CCD) video camera (Pieper FK 6990A-IQ) Creating a video file via a camera-coupled recorder (Panasonic DMR-EX99V).

The observation of the cremaster muscle was done with the aid of a water immersion objective with 20x Magnification factor (Olympus LUC Plan FLN) and also coupled to the camera Screen (Ikegami).

For contrast-intensification of plasma, 100 µl fluorescent dye Rhodamine 6G was injected then followed with flushing of 0.9 % NaCl. The prepared stem cells were put in an insulin syringe and attached to the femoral catheter. Thereafter microcirculation of cremaster muscle could be visualized after focusing and clarification of the image. Then six different clear postcapillary venules randomly selected under 20x blue color filter. The recording was started at this moment. The first selected venule was focused, and the stem cells were injected (Fig. 6). The recording was done for one minute for later analysis. The same recording was repeated for the other venules. After recording all six venules, the second step of registration was commenced. Recording started from the first venule for one minute, and respectively the rest venules were recorded for further digital analysis. The first round of recording was to view the rolling cells. The second round of recording was to visualize the adherent cells. The intravital microscopy procedure was recorded on a DVD and labeled with related information. After completion of the experiment, the cremaster muscle was removed. Before preservation the probe was immersed in PBS in order to be washed from blood and hair remnants then dried and after that placed in 1.5 ml tube. After that, it was immersed in liquid nitrogen for less than 10 sec. The cremaster muscle was preserved in – 80 °C for later investigations. Finally, the animal was sacrificed through cervical dislocation.

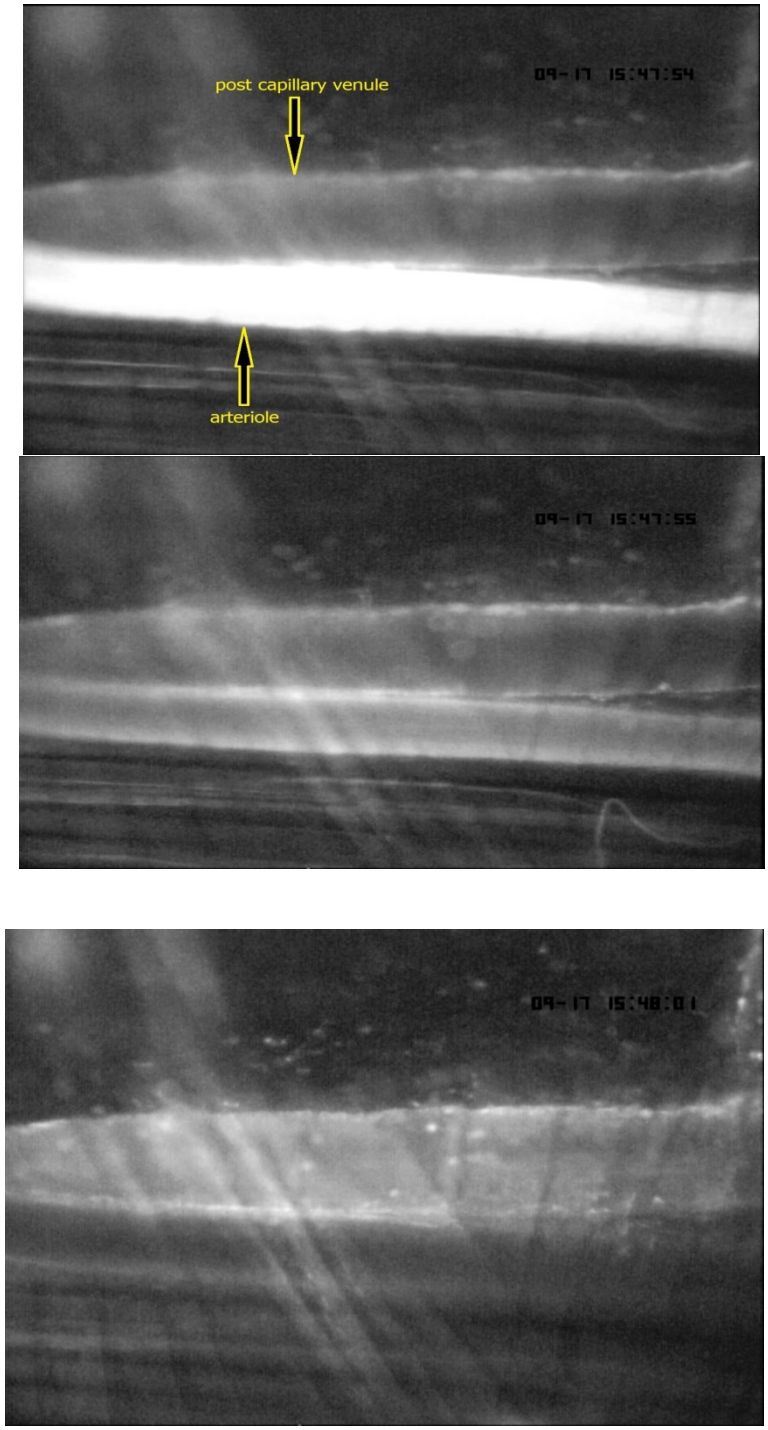


Fig. 6: Representative shot of intravital microscopy showing cremasteric microvasculature, namely arteriole and venule immediately after injection of fluorescently stained stem cells (picture captured under 20x Olympus LUC Plan FLN-water immersion objective).

3.7 RNA Isolation

Total RNA was isolated from explanted cremaster muscle tissue through TRIZOL® Reagent (Life Technologies, Darmstadt, Germany) as follows:

Reagents:

- TRIzol Reagent
- Chloroform (trichloromethane, methyl trichloride)
- Isopropyl alcohol (2-Propanol)
- 75% ethanol (in diethylpyrocarbonate (DEPC)- treated water)
- RNAse-free water (non-DEPC treated) or 0.5% SDS (sodium dodecyl sulfate) solution

Before starting with the procedure, the centrifuge was set at 4 °C.

- Homogenization:
  - The frozen cremaster muscle was degraded means mortar to make a powder-like substance with the aid of liquid nitrogen
  - The TRIzol reagent was added to the dish (1 ml per 3.5 cm diameter dish)
  - Pipetting was done a few times, and the dish was incubated for 5 min at room temperature
  - The solution then transferred to 1.5 ml tube
- Phase separation:
  - Centrifugation was done at 12.000 x G for 10 min at 4 °C
  - The supernatant containing RNA was collected and transferred to 1.5 ml tube
  - Chloroform was added to homogenate (0.2 ml per 1 ml TRIzol used for homogenization)
  - Tubes were shaken vigorously by hand for 15 sec
  - Then incubation room 3 min at room temperature was done
  - Thereafter the solution was centrifuged at 12.000 x G for 15 min. at °C
  - A colorless aqueous upper phase was appeared containing RNA only (around 60% of the total volume of homogenization)
- RNA precipitation:
  - The aqueous phase was transferred to a fresh 1.5 ml tube

- Isopropyl alcohol was added and mixed (0.5 ml per 1 ml TRIzol reagent used for homogenization)
- The mixture was incubated for 10 min at room temperature
- After that, the solution was centrifuged at 12.000 x G for 10 min at 4 °C

❖ RNA wash:

- Gel-like pellet supernatant was removed
- Washing was done with 75% ethanol (1 ml per 1 ml TRIzol reagent used for homogenization; 100% Ethanol was diluted in non-DEPC treated water
- Shaking was done using Vortex
- Centrifugation at 7500 x G for 5 min at 4 °C
- The supernatant was removed carefully

❖ Redissolving the RNA:

- Air-dry pellet for 5-10 min
- The RNA pellet dissolved by resuspension with RNAse-free water (> 15 µl)

3.8 cDNA Preparation

"Samples were analyzed with NanoDrop1000 (Thermo Scientific, Karlsruhe, Germany) to demonstrate consistent quality and to determine RNA concentration" (Donndorf, Abubaker, Vollmar, Rimmbach, Steinhoff, & Kaminski, 2017).
"Reverse transcription of RNA for first-strand cDNA synthesis was done using High Capacity cDNA Reverse Transcription Kit (Life Technologies)" (Donndorf, Abubaker, Vollmar, Rimmbach, Steinhoff, & Kaminski, 2017).

3.9 Real-time PCR

"cDNA was then analyzed using quantitative real-time reverse-transcription polymerase chain reaction (PCR) in Taqman gene expression assays (Life Technologies).

Amplification and detection were performed with the StepOnePlus™ Real-Time PCR System (Life Technologies) in TaqMan Universal Master Mix (Life Technologies) according to the instructions of the manufacturer (Life Technologies). cDNA extracts were tested in duplicates, and negative controls were included in each assay. Cycle thresholds (C_T) for single reactions were determined with StepOne™ Software 2.0 (Applied Biosystems), and the target genes were normalized against RPLP0 housekeeping gene (formula: $\Delta C_T = C_{T\ target} - C_{T\ RPLP0}$). Resulting ΔC_T of duplicates was averaged, and $\Delta\Delta C_T$ were obtained using the control group as a calibrator sample (formula: $\Delta\Delta C_T = \Delta C_{T\ sample} - \Delta C_{T\ calibrator\ sample}$). In our study, the $2^{-\Delta\Delta Ct}$ method was employed to analyze the changes in TNF-α gene expression within the experimental groups" (Donndorf, Abubaker, Vollmar, Rimmbach, Steinhoff, & Kaminski, 2017).

3.10 Digital Analysis of microcirculatory interaction of c-kit$^+$ stem cells in vivo

"C-kit$^+$ cell behavior along the endothelial lining was considered as 'rolling' when a more than 50% reduction of cell velocity in combination with the typical cellular 'stick and release' motion was present. The rolling cells were expressed as percentage of all passing cells along a predefined venular distance during 1 min of observation. All the cells that showed random brief tethering phenomena were not considered as rolling cells. Firm adhesion was recorded when cells did not move for more than 30 s. The number of firmly adherent c-kit$^+$ cells was calculated in relation to the endothelial surface of the predefined venules (diameter × length × π) and was expressed as adherent cells/mm^2 endothelial surface.

In order to provide similar microcirculatory conditions within the experimental groups, several parameters were measured and taken into careful consideration. Red blood cell velocity profile was verified using the line shift method on intravital microscopy recordings (CapImage Software, Zeintl, Heidelberg, Germany). The analyses of microcirculation also included the measurement of vessel diameter and wall shear rates based on the Newtonian definition $\gamma=8 \times v/d$, where 'v' represents the red blood cell velocity divided by 1.6 according to the Baker-Wayland factor (Baker and Wayland,

1974) and ‘d’ represents the single vessel diameter" (Donndorf, Abubaker, Vollmar, Rimmbach, Steinhoff, & Kaminski, 2017).

3.11 Statistical analysis

"All statistical analyses were performed using Sigma Stat software version 3.0 (SPSS Inc., Chicago, IL, USA). Results are given as mean ± S.E.M. Comparisons of the groups were executed using one-way analysis of variance (ANOVA) with subsequent post hoc multiple Holm–Sidak analysis. In case the data failed normality testing, the nonparametric Kruskal– Wallis or post hoc multiple Dunn tests were employed. P-values <0.05 were considered statistically significant" (Donndorf, Abubaker, Vollmar, Rimmbach, Steinhoff, & Kaminski, 2017).

4. Results

4.1 Rolling as an initiation in interaction of stem cells with the endothelium

4.1.1 Rolling in Group 1 (*Control-WT*, n=6)

The percentage of rolling bone marrow stem cells in the postcapillary venules of the cremaster muscle in the control group without exposure to ischemia and reperfusion was reaching to 17.3±3.9% (Fig. 7).

4.1.2 Rolling in Group 2 (*IR-WT*, n=6)

The percentage of rolling of stem cells in the postcapillary venules of the cremaster muscle exposed to 15 min ischemia followed by 15 min reperfusion was significantly higher (67.6±2.3% in *IR-WT* vs. 17.3±3.9% in *Control-WT*, $p<0.05$) (Fig. 7).

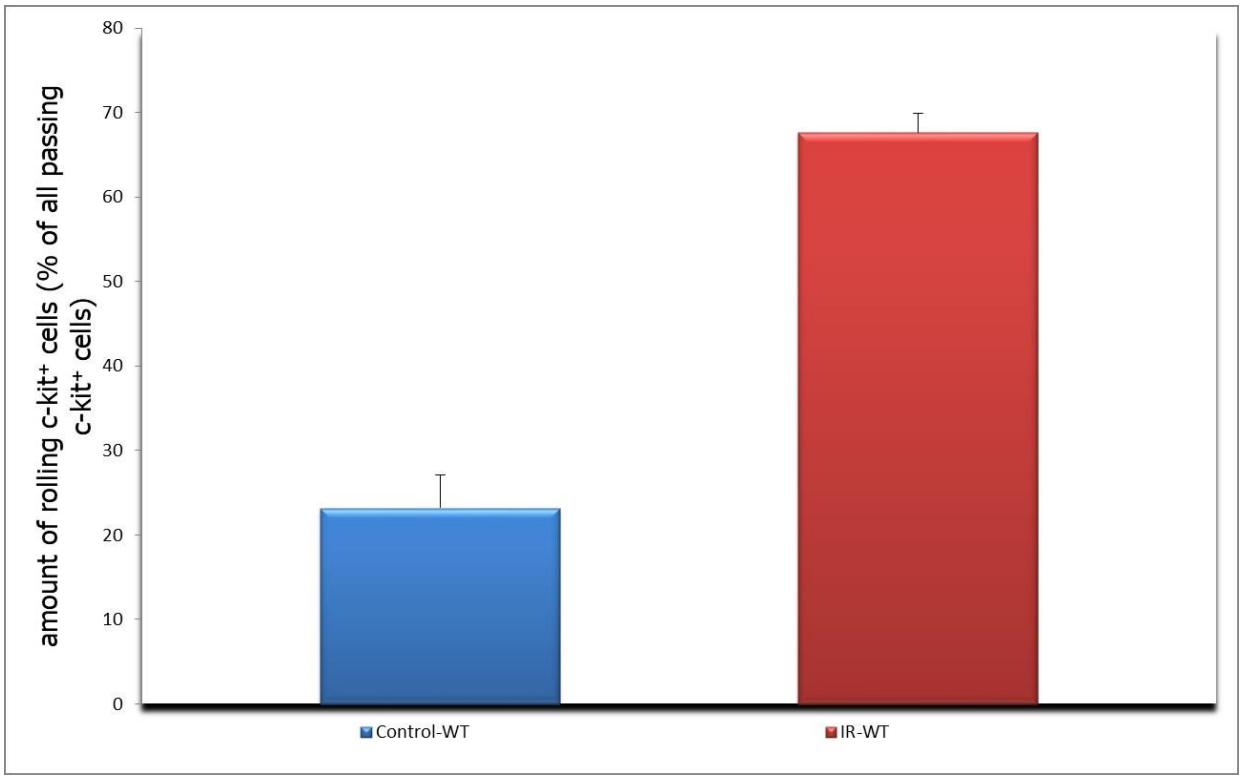


Fig. 7: Quantitative analysis of c-kit$^+$ cells showing rolling in control groups.

4.1.3 Rolling in Group 3 (*IR-TLR2-ko*, n=6)

The percentage of rolling stem cells after ischemia and reperfusion in TLR2-knockout animals was 46.4± 4.9% vs. 67.6±2.3% in *IR-WT*, $p<0.05$ (Fig. 8).

4.1.4 Rolling in Group 4 (*IR-TLR4-ko*, n=6)

The percentage of c-kit$^+$ cells after ischemia and reperfusion in TLR4-knockout mice showing endothelial rolling was 45.3± 4.8% vs. 67.6±2.3% in *IR-WT*, $p<0.05$ (Fig. 8).

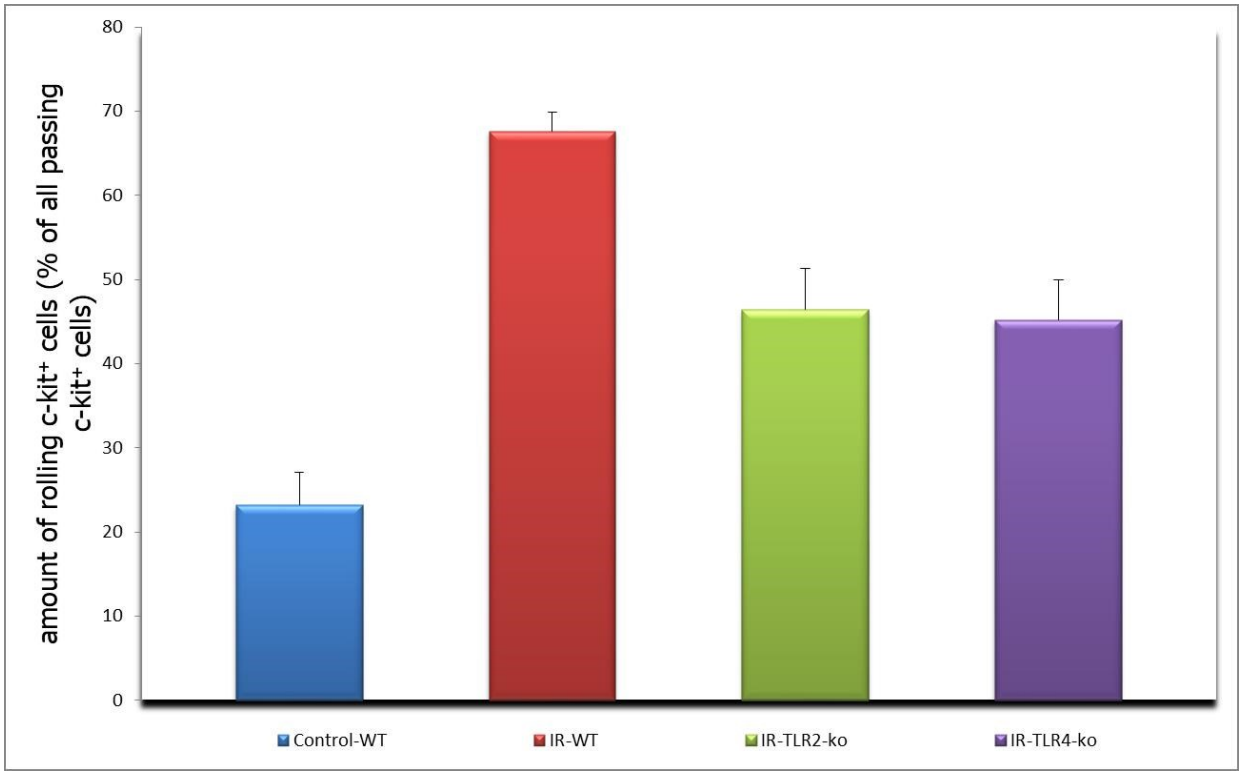


Fig. 8: Quantitative analysis of c-kit$^+$ cells showing rolling in control groups and TLR knockout groups.

4.1.5 Rolling in Group 5 (*IR-MyD88-ko*, n=6)

The percentage of rolling progenitor cells after ischemia and reperfusion in MyD88-knockout mice was 47.4±4.1% vs. 67.6±2.3% in *IR-WT*, $p<0.05$ (Fig. 9).

4.1.6 Rolling in Group 6 (*IR-TRIF-ko*, n=6)

The percentage of rolling stem cells after ischemia and reperfusion in TRIF knockout mice was 45.8±3.1% vs. 67.6±2.3% in *IR-WT*, $p<0.05$ (Fig. 9).

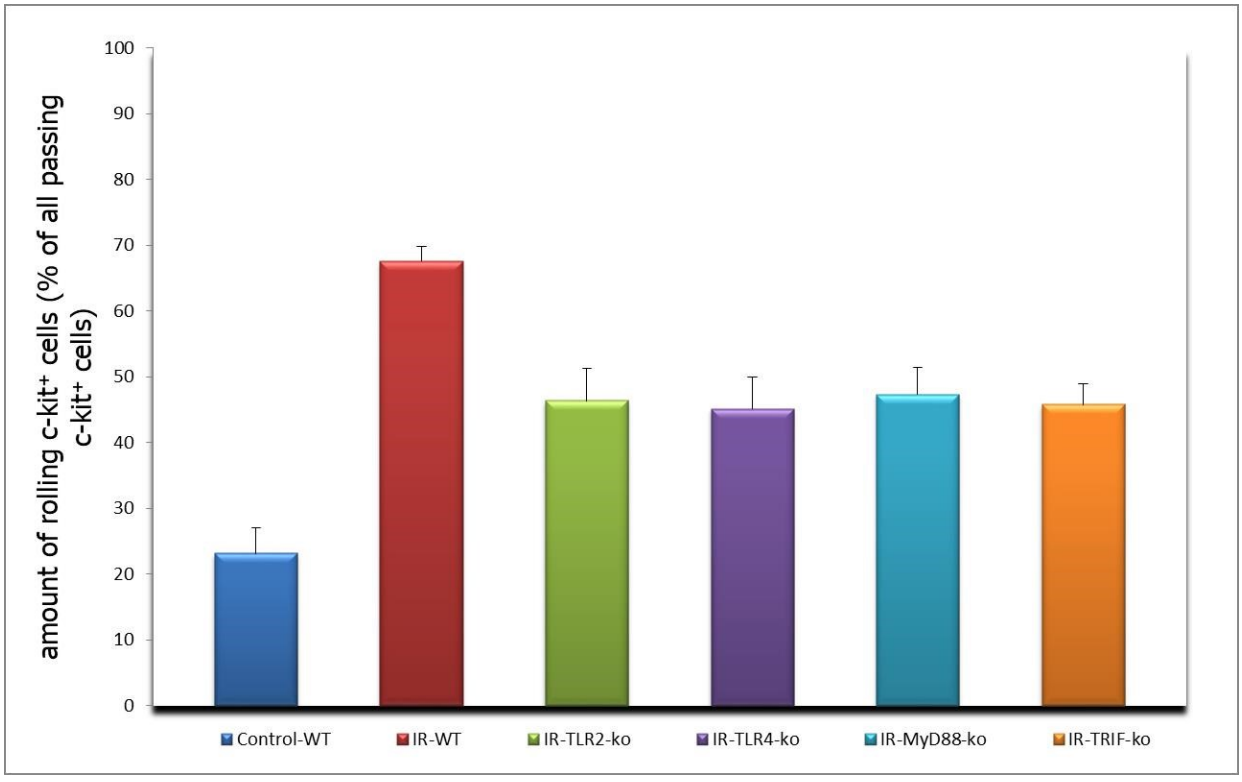


Fig. 9: Quantitative analysis of c-kit$^+$ cells showing rolling in experimental groups.

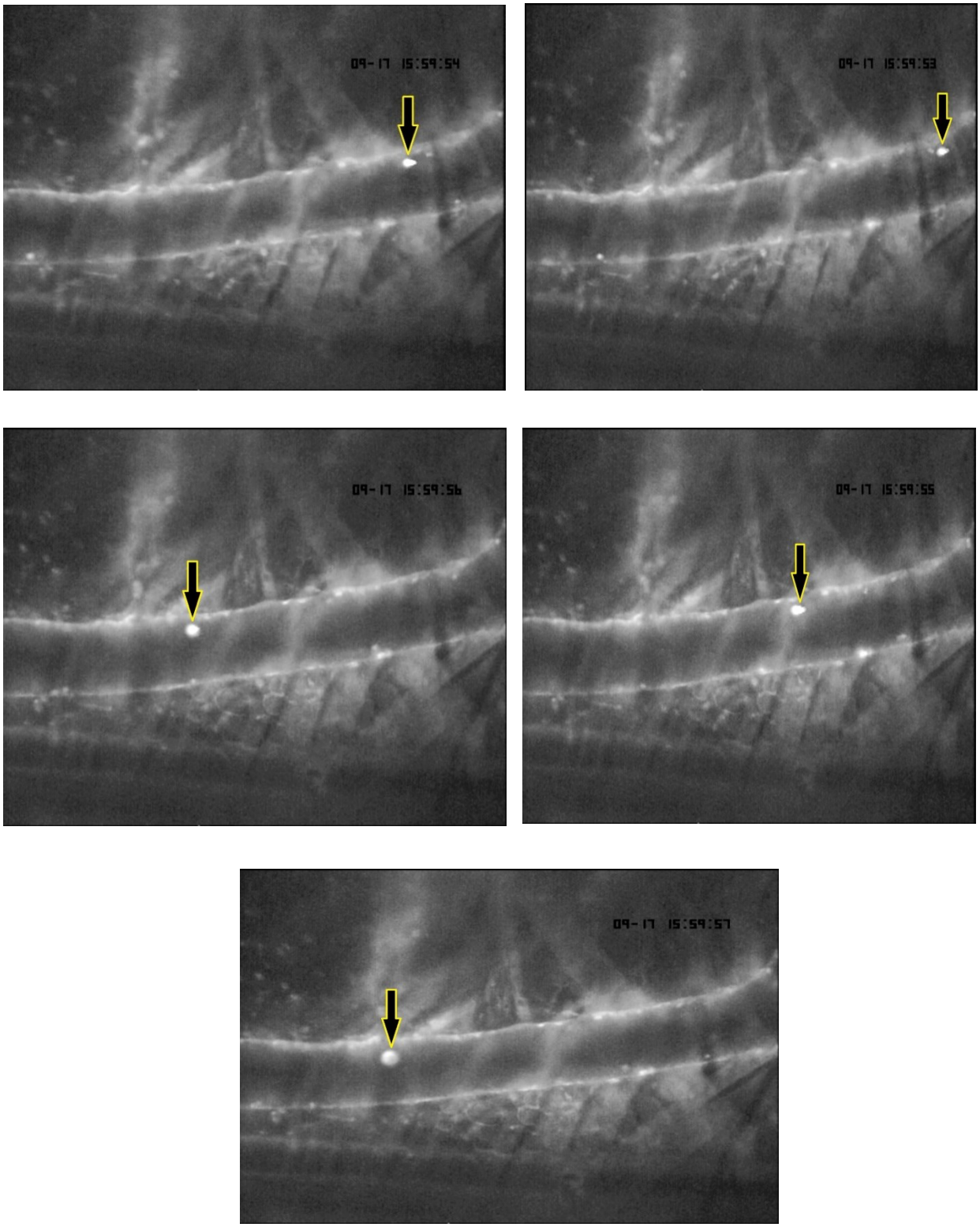


Fig. 10: Representative picture of intravital microscopy showing rolling stem cell in postcapillary venule of the cremaster muscle (picture captured under 20x Olympus LUC Plan FLN- water immersion objective).

4.2 Firm endothelial adhesion

4.2.1 Adhesion in Group 1 (*Control-WT*, n=6)

The amount of firm adherent c-kit$^+$ cells in postcapillary venules of cremaster muscle in the negative control group wildtype animals was 5.6±1.3 cells/mm2 (Fig. 11).

4.2.2 Adhesion in Group 2 (*IR-WT*, n=6)

Following application of 15 min ischemia and 15 min reperfusion in wildtype mice, the number of bone marrow stem cells exhibiting firm adhesion was 30.1±9.9 cells/mm2 vs. 5.6±1.3 cells/mm2 in *Control-WT*, $p<0.05$ (Fig. 11).

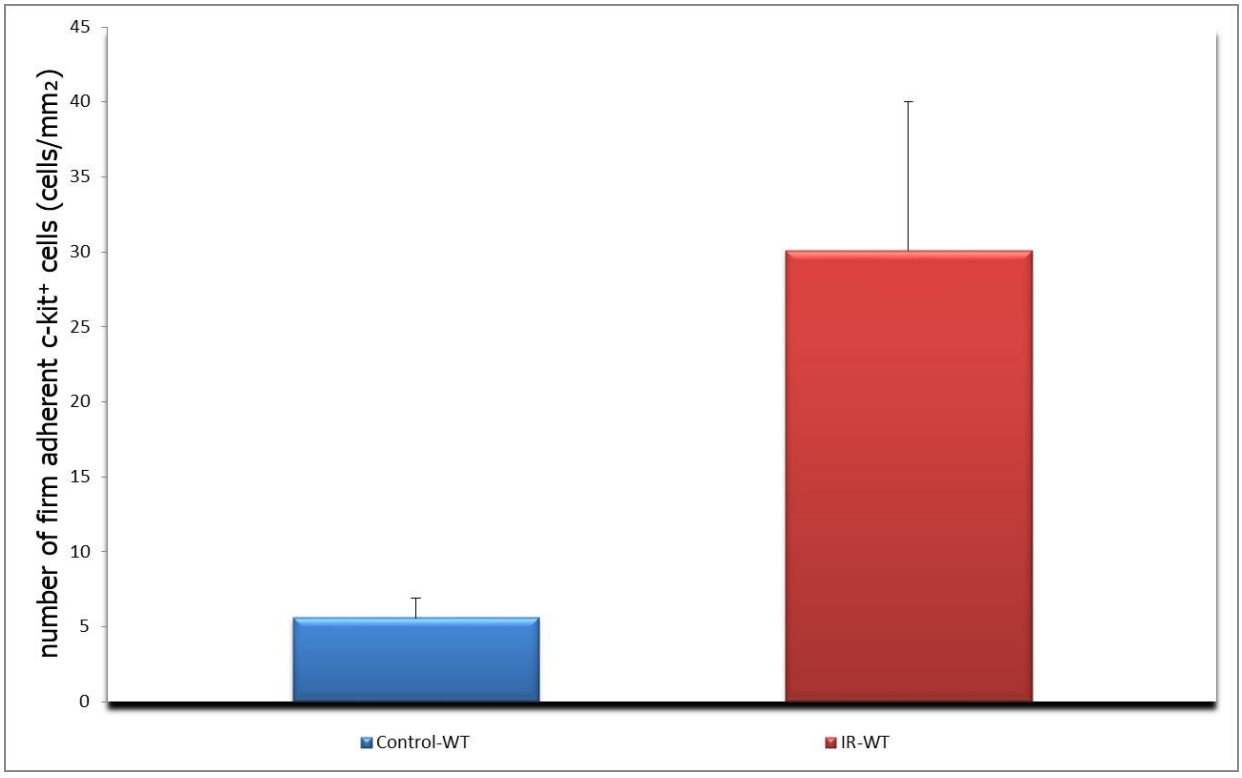


Fig. 11: Quantitative analysis of c-kit$^+$ cells showing firm adhesion in control groups.

4.2.3 Adhesion in Group 3 (*IR-TLR2-ko*, n=6)

The number of bone marrow stem cells exhibiting firm adhesion in TLR2-Knockout mice after ischemia and reperfusion was 16.4±4.0 cells/mm2 vs. 30.1±9.9 cells/mm2 in *IR-WT*, p<0.22 (Fig. 12).

4.2.4 Adhesion in Group 4 (*IR-TLR4-ko*, n=6)

The number of c-kit$^+$ cells exhibiting firm adhesion in TLR4-Knockout mice after ischemia and reperfusion was 14.6±4.4 cells/mm2 vs. 30.1±9.9 cells/mm2 in *IR-WT*, p<0.22 (Fig. 12).

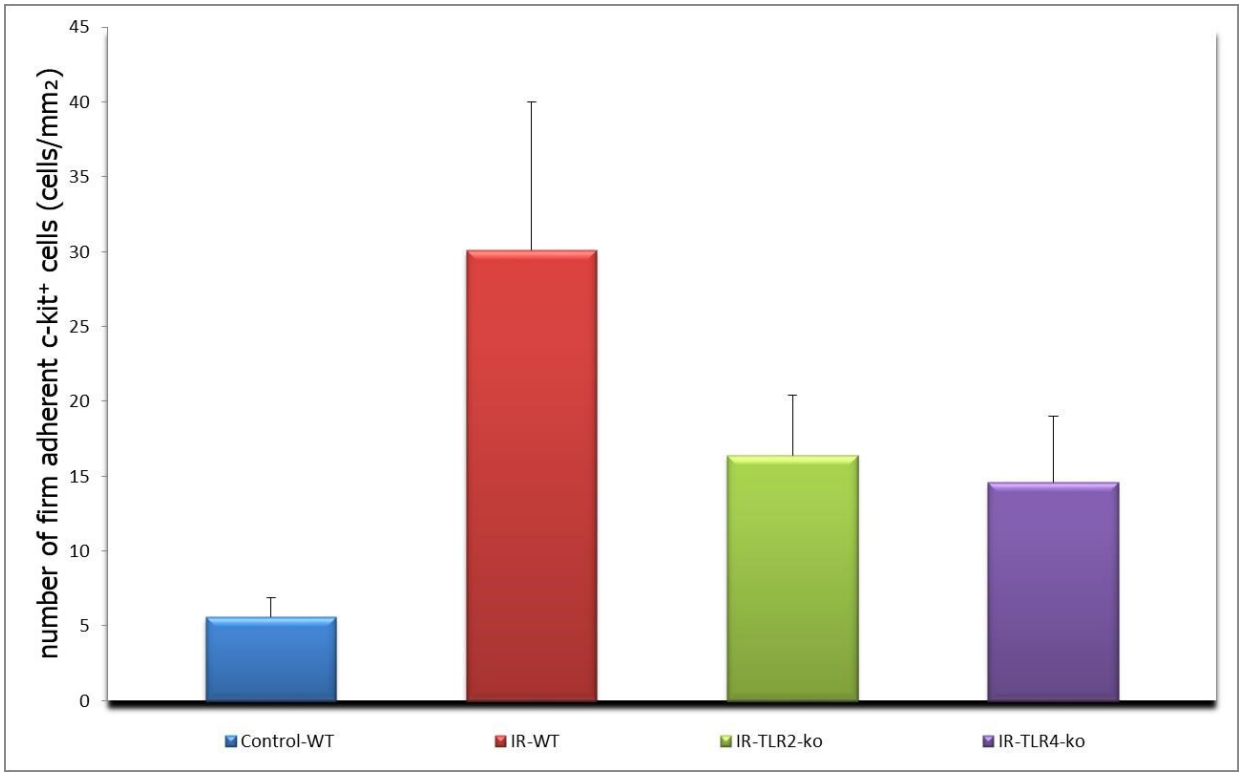


Fig. 12: Quantitative analysis of c-kit$^+$ cells showing firm adhesion in wildtype mice and TLR knockout groups.

4.2.5 Adhesion in Group 5 (*IR-MyD88-ko*, n=6)

The number of c-kit$^+$ cells exhibiting firm adhesion in MyD88- knockout mice after ischemia and reperfusion was 9.2±2.2 cells/mm2 vs. 30.1±9.9 cells/mm2 in *IR-WT*, p<0.05 (Fig. 13).

4.2.6 Adhesion in Group 6 (*IR-TRIF-ko*, n=6)

The number of bone marrow stem cells exhibiting firm adhesion in TRIF-knockout animals after ischemia and reperfusion was 14.1±4.6 cells/mm2 vs. 30.1±9.9 cells/mm2 in *IR-WT*, p<0.08 (Fig. 13).

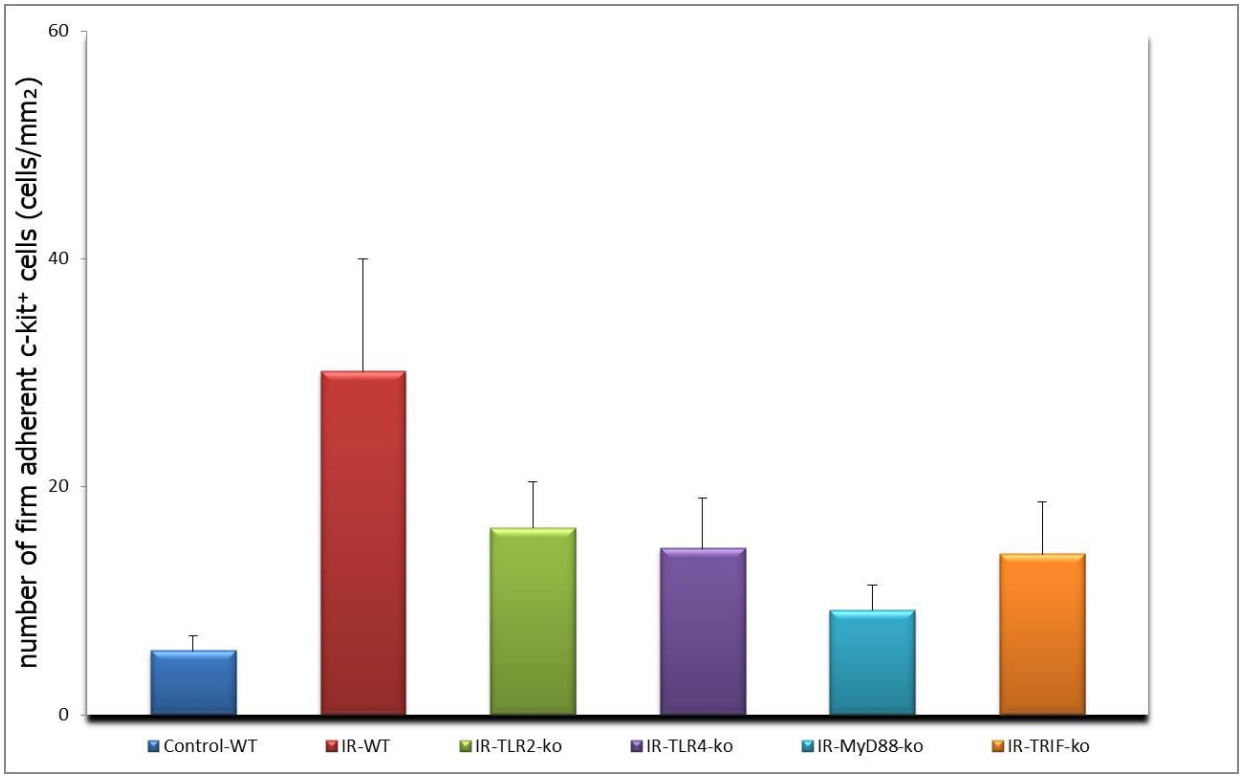


Fig. 13: Quantitative analysis of c-kit$^+$ cells showing firm adhesion in experimental groups.

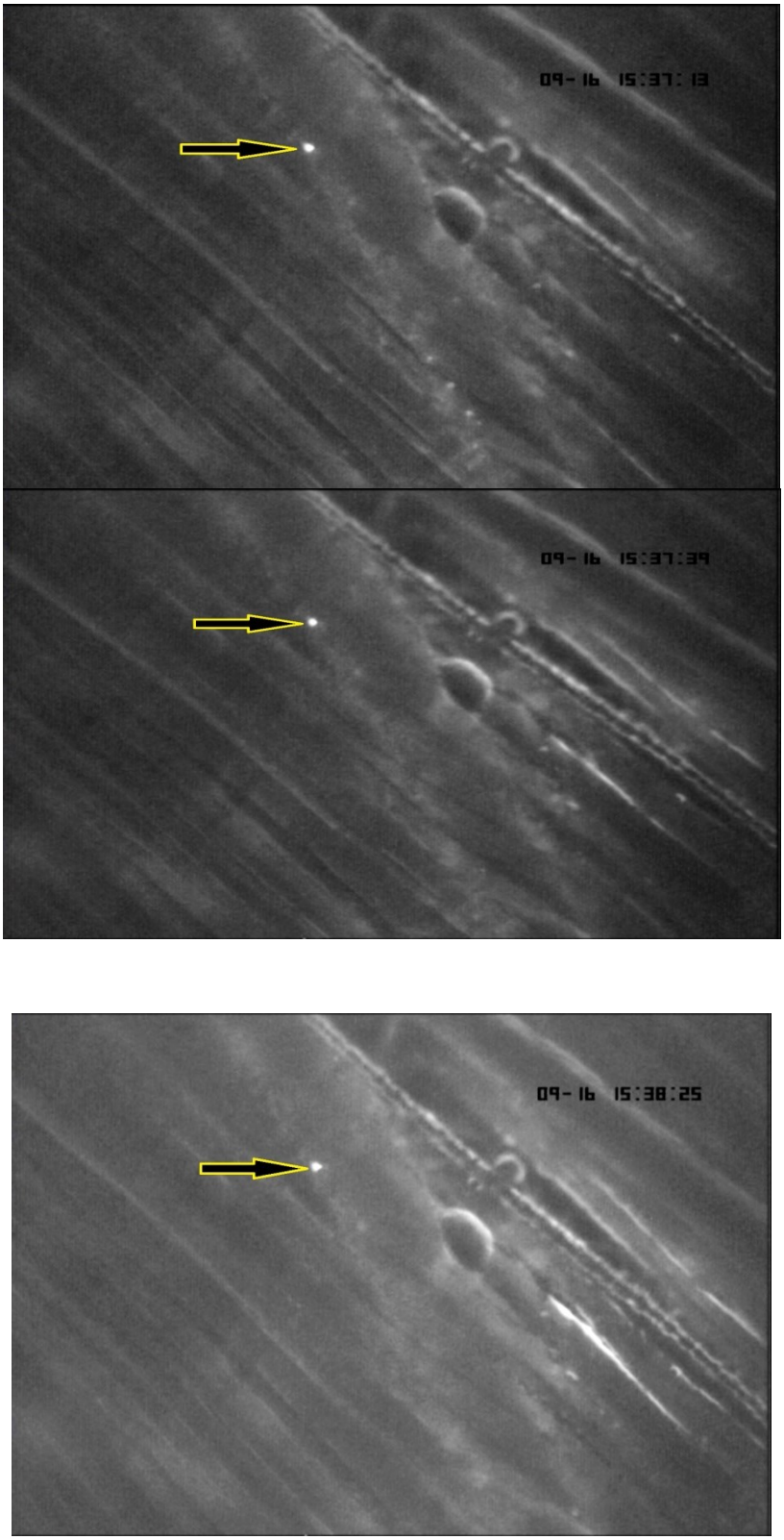


Fig. 14: Representative Picture of Intra-vital microscopy exhibiting adherent stem cell in postcapillary venule of the cremaster muscle (picture captured under 20x Olympus LUC Plan FLN- water immersion objective).

4.3 Real-time PCR to quantify the Expression of TNF-α

4.3.1 Real-time PCR in Group 1 (*Control-WT, n=3*)

The average mRNA expression level of TNF-α in the Control group without ischemia and reperfusion was arbitrarily given the value 0 (Fig. 15).

4.3.2 Real-time PCR in Group 2(*IR-WT, n=3*)

The average mRNA expression level of TNF-α in wild mice treated with 15 min ischemia and 15 min reperfusion was 1.81 ± 0.3 fold higher with $p<0.05$ as compared to the untreated control group (*Control-WT*) (Fig. 15).

4.3.3 Real-time PCR in Group 3 (*IR-TLR2-ko, n=3*)

The average mRNA expression level of TNF-α in TLR2-knockout mice after ischemia and reperfusion was 0.86 ± 0.39 fold higher as in the control group (*Control-WT*) without reaching statistical significance (Fig. 15).

4.3.4 Real-time PCR in Group 4 (*IR-TLR4-ko, n=3*)

The average mRNA expression level of TNF-α in TLR4-knockout mice after ischemia and reperfusion was 2.45 ± 0.47 fold higher as in the control group (*Control-WT*) with $p<0.05$ (Fig. 15).

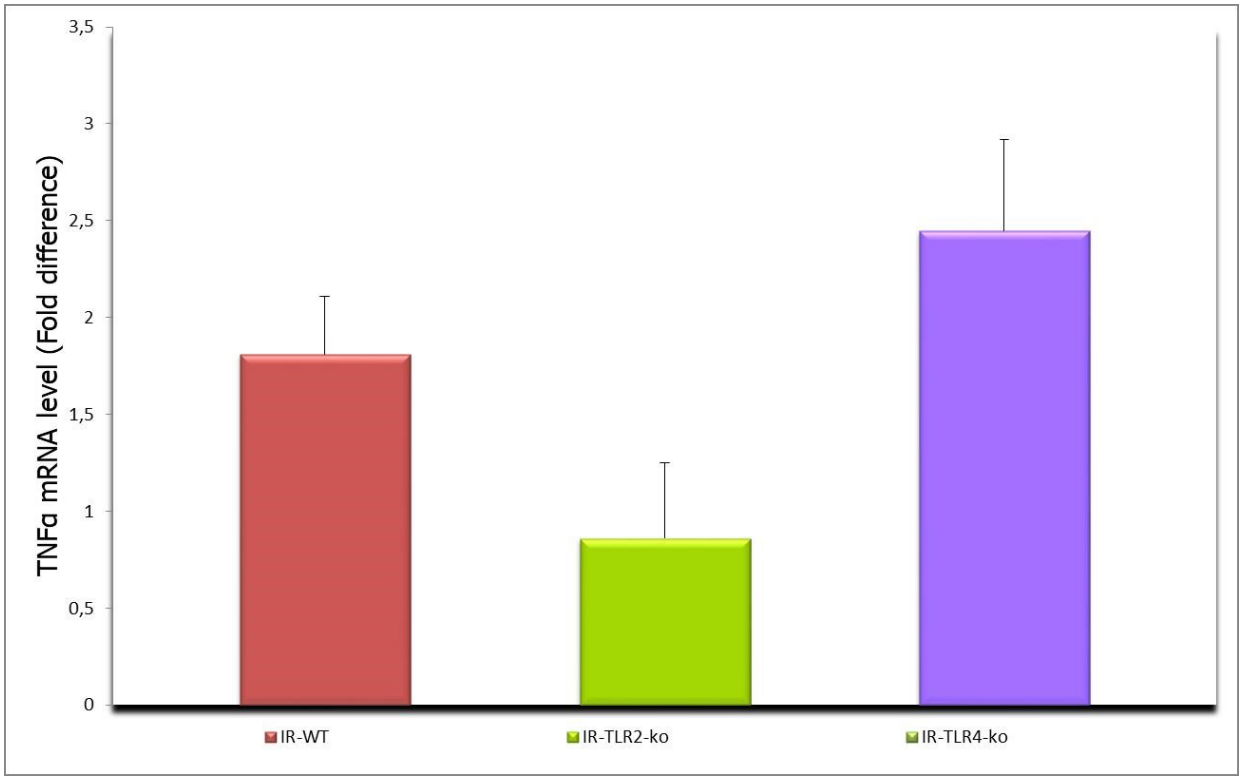


Fig. 15: Quantitative real-time PCR analysis for TNF-α gene expression in wild type mice and TLR knockout groups. The average mRNA expression level of TNF-α in control tissue was arbitrarily given the value 0 (line).

4.3.5 Real-time PCR in Group 5 (*IR-MyD88-ko, n=3*)

The average mRNA expression level of TNF-α in MyD88-knockout mice after ischemia and reperfusion was 0.91±0.1 folds higher in control group (*Control-WT*) resulting in $p<0.05$ as compared to the positive control group (*IR-WT*) (Fig. 16).

4.3.6 Real-time PCR in Group 6 (*IR-TRIF-ko,* n=3)

The average mRNA expression level of TNF-α in TRIF-knockout mice after ischemia and reperfusion was 4.09±0.73 folds higher with $p<0.05$ as compared to control group (*Control-WT*) (Fig. 16).

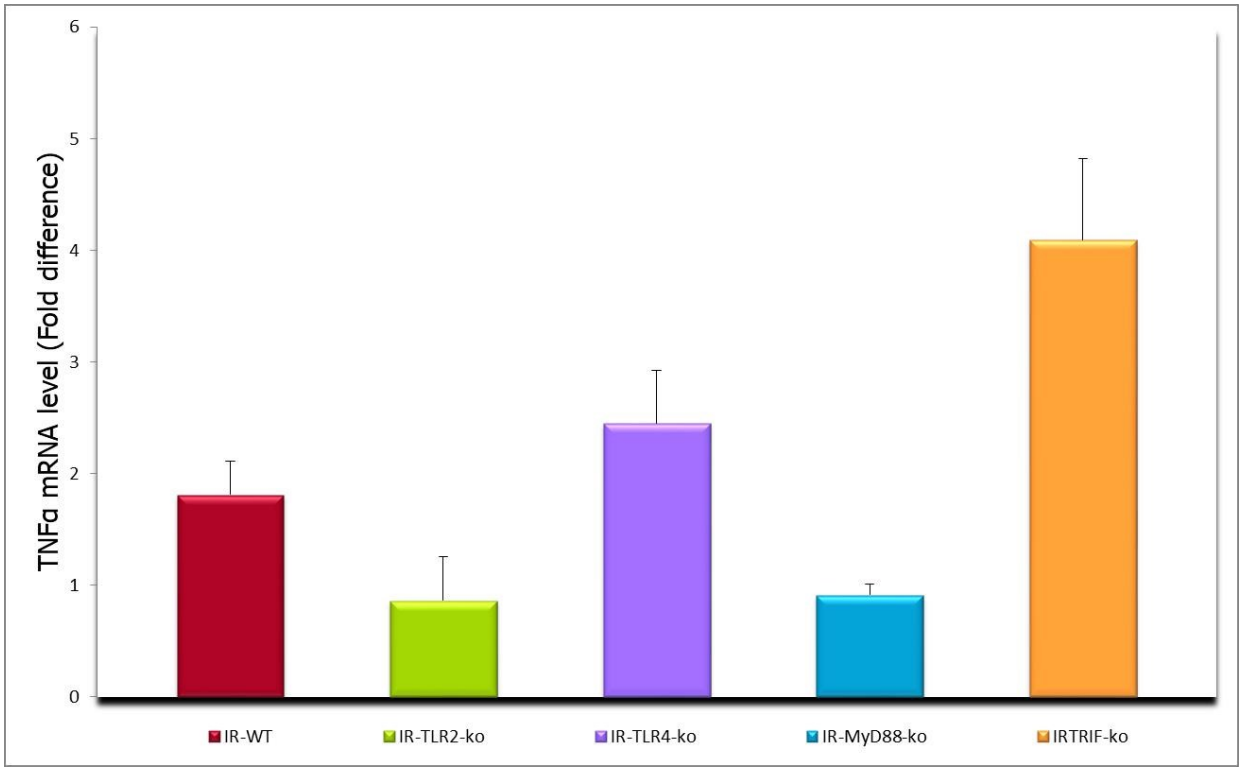


Fig. 16: Quantitative real-time PCR analysis for TNF-α gene expression in experimental groups.

5. Discussion

5.1 Experimental model

WHO report showed that in 2016, noncommunicable diseases are responsible for around 71% of deaths globally. Cardiovascular diseases, chronic respiratory diseases, cancer, and diabetes are the most common mortality causes of this group. Around 17.9 million deaths occurred due to cardiovascular diseases, which are making 44% of all noncommunicable diseases (World Health Organization, 2018). Ischemic heart disease is the major killer in all socioeconomic groups (Nowbar, Gitto, Howard, Francis, & Al-Lamee, 2019). One of the significant complications of ischemic heart disease is ischemic cardiomyopathy and heart failure, in which the loss of myocardium, resulting in ceasing the functional performance of the heart to pump the blood. Management of patients with heart failure is complex, and upon late advancement of the disease reluctant to therapy, the heart must be replaced through heart transplantation (Bhandari, B., & Masood, W., 2019).

Emerging stem cell therapy brought hope in treating patients with ischemic heart failure (Rosenstrauch, D., 2005). Despite recorded beneficial effects, the exact underlying mechanisms yet need more detailed studies. It is essential to conduct researches to maximize the positive effects of stem cells. Proper homing seems to be the initial step.

For this purpose in our study, we analyzed Stem cell-endothelial interaction exhibited in terms of stem cell rolling and adhesion in cremaster muscle microvasculature using fluorescent intravital microscopy.

Using the intravital microscopy in visualizing the cremaster muscle microvasculature has been applied primarily in immunological studies demonstrating the leukocyte-endothelial interaction (Secklehner, Lo Celso, & Carlin, 2017). Studying interplay between stem cells and target endothelial via cremaster muscle intravital microscopy

has been used in numerous studies in our research groups (Donndorf et al., 2013; Kaminski et al., 2008; Furlani et al., 2012).

In treating myocardial ischemia reestablishment of blood supply to the ischemic region is leading paradoxically to reperfusion injury. Immune system components like TLRs play a crucial rule in mediating ischemia-reperfusion injury (Zuidema & Zhang, 2010). Additionally, TLRs found to be important in mediating stem cell-related regeneration after ischemia-reperfusion injury (Abarbanell et al., 2010).

In this series of experiments, we modified the cremaster muscle model by adding a sequence of 15 min ischemia, followed by 15 min of reperfusion. One of the primary goals of this research was to explain the role of TLRs and their intracellular signaling pathways in the regulation of stem cell homing. Therefore we used selected knockout animals for each TLR2, TLR4, MyD88, and TRIF, in order to establish possible holistic approach in the interpretation of crosstalk between stem cell homing and vital members of the immune system after ischemia-reperfusion injury.

5.2 Stem cells interaction in control groups

C-kit$^+$ stem cell-endothelial interaction happened mainly in postcapillary venules of cremaster microvasculature. This interaction found to be scarce in the untreated control group. The results showed that Stem cell rolling accounted for about 17.3±3.9%. Moreover, the amount of c-kit$^+$ cells exhibiting firm endothelial adhesion was 5.6±1.3 cells/mm2.

The experiments confirmed our basic idea that in the case of ischemia-reperfusion injury stem cell-endothelial interaction might be enhanced. We found around 3,9 fold increase in stem cell rolling (17.3±3.9% in control-WT vs. 67.6±2.3% in IR-WT with $p< 0.05$) and also a remarkable increase in stem cell firm adhesion (5.6±1.3 cells/mm2 in control-WT vs. 30.1±9.9 cells/mm2, $p<0.05$).

Stem cell interaction increases in some way like the perpetuation of leukocyte interaction after ischemia-reperfusion injury (Ozmen, Ayhan, Demir, Siemionow, & Atabay, 2008). It is not surprising that part of the inflammatory response is stem cell recruitment to promote regeneration and repair mechanisms (Ratajczak et al., 2010). The increased level of inflammatory mediators such as TNF-α play a critical role in inducing leukocyte rolling and adhesion (Leick, Azcutia, Newton, & Luscinskas, 2014); likewise, the stem cell migration depends on these mediators (Kaminski et al., 2008).

Interestingly our results showed that TNF-α mRNA expression boosted after application of a sequence of 15 min ischemia followed by 15 min reperfusion to about 1,81 ± 0,3 fold with $p<0.05$. It is well known that TNF-α is an important factor in homing of stem cell through modifying the expression of adhesive molecules like ICAM-1 and P-Selectin (Furlani et al., 2012). Our results are comparable with other studies in demonstrating the crucial role of TNF-α in mediating stem cell homing (Prisco et al., 2016; Kaminski et al., 2008).

5.3 Stem cell-endothelial cells interaction in knockout animal groups

Our results exhibited that early stem cell interaction with endothelial cells was reduced in TLR receptor 2 and 4 knockout mice. We also observed an apparent decrease in late stem cells interaction in the form of firm adhesion in the absence of these receptors showing the importance of these members of innate immunity in stem cells homing. This finding is in line with the results of earlier study regarding importance of Toll-like receptor 2 and 4 for stem cell homing in the context of induction via HMGB-1 (Furlani et al., 2012).

Based on knowing two substantial toll-like receptor secondary messengers, MyD88 and TRIF, we intended to analyze further which messenger influence stem cells-migration to the site of injury after ischemia and reperfusion by using MyD88 and TRIF knockout mice. Data showed significant depletion of firm stem cell adhesion to endothelial cells absent to endothelial MyD88 pathway. Our results are giving distinctive importance to

MyD88 pathway in influencing stem cell migration to the site of injury after ischemia and reperfusion. This result is parallel to newly demonstrated finding from Downes et al., which reported the neuroprotective effect of TLR-MyD88-dependent signaling after a stroke produced via reversible occlusion of middle-cerebral artery of mice (Downes et al., 2013).

Both adverse and beneficial influences of TLRs after a myocardial ischemia-reperfusion injury have been reported. Feng et al. (2010) found that MyD88 signaling in bone marrow-derived circulating cells produce induction of myocardial ischemia and reperfusion insult through neutrophil recruitment (Feng et al., 2010), whereas Wang et al. reported the cardioprotective effect of TLR4 - MyD88 in ischemia-reperfusion injury mediated through iNOS- and sGC-dependent mechanisms (Wang et al., 2011).

A protective role of TLRs becomes even more prominent in the framework of stem cell application. Preconditioning of stem cells through activation of TLRs via ligands before transplantation enhances survival of these cells, which is a crucial part in stem cell homing (Ha, Liu, Kelley, Williams, & Li, 2013). Application of TLR ligands like HMGB-1 in combination with mesenchymal stem cell (MSCs) transplantation induces de novo vasculogenesis in myocardial infarcted and boundary zone after acute myocardial infarction in rats (Jiang, Wang, Jiang, Niu, & Zhang, 2016).

Our data is in controversy with some studies which registered decreased area of myocardial damage in TLR2, TLR4 and MyD88 knockout mice (Arslan et al., 2010; Chong et al., 2004; Feng et al., 2008). In order to assimilate these seemingly contradicting results, it is essential to take into consideration several aspects. One aspect is that both circulating cells and target endothelial cells are expressing TLRs. One of the particular goals of our research was to analyze the impact of endothelial-TLRs. Arslan et al. (2010) showed that circulating TLR2 promotes myocardial ischemia-reperfusion injury and blockage of TLR2 with OPN-301 minimizes cardiac damage and improves the protection of the cardiac performance, whereas in our experiments endothelial TLRs showed some positive influences in the context of ischemia and reperfusion injury through mediating homing of stem cells.

Another meaningful aspect of explanation is, that studies, which showed a reduction in infarct size using genetic deletion were concerned about the limitation of inflammation in order to reduce inflammatory damage and less concerned about repair mechanism, which is one of the primary goals of inflammation to restore functional ability of the damaged organ. In our study, we tried to expose light on the regenerative side after ischemia-reperfusion injury.
Moreover, Mersmann et al. exhibited that deletion of TLR2 in the long term produces a detrimental effect on myocardial remodeling as a long term effect after myocardial ischemia-reperfusion injury (Mersmann et al., 2010). Additionally, Kim et al. exhibited that myocardial ischemia and reperfusion in inactive TLR4 resulted in smaller infarcted area and lowering the production of cytokines without salvation of cardiac performance (Kim et al., 2007). One important reason could be impairment of regenerative process through lack of competent TLRs.

Reduction of inflammation on one side may be beneficial to downsize the extent of damage caused by inflammatory cell invasion, but on the other side may exert a negative effect on healing process which is part of the inflammatory process. Furthermore, suppression of cellular recruitment to the ischemic damaged area in the reperfusion phase might involve the stem cells resulting in impairment of myocardial regeneration. That is why a more detailed understanding of inflammation, regeneration and close interconnection is required to implement more selective therapy without compromising the homeostatic system to achieve lesser damage and improve regeneration in order to preserve the functional capacity of the heart.

6. Summary

The molecular pathophysiology behind stem cell homing is not well understood. TLRs are known as an essential family member of innate immune system and crucial regulator of inflammatory response after ischemia and reperfusion injury. These receptors are recently recognized as a new player in stem cell trafficking.

In our study, we aimed to identify the impact of TLR2 and TLR4 and their intracellular signaling routes namely MyD88 and TRIF pathways on stem cell homing.

We utilized a well-established intravital microscopic examination of the cremaster microvasculature. Through the application of 15 min ischemia followed by 15 min reperfusion of cremaster muscle, we intended to add more similarity to myocardial injury following ischemia and reperfusion.

Our results showed that ischemia-reperfusion enhances stem cell-endothelial interaction in terms of stem cell rolling and adhesion. This enhancement was accompanied by increased expression of TNF-α mRNA, a cytokine produced after TLR stimulation.

The c-kit$^+$ stem cells rolling ability was significantly reduced after ischemia and reperfusion in TLR2 and TLR4 knockout animals. Additionally a noticeable reduction in stem cell-endothelial firm adhesion was observed in animals lacking to TLR2 and TLR4. Our Data explains the decisive role of TLR2 and TLR4 in stem cell homing following ischemia-reperfusion.

Our results exhibited the importance of intracellular signaling pathways of TLRs in the induction of stem cell homing and showed peculiar significance of MyD88 pathway in regulating stem cell trafficking after ischemia and reperfusion.

The clinical application of our research is that the suppression of TLRs and associated signaling pathways through anti-inflammatory medications might exert a drawback effect on regenerative mechanisms via disturbing proper stem cell homing. It appears to be reasonable that the existence of competent or enhanced TLRs might improve stem cell engraftment and potentiate tissue repair in patients undergoing stem cell therapy.

7. Conclusion

1- Artificially applied c-kit^{+} cells interact with the target organ endothelium.

2- Ischemia and reperfusion enhance stem cell-endothelial cell interplay.

3- This stem cell-endothelial interaction mediated through TLR2 and TLR4.

4- Intracellular signaling of TLRs plays an essential role in stem cell trafficking, with emphasis on the MyD88 signaling pathway.

5- Therapeutic inhibition of TLRs and related signaling may hinder the reparative process.

6- Genetic analysis might be necessary for individuals suffering from ischemic heart disease undergoing stem cell therapy to assure the presence of intact TLRs.

8. Zusammenfassung in Deutsch

Die molekulare Pathophysiologie hinter dem Stammzell-Homing ist bisher noch nicht ausreichend untersucht. TLRs sind als wichtiges Familienmitglied des angeborenen Immunsystems und als entscheidender Regulator der Entzündungsreaktion nach Ischämie und Reperfusion bekannt. Diese Rezeptoren wurden kürzlich als neue Akteure im Stammzell-Homing anerkannt.

Die Fragestellung der vorliegenden Studie war daher den Einfluss von TLR2 und TLR4 und ihrer intrazellulären Signalwege, nämlich MyD88 und TRIF, auf das Homing von Stammzellen zu identifizieren.

Hierfür wurde eine etablierte intravitalmikroskopische Untersuchung der Cremaster-Mikrovaskulatur verwendet. Durch die Anwendung einer 15-minütigen Ischämie gefolgt von einer 15-minütigen Reperfusion des Cremaster-Muskels beabsichtigten wir unserem Model mehr Ähnlichkeit zu Myokardverletzung nach Ischämie und Reperfusion zu verleihen.

Unsere Ergebnisse zeigten, dass die Ischämie-Reperfusion die Stammzell-Endothel-Wechselwirkung im Hinblick auf das Rollen und die Adhäsion von Stammzellen steigerte. Diese Verstärkung wurde von einer erhöhten Expression von TNF-α-mRNA begleitet, ein Zytokin, das nach TLR-Stimulation produziert wird.

Die Rollfähigkeit von c-kit+-Stammzellen war nach Ischämie und Reperfusion bei TLR2- und TLR4-Knockout-Tieren signifikant verringert. Zusätzlich wurde bei Tieren, denen TLR2 und TLR4 fehlten, eine merkliche Verringerung der festen Adhäsion zwischen Stammzellen und Endothelzellen beobachtet. Unsere Daten erklären die entscheidende Rolle von TLR2 und TLR4 beim Stammzell-Homing nach Ischämie-Reperfusion.

Unsere Ergebnisse zeigten die Bedeutung der intrazellulären Signalwege von TLRs bei der Induktion des Stammzell-Homing und eine besondere Betonung des MyD88-Signalwegs bei der Regulierung der Wechselwirkung zwischen Stammzellen und Endothelzellen nach Ischämie und Reperfusion.

Die klinische Anwendung unserer Forschung besteht darin, dass die Unterdrückung von TLRs durch entzündungshemmende Medikamente die Regenerationsmechanismen verhindern könnte, indem das ordnungsgemäße Homing der Stammzellen gestört wird. Es erscheint berechtigt, dass das Vorhandensein kompetenter oder verstärkter TLRs

die Stammzelltransplantation verbessern und die Gewebereparatur bei Patienten, die sich einer Stammzelltherapie unterziehen, potenzieren könnte.

9. References

Abarbanell, A. M., Wang, Y., Herrmann, J. L., Weil, B. R., Poynter, J. A., Manukyan, M. C., & Meldrum, D. R. (2010). Toll-like receptor 2 mediates mesenchymal stem cell-associated myocardial recovery and VEGF production following acute ischemia-reperfusion injury. *American Journal of Physiology-Heart and Circulatory Physiology*, *298*(5), H1529-H1536. doi:10.1152/ajpheart.01087.2009.

Arslan, F., De Kleijn, D. P., & Pasterkamp, G. (2011). Innate immune signaling in cardiac ischemia. *Nature Reviews Cardiology*, *8*(5), 292-300. doi:10.1038/nrcardio.2011.38.

Arslan, F., Smeets, M. B., O'Neill, L. A., Keogh, B., McGuirk, P., Timmers, L., … De Kleijn, D. P. (2010). Myocardial Ischemia/Reperfusion Injury Is Mediated by Leukocytic Toll-Like Receptor-2 and Reduced by Systemic Administration of a Novel Anti–Toll-Like Receptor-2 Antibody. *Circulation*, *121*(1), 80-90. doi:10.1161/circulationaha.109.880187.

Arumugam, T. V., Okun, E., Tang, S., Thundyil, J., Taylor, S. M., & Woodruff, T. M. (2009). TOLL-LIKE RECEPTORS IN ISCHEMIA-REPERFUSION INJURY. *Shock*, *32*(1), 4-16. doi:10.1097/shk.0b013e318193e333.

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., … Isner, J. M. (1999). Bone Marrow Origin of Endothelial Progenitor Cells Responsible for Postnatal Vasculogenesis in Physiological and Pathological Neovascularization. *Circulation Research*, *85*(3), 221-228. doi:10.1161/01.res.85.3.221.

Baez, S. (1973). An open cremaster muscle preparation for the study of blood vessels by in vivo microscopy. *Microvascular Research*, *5*(3), 384-394. doi:10.1016/0026-2862(73)90054-x.

Baker, M., & Wayland, H. (1974). On-line volume flow rate and velocity profile measurement for blood in microvessels. *Microvascular Research*, *7*(1), 131-143. doi:10.1016/0026-2862(74)90043-0.

Beltrami, A. P., Barlucchi, L., Torella, D., Baker, M., Limana, F., Chimenti, S., … Anversa, P. (2003). Adult Cardiac Stem Cells Are Multipotent and Support Myocardial Regeneration. *Cell*, *114*(6), 763-776. doi:10.1016/s0092-8674(03)00687-1.

Bergmann, O., Bhardwaj, R. D., Bernard, S., Zdunek, S., Barnabé-Heider, F., Walsh, S., … Frisén, J. (2009). Evidence for Cardiomyocyte Renewal in Humans. *Science*, *324*(5923), 98-102. doi:10.1126/science.1164680.

Bhandari, B., & Masood, W. (2019). *Ischemic Cardiomyopathy.* [Updated 2019 Jun 3]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2019 Jan-. Retrieved from https://www.ncbi.nlm.nih.gov/books/NBK537301/.

Borlongan, C. V. (2011). Bone marrow stem cell mobilization in stroke: a 'bonehead' may be good after all! *Leukemia*, *25*(11), 1674-1686. doi:10.1038/leu.2011.167.

Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., & Williamson, B. (1975). An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings*

of the National Academy of Sciences, *72*(9), 3666-3670. doi:10.1073/pnas.72.9.3666.

Chen, C., Liu, L., Zhou, J., Wang, X., Han, M., Jiao, X., … Yuan, X. (2017). Up-Regulation of HMGB1 Exacerbates Renal Ischemia-Reperfusion Injury by Stimulating Inflammatory and Immune Responses through the TLR4 Signaling Pathway in Mice. *Cellular Physiology and Biochemistry*, *41*(6), 2447-2460. doi:10.1159/000475914.

Chen, Y., Zhang, Q., Liao, Y., Cao, Z., Du, Y., Xia, J., … Chen, Z. (2011). Effect of Tumor Necrosis Factor-α on Neutralization of Ventricular Fibrillation in Rats with Acute Myocardial Infarction. *Mediators of Inflammation*, *2011*, 1-8. doi:10.1155/2011/565238.

Chong, A. J., Shimamoto, A., Hampton, C. R., Takayama, H., Spring, D. J., Rothnie, C. L., … Verrier, E. D. (2004). Toll-like receptor 4 mediates ischemia/reperfusion injury of the heart. *The Journal of Thoracic and Cardiovascular Surgery*, *128*(2), 170-179. doi:10.1016/j.jtcvs.2003.11.036.

Chu, W. (2013). Tumor necrosis factor. *Cancer Letters*, *328*(2), 222-225. doi:10.1016/j.canlet.2012.10.014.

Chugh, A. R., Beache, G. M., Loughran, J. H., Mewton, N., Elmore, J. B., Kajstura, J., … Bolli, R. (2012). Administration of cardiac stem cells in patients with ischemic cardiomyopathy: the SCIPIO trial: surgical aspects and interim analysis of myocardial function and viability by magnetic resonance. *Circulation*, *126*(11_suppl_1), S54-S64. doi:10.1161/circulationaha.112.092627.

Cicha, I., & Urschel, K. (2015). TNF-α in the cardiovascular system: from physiology to therapy. *International Journal of Interferon, Cytokine and Mediator Research*, 9. doi:10.2147/ijicmr.s64894.

Donndorf, P., Abubaker, S., Vollmar, B., Rimmbach, C., Steinhoff, G., & Kaminski, A. (2017). Therapeutic progenitor cell application for tissue regeneration: Analyzing the impact of toll-like receptor signaling on c-kit + cell migration following ischemia-reperfusion injury in vivo. *Microvascular Research*, *112*, 87-92. doi:10.1016/j.mvr.2017.03.011

Donndorf, P., Kaminski, A., Tiedemann, G., Kundt, G., & Steinhoff, G. (2012). Validating intramyocardial bone marrow stem cell therapy in combination with coronary artery bypass grafting, the PERFECT Phase III randomized multicenter trial: study protocol for a randomized controlled trial. *Trials*, *13*(1). doi:10.1186/1745-6215-13-99

Donndorf, P., Useini, D., Lux, C. A., Vollmar, B., Delyagina, E., Laupheimer, M., … Steinhoff, G. (2013). Analyzing Migratory Properties of Human CD133+Stem Cells in Vivo after Intraoperative Sternal Bone Marrow Isolation. *Cell Transplantation*, *22*(9), 1627-1635. doi:10.3727/096368912x657729

Downes, C. E., Wong, C. H., Henley, K. J., Guio-Aguilar, P. L., Zhang, M., Ates, R., … Crack, P. J. (2013). MyD88 Is a Critical Regulator of Hematopoietic Cell-Mediated Neuroprotection Seen after Stroke. *PLoS ONE*, *8*(3), e57948. doi:10.1371/journal.pone.0057948.

Eltzschig, H. K., & Eckle, T. (2011). Ischemia and reperfusion—from mechanism to translation. *Nature Medicine*, *17*(11), 1391-1401. doi:10.1038/nm.2507.

Epelman, S., Liu, P. P., & Mann, D. L. (2015). Role of innate and adaptive immune mechanisms in cardiac injury and repair. *Nature Reviews Immunology*, *15*(2), 117-129. doi:10.1038/nri3800.

Feng, Y., Zhao, H., Xu, X., Buys, E. S., Raher, M. J., Bopassa, J. C., … Chao, W. (2008). Innate immune adaptor MyD88 mediates neutrophil recruitment and myocardial injury after ischemia-reperfusion in mice. *American Journal of Physiology-Heart and Circulatory Physiology*, *295*(3), H1311-H1318. doi:10.1152/ajpheart.00119.2008.

Feng, Y., Zou, L., Si, R., Nagasaka, Y., & Chao, W. (2010). Bone marrow MyD88 signaling modulates neutrophil function and ischemic myocardial injury. *American Journal of Physiology-Cell Physiology*, *299*(4), C760-C769. doi:10.1152/ajpcell.00155.2010.

Ferrari, G. (1998). Muscle Regeneration by Bone Marrow-Derived Myogenic Progenitors. *Science*, *279*(5356), 1528-1530. doi:10.1126/science.279.5356.1528.

Frantz, S., Ertl, G., & Bauersachs, J. (2007). Mechanisms of Disease: Toll-like receptors in cardiovascular disease. *Nature Clinical Practice Cardiovascular Medicine*, *4*(8), 444-454. doi:10.1038/ncpcardio0938.

Furlani, D., Donndorf, P., Westien, I., Ugurlucan, M., Pittermann, E., Wang, W., … Ma, N. (2012). HMGB-1 induces c-kit+ cell microvascular rolling and adhesion via both toll-like receptor-2 and toll-like receptor-4 of endothelial cells. *Journal of Cellular and Molecular Medicine*, *16*(5), 1094-1105. doi:10.1111/j.1582-4934.2011.01381.x.

Ha, T., Liu, L., Kelley, J., Williams, D., & Li, C. (2013). Role of Toll-Like Receptors in Myocardial Ischemia/Reperfusion Injury. *Cardiovascular Diseases*, 123-142. doi:10.1201/b14663-11.

Hansson, G. K., & Edfeldt, K. (2005). Toll To Be Paid at the Gateway to the Vessel Wall. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *25*(6), 1085-1087. doi:10.1161/01.atv.0000168894.43759.47.

Hare, J. M., Fishman, J. E., Gerstenblith, G., DiFede Velazquez, D. L., Zambrano, J. P., Suncion, V. Y., … Heldman, A. W. (2012). Comparison of Allogeneic vs Autologous Bone Marrow–Derived Mesenchymal Stem Cells Delivered by Transendocardial Injection in Patients With Ischemic Cardiomyopathy. *JAMA*, *308*(22), 2369. doi:10.1001/jama.2012.25321.

Hoebe, K., Du, X., Georgel, P., Janssen, E., Tabeta, K., Kim, S. O., … Beutler, B. (2003). Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature*, *424*(6950), 743-748. doi:10.1038/nature01889.

Hotchkiss, R. S., Strasser, A., McDunn, J. E., & Swanson, P. E. (2009). Cell Death. *New England Journal of Medicine*, *361*(16), 1570-1583. doi:10.1056/nejmra0901217.

Janssens, S., Dubois, C., Bogaert, J., Theunissen, K., Deroose, C., Desmet, W., … Van de Werf, F. (2006). Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. *The Lancet*, *367*(9505), 113-121. doi:10.1016/s0140-6736(05)67861-0.

Jiang, B., & Liao, R. (2010). The Paradoxical Role of Inflammation in Cardiac Repair and Regeneration. *Journal of Cardiovascular Translational Research*, *3*(4), 410-416. doi:10.1007/s12265-010-9193-7.

Jiang, Y., Wang, X., Jiang, X., Niu, S., & Zhang, L. (2016). Influence of HMGB1 and MSCs transplantation on rat cardiac angiogenesis with acute myocardial infarction. *Pakistan Journal of Pharmaceutical Sciences*, *29*, 1391-1396.

Kaminski, A., Ma, N., Donndorf, P., Lindenblatt, N., Feldmeier, G., Ong, L., … Steinhoff, G. (2008). Endothelial NOS is required for SDF-1α/CXCR4-mediated peripheral endothelial adhesion of c-kit+ bone marrow stem cells. *Laboratory Investigation*, *88*(1), 58-69. doi:10.1038/labinvest.3700693.

Kataoka, K., Medina, R. J., Kageyama, T., Miyazaki, M., Yoshino, T., Makino, T., & Huh, N. (2003). Participation of Adult Mouse Bone Marrow Cells in Reconstitution of Skin. *The American Journal of Pathology*, *163*(4), 1227-1231. doi:10.1016/s0002-9440(10)63482-7.

Kawai, T., & Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology*, *11*(5), 373-384. doi:10.1038/ni.1863.

Keith, M. C., & Bolli, R. (2015). "String Theory" of c-kit pos Cardiac Cells. *Circulation Research*, *116*(7), 1216-1230. doi:10.1161/circresaha.116.305557.

Kim, S. C., Ghanem, A., Stapel, H., Tiemann, K., Knuefermann, P., Hoeft, A., … Baumgarten, G. (2007). Toll-like receptor 4 deficiency: smaller infarcts, but no gain in function. *BMC physiology*, *7*(5). doi:10.1186/1472-6793-7-5.

Laflamme, M. A., & Murry, C. E. (2011). Heart regeneration. *Nature*, *473*(7347), 326-335. doi:10.1038/nature10147.

Lagasse, E., Connors, H., Al-Dhalimy, M., Reitsma, M., Dohse, M., Osborne, L., … Grompe, M. (2000). Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nature Medicine*, *6*(11), 1229-1234. doi:10.1038/81326.

Leick, M., Azcutia, V., Newton, G., & Luscinskas, F. W. (2014). Leukocyte recruitment in inflammation: basic concepts and new mechanistic insights based on new models and microscopic imaging technologies. *Cell and Tissue Research*, *355*(3), 647-656. doi:10.1007/s00441-014-1809-9.

Limana, F., Germani, A., Zacheo, A., Kajstura, J., Di Carlo, A., Borsellino, G., … Capogrossi, M. C. (2005). Exogenous High-Mobility Group Box 1 Protein Induces Myocardial Regeneration After Infarction via Enhanced Cardiac C-Kit + Cell Proliferation and Differentiation. *Circulation Research*, *97*(8). doi:10.1161/01.res.0000186276.06104.04.

Lyngbæk, S., Schneider, M., Hansen, J. L., & Sheikh, S. P. (2007). Cardiac regeneration by resident stem and progenitor cells in the adult heart. *Basic*

Research in Cardiology, *102*(2), 101-114. doi:10.1007/s00395-007-0638-3.

Mersmann, J., Habeck, K., Latsch, K., Zimmermann, R., Jacoby, C., Fischer, J. W., … Zacharowski, K. (2010). Left ventricular dilation in toll-like receptor 2 deficient mice after myocardial ischemia/reperfusion through defective scar formation. *Basic Research in Cardiology*, *106*(1), 89-98. doi:10.1007/s00395-010-0127-y.

Nowbar, A. N., Gitto, M., Howard, J. P., Francis, D. P., & Al-Lamee, R. (2019). Mortality From Ischemic Heart Disease. *Circulation: Cardiovascular Quality and Outcomes*, *12*(6). doi:10.1161/circoutcomes.118.005375.

Orlic, D., Kajstura, J., Chimenti, S., Jakoniuk, I., Anderson, S. M., Li, B., … Anversa, P. (2001). Bone marrow cells regenerate infarcted myocardium. *Nature*, *410*(6829), 701-705. doi:10.1038/35070587.

Ozmen, S., Ayhan, S., Demir, Y., Siemionow, M., & Atabay, K. (2008). Impact of gradual blood flow increase on ischaemia–reperfusion injury in the rat cremaster microcirculation model. *Journal of Plastic, Reconstructive & Aesthetic Surgery*, *61*(8), 939-948. doi:10.1016/j.bjps.2007.05.017.

Park, S. W., Kim, M., Brown, K. M., D'Agati, V. D., & Lee, H. T. (2011). Paneth cell-derived interleukin-17A causes multiorgan dysfunction after hepatic ischemia and reperfusion injury. *Hepatology*, *53*(5), 1662-1675. doi:10.1002/hep.24253.

Prisco, A. R., Hoffmann, B. R., Kaczorowski, C. C., McDermott-Roe, C., Stodola, T. J., Exner, E. C., & Greene, A. S. (2016). Tumor Necrosis Factor α Regulates

Endothelial Progenitor Cell Migration via CADM1 and NF-kB. *STEM CELLS*, *34*(7), 1922-1933. doi:10.1002/stem.2339.

Ratajczak, M. Z., Kim, C. H., Wojakowski, W., Janowska-Wieczorek, A., Kucia, M., & Ratajczak, J. (2010). Innate immunity as orchestrator of stem cell mobilization. *Leukemia*, *24*(10), 1667-1675. doi:10.1038/leu.2010.162.

Rosenstrauch, D., Poglajen, G., Zidar, N., & Gregoric, I. D. (2005). Stem cell therapy for ischemic heart failure. *Texas heart institute Journal*, *32*(3), 339–347. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1336704/.

Rossini, A., Zacheo, A., Mocini, D., Totta, P., Facchiano, A., Castoldi, R., … Germani, A. (2008). HMGB1-stimulated human primary cardiac fibroblasts exert a paracrine action on human and murine cardiac stem cells. *Journal of Molecular and Cellular Cardiology*, *44*(4), 683-693. doi:10.1016/j.yjmcc.2008.01.009.

Sahin, A. O., & Buitenhuis, M. (2012). Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. *Cell Adhesion & Migration*, *6*(1), 39-48. doi:10.4161/cam.18975.

Secklehner, J., Lo Celso, C., & Carlin, L. M. (2017). Intravital microscopy in historic and contemporary immunology. *Immunology and Cell Biology*, *95*(6), 506-513. doi:10.1038/icb.2017.25.

Sobhani, A., Khanlarkhani, N., Baazm, M., Mohammadzadeh, F., Najafi, A., Mehdinejadiani, S., & Aval, F. S. (2017). Multipotent stem cell and current application. *Acta Medica Iranica*, *55*(1), 6-23. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/28188938.

Takeishi, Y., & Kubota, I. (2009). Role of Toll-like receptor mediated signaling pathway in ischemic heart. *Frontiers in Bioscience*, *Volume*(14), 2553. doi:10.2741/3397.

Takeuchi, O., & Akira, S. (2010). Pattern Recognition Receptors and Inflammation. *Cell*, *140*(6), 805-820. doi:10.1016/j.cell.2010.01.022.

Tian, M., Yuan, Y., Li, J., Gionfriddo, M. R., & Huang, R. (2015). Tumor necrosis factor-α and its role as a mediator in myocardial infarction: A brief review. *Chronic Diseases and Translational Medicine*, *1*(1), 18-26. doi:10.1016/j.cdtm.2015.02.002.

Timmers, L., Pasterkamp, G., De Hoog, V. C., Arslan, F., Appelman, Y., & De Kleijn, D. P. (2012). The innate immune response in reperfused myocardium. *Cardiovascular Research*, *94*(2), 276-283. doi:10.1093/cvr/cvs018.

Tsung, A., Sahai, R., Tanaka, H., Nakao, A., Fink, M. P., Lotze, M. T., … Billiar, T. R. (2005). The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *The Journal of Experimental Medicine*, *201*(7), 1135-1143. doi:10.1084/jem.20042614.

Wang, E., Feng, Y., Zhang, M., Zou, L., Li, Y., Buys, E. S., … Chao, W. (2011). Toll-like Receptor 4 Signaling Confers Cardiac Protection against Ischemic Injury via Inducible Nitric Oxide Synthase- and Soluble Guanylate Cyclase-dependent Mechanisms. *Anesthesiology*, *114*(3), 603-613. doi:10.1097/aln.0b013e31820a4d5b.

Wang, W., Chen, X., Houser, S., & Zeng, C. (2013). Potential of cardiac stem/progenitor cells and induced pluripotent stem cells for cardiac repair in ischaemic heart disease. *Clinical Science*, *125*(7), 319-327. doi:10.1042/cs20130019.

Weissman, I. L. (2000). Stem Cells. *Cell*, *100*(1), 157-168. doi:10.1016/s0092-8674(00)81692-x.

Wojakowski, W., Landmesser, U., Bachowski, R., Jadczyk, T., & Tendera, M. (2012). Mobilization of stem and progenitor cells in cardiovascular diseases. *Leukemia*, *26*(1), 23-33. doi:10.1038/leu.2011.184.

World Health statistics. (2018). *monitoring health for the SDGs, sustainable development goals*(Licence: CC BY-NC-SA 3.0 IGO). Retrieved from World Health Organization website: https://apps.who.int/iris/bitstream/handle/10665/272596/9789241565585-eng.pdf?ua=1.

Ye, X., Kong, D., Wang, J., Ishrat, T., Shi, H., Ding, X., … Hua, F. (2016). MyD88 contributes to neuroinflammatory responses induced by cerebral ischemia/reperfusion in mice. *Biochemical and Biophysical Research Communications*, *480*(1), 69-74. doi:10.1016/j.bbrc.2016.10.007.

Zhang, H., Wang, H., Li, N., Duan, C., & Yang, Y. (2013). Cardiac progenitor/stem cells on myocardial infarction or ischemic heart disease: what we have known from current research. *Heart Failure Reviews*, *19*(2), 247-258. doi:10.1007/s10741-013-9372-0.

Zhang, Y., Mignone, J., & MacLellan, W. R. (2015). Cardiac Regeneration and Stem Cells. *Physiological Reviews*, *95*(4), 1189-1204. doi:10.1152/physrev.00021.2014.

Zuidema, M. Y., & Zhang, C. (2010). Ischemia/reperfusion injury: The role of immune cells. *World Journal of Cardiology*, *2*(10), 325. doi:10.4330/wjc.v2.i10.325.

10. Self-declaration

I hereby declare, that I have made this present work without any unauthorized help of third parties and the use of other than the specified aids. The ideas taken directly or indirectly from external sources are identified as such.
The Dissertation has not yet been submitted either in Germany or abroad in the same or similar form.

Woerth am Rhein, 13.02.2020

Saifullah Abubaker

11. Acknowledgment

I owe my deepest gratitude to Prof. Dr. med. Gustav Steinhoff for granting me this work and with his guidance and profound help enabled the completion of this project.
I wish to express my sincere gratitude to Priv.-Doz. Dr. med. Peter Donndorf, for his continuous support throughout my research with patience and immense knowledge.
I am deeply indebted to my colleagues from FKGO-Research Laboratory for their generosity in providing the necessary information and supportive attitude during the whole of this endeavor.
I would like to thank Prof. Dr. med. Brigitte Vollmar and her wonderful Team in the institute for experimental surgery in Rostock for their remarkable help allowed me to carry out the experiments.
It gives me great pleasure in acknowledging Prof. Dr. med. Dr. h.c. Uwe Mehlhorn for his persistent encouragement in accomplishment of this thesis.
I would like to express my great thanks to Dr. Hunar Ahmed for his kind proofreading and valuable tips.
My special thanks go to Dr. med. Hosam Homedan, without his motivating words and inspiration this dissertation would have remained a dream.
Many thanks and appreciations go to friends and people who have helped me throughout my journey.
I am greatly indebted to my father and mother, Salah and Jazaer, for your unlimited love. From the day I opened my eyes, I see your care surrounds me. I can never thank you enough.
My joy knows no bounds in expressing my thanks to my brother, Mustafa, and his family, my sister, Sidra and her family and my younger sister, Renas. Thank you for your constant support throughout my life.
I owe an enormous debt of gratitude to my beloved wife, Rangen, for your sacrifice and unconditional support. I would never have reached this point without your love.
I am immensely beholden to my children, Nuha and Elias for giving me your time during this process. Through the struggles you have been a constant source of joy.
Above all I would like to thank the Almighty God, without his grace and blessings this work could not become a reality.

12. Curriculum Vitae

The CV has been removed for personal data protection reasons

13. Thesis

1. The mechanisms underlying stem cell homing and its interaction in the target organ are not conclusively understood

2. Using a well-established method utilizing the intravital microscopic examination of the cremaster muscle is allowing insight into the stem cell interaction in vivo

3. Applying ischemia and reperfusion in the cremaster muscle model adding more similarity to the disease picture

4. C-kit^{+} stem cells rolling and adhesion occurred mainly in the postcapillary venules of the cremaster muscle

5. Stem cell-endothelial interaction revealed to be rare in the untreated group

4. C-kit^{+} cells rolling and adhesion in the affected tissue were raised after ischemia and reperfusion

5. Among the members of the immune system, the toll-like receptors influence the regenerative mechanisms

6. TLR2 and TLR4 are playing a crucial role in stem cell homing

7. Intracellular signaling pathways of TLRs induce stem cell-endothelial interplay with particular importance of MyD88

8. The clinical implication of our study is broad anti-inflammatory or inhibition of TLRs and their intracellular signaling pathways may hinder the regeneration

9. In the context of stem cell transplantation presence of intact or enhanced TLR might improve progenitor cell survival leading to potentiation of the engraftment and successful tissue repair

14. Scientific publications

- Donndorf, P., **Abubaker, S.**, Vollmar, B., Rimmbach, C., Steinhoff, G., & Kaminski, A. (2017). Therapeutic progenitor cell application for tissue regeneration: Analyzing the impact of toll-like receptor signaling on c-kit + cell migration following ischemia-reperfusion injury in vivo. *Microvascular Research*, *112*, 87-92. doi:10.1016/j.mvr.2017.03.011

- Donndorf, P., **Abubaker, S.**, Kaminski, A., Vollmar, B., & Steinhoff, G. (2015). Toll-like Receptor Signalling Mediates C-kit+ Cell Migration in vivo Following Ischemia-reperfusion Injury via the Myd88 Pathway. *Circulation*, *132*(suppl_3), A12135-A12135.