Mast cells contribute to arteriogenesis in a PI3 Kinase γ-dependent manner

Inauguraldissertation
zur Erlangung des Grades eines Doktors der Medizin
des Fachbereichs Medizin
der Justus-Liebig-Universität Gießen

vorgelegt von Mauer, Annika Heike Beatrix
aus Bergisch Gladbach

Gießen 2014

Aus dem Institut für Biochemie
Geschäftsführender Direktor: Prof. Dr. Lienhard Schmitz

1. **Gutachter:** Prof. K.T. Preissner
2. **Gutachter:** Prof. Rohrbach

Tag der Disputation: 07.05.2015

To my family

Success is not final,
Failure is not fatal:
It is the courage to continue that counts

Winston Churchill

Table of Contents

1.0 Introduction - 1 -

1.1 Vascular occlusive disease - 1 -

1.1.1 Angiogenesis versus Arteriogenesis - 2 -

1.1.2 Arteriogenesis - 3 -

1.1.3 Future goals in arteriogenesis - 5 -

1.1.4 Mast cells in arteriogenesis - 6 -

1.2 Mast cells - 7 -

1.2.1 Historical perspective - 7 -

1.2.2 Cells with special features - 7 -

1.2.3 Peritoneal and bone marrow derived mast cells - 10 -

1.2.4 Mediators released by mast cells - 11 -

1.2.5 Mechanism of mast cell degranulation by IgE activation - 14 -

1.2.6 Mast cell activation by compound 48/80 and ionomycin - 15 -

1.3 The Phosphatidylinositol-3-Kinases (PI3K) - 17 -

2.0 Aim - 19 -

3.0 Material - 20 -

3.1 Animals and cell lines - 20 -

3.2 Apparatus and Materials - 21 -

3.3 Buffers and solutions 23

3.3.1 Cell Culture media - 27 -

3.4 Anesthetics - 28 -

3.5 Antibodies - 29 -

3.6 Kits - 29 -

4.0 Methods - 30 -

4.1 *In vivo* studies - 30 -

4.1.1 Femoral artery ligation - 30 -

4.1.2 Different treatments of animals for in vivo studies - 31 -

4.1.3 Laser Doppler Imaging (LDI) - 32 -

4.2 In vitro studies - 34 -

4.2.1 Cell culture - 34 -

4.2.2 Cultivation and passaging of eukaryotic cells - 34 -

4.2.3 Isolation of primary peritoneal mast cells - 35 -

4.2.4 Isolation of primary bone marrow derived mast cells (BMMCs) - 36 -

4.2.5 Characterisation of mast cells by flow cytometry - 36 -

4.2.6 Isolation of primary murine vascular smooth muscle cells (MVSMCs) - 37 -

4.2.7 Characterisation of smooth muscle cells - 37 -

4.2.8 Cultivation of the murine cardiac endothelial cell line - 38 -

4.2.9 Cryo-conservation, storage and thawing of the cells - 38 -

4.3 Biochemical and molecular biological methods - 39 -

4.3.1 Protein Assay - 39 -

4.3.2 Proliferation Assay .... - 39 -
4.3.3 Mast cell degranulation .... - 40 -
4.3.4 Inhibition of mast cell degranulation .... - 40 -
4.3.5 Determining the β-hexosaminidase activity in mast cell supernatants .... - 41 -
4.3.6 RNA isolation from mast cell supernatants .... - 42 -
4.3.7 RNA isolation from cell lysates and photometric quantification .... - 43 -
4.3.8 Determination of RNase activity in mast cell supernatants .... - 43 -
4.3.9 cDNA-synthesis .... - 44 -
4.3.10 Quantitative real-time Polymerase Chain Reaction (qRT-PCR) .... - 45 -

4.4 Statistical analysis .... - 47 -

5.0 Results ________ - 47 -

5.1 Characterisation of the cell types .... - 47 -
5.1.1 Characterisation of mast cells by flow cytometry .... - 47 -
5.1.2 Characterisation of smooth muscle cells .... - 48 -

5.2 Involvement of PI3Kγ-dependent mast cell degranulation in arteriogenesis .... - 49 -

5.3 The effect of Leukotriene synthesis inhibition on arteriogenesis .... - 52 -

5.4 Mast cell stimulation or inhibition *in vitro* in dependence of PI3Kγ .... - 53 -
5.4.1 Determination of most efficient mast cell degranulation .... - 53 -
5.4.2 Dose dependency of mast cell degranulation induced by ionomycin .... - 54 -
5.4.3 Determination of the most appropriate incubation time .... - 55 -
5.4.4 The effect of cromolyn on mast cell degranulation .... - 56 -

5.5 The involvement of the PI3Kγ in the process of mast cell degranulation .... - 56 -
5.5.1 Involvement of PI3Kγ activation in -/- mast cell degranulation .... - 57 -

5.6 Release of nucleic acids from mast cells .... - 58 -
5.6.1 RNase activity released from activated mast cells .... - 58 -
5.6.2 RNA-release by activated mast cells .... - 59 -

5.7 Smooth muscle- and endothelial cell proliferation in response to .... - 61 -
supernatants derived from activated mast cells .... - 61 -
5.7.1 Influence of mast cell mediators on the proliferation of smooth muscle cells .... - 61 -
5.7.2 Proliferation of smooth muscle cells in response to supernatants derived from activated ... - 62 -
peritoneal mast cells .... - 62 -
5.7.3 MVSMC proliferation in response to supernatants derived from wild type bone marrow ... - 63 -
derived mast cells .... - 63 -
5.7.4 Proliferation of smooth muscle cells in response to supernatants derived from PI3Kγ .... - 64 -
-/- mast cells .... - 64 -
5.7.5 Proliferation of smooth muscle cells in response to supernatants derived from .... - 64 -
PI3Kγ-inhibited mast cells .... - 64 -

5.8 Effect of mast cell supernatants on endothelial cell proliferation .... - 65 -
5.8.1 Proliferation of bovine endothelial cells in response to supernatants derived from .... - 66 -
activated peritoneal mast cells .... - 66 -
5.8.2 Endothelial cell proliferation in response to supernatants derived from wild type bone .... - 66 -
marrow mast cells .... - 66 -
5.8.3 Proliferation of endothelial cells in response to supernatants derived from PI3Kγ .... - 67 -
inhibited mast cells .... - 67 -
5.8.4 Influence of RNase 1 on the proliferation of smooth muscle and endothelial cells in .... - 69 -

response to supernatants derived from activated mast cells .... - 69 -
5.9 Alteration of gene expression in vascular cells after treatment with activated..... - 70 -
mast cell supernatants .... - 70 -
5.9.1 PDGF-BB gene expression in smooth muscle cells .... - 70 -
5.9.2 MCP-1 gene expression in endothelial cells .... - 71 -
5.9.3 Thrombomodulin (TM) gene expression in endothelial cells .... - 72 -

6.0 Discussion ________ - 73 -

6.1 *In vivo* studies .... - 73 -
6.1.1 Mast cells and arteriogenesis .... - 73 -
6.1.2 PI3Kγ and mast cell degranulation in arteriogenesis .... - 74 -

6.2 *In vitro* studies .... - 74 -
6.2.1 Mast cell degranulation .... - 75 -
6.2.2 Mast cell-related release of RNA and its supposed function .... - 75 -
6.2.3 Effect of mast cell mediators on vascular cell proliferation .... - 77 -
6.2.4 Proliferation studies .... - 77 -
6.2.5 Growth factor release by mast cells .... - 78 -
6.2.6 Future research .... - 80 -

7.0 Conclusion ________ - 82 -

8.0 Appendix ________ - 83 -

8.1 Abstract .... - 83 -

8.2 Zusammenfassung .... - 84 -

8.3 List of abbreviations .... - 85 -

8.4 Figures and Tables .... - 90 -

8.5 References .... - 92 -

8.6 List of publications .... - 101 -

1.0 Introduction

1.1 Vascular occlusive disease

Cardiovascular diseases (CVD) are still number one cause of death globally. In 2008 approximately 17.3 million people died from CVD, 8.3 million due to coronary heart disease; by 2030 these numbers are predicted to increase to 23.3 million deaths worldwide and to remain the single leading cause of death (World Health Organisation, 2011).

Acute coronary syndrome is the general term for a group of disorders including STEMI (myocardial infarction with persistent ST-elevations), NSTEMI (ST/T-abnormalities) and the unstable angina (Hamm et al., 2011). Patients with NSTEMI and unstable angina can be treated with elective percutaneous coronary intervention (PCI) or conservatively, respectively, whereas patients suffering from STEMI need emergency PCI or thrombolysis if PCI is not available (Bax et al., 2008). In 70% of patients, PCI involves stent implantation (Lowe et al., 2012). Historically coronary-artery bypass grafting (CABG) was the treatment of choice, which has changed to PCI nowadays commonly used to treat complex coronary artery disease (Serruys et al., 2009). PCI with stent implantation reduces re-stenosis and the rate of re-intervention compared to percutaneous transluminal coronary angioplasty (PTCA), which involves balloon dilation of the vessel (Lowe et al., 2012). However, in-stent stenosis still is a relatively common complication with an incidence of approximately 10-50%, becoming more and more important (Lowe et al., 2012).

Patients with re-stenosis will require bypass surgery and in patients with multivessel CAD the more invasive bypass surgery still is superior to PCI (Serruys et al., 2009). In-hospital mortality of PCI is approximately 1.5% in STEMI patients (Peterson et al., 2010) and for CABG surgery 1.7% (Bauriedel, Skowasch and Lüderitz, 2007).

The acute occlusion of vessels normally leads to reduced tissue perfusion and as a result to cell death and necrosis. However, it was observed that gradually developing stenoses lead to the growth of pre-existing arteriolar connections, creating a bypass circulation taking over the function of the occluded vessel partially, sometimes completely (Troidl and Schaper, 2012). These patients benefited from a slow progression of thrombus formation and of compensating collateral arteries improving the symptoms of

myocardial ischemia (Schaper et al., 1999). In peripheral tissues even acute occlusions may not lead to tissue destruction but may return to full function within days or weeks due to collateral development (Cai and Schaper, 2008). Restoration of tissue perfusion in patients with critical ischemia attributable to coronary artery disease or peripheral artery disease is a major therapeutic goal (Kubo et al., 2009) as the risk being associated with surgery or intervention could be avoided.

Neovascularisation is a term used to describe the installation of new vascular structures in previously avascular areas (Coman et al., 2010). It is a general term for three distinct mechanisms: vasculogenesis, angiogenesis and arteriogenesis (Keeley et al., 2008).

Vasculogenesis is the *de novo* formation of blood vessels by endothelial progenitor cells (EPCs) (Keeley et al., 2008). It takes place during the embryonic development by formation of *de novo* vessels from angioblasts (Cai and Schaper, 2008). During the postpartum period vasculogenesis can be mediated by bone marrow-derived endothelial progenitors, however, it is discussed controversial whether it indeed contributes to neovascularisation in the adult (Keeley et al., 2008).

1.1.1 Angiogenesis versus Arteriogenesis

The term angiogenesis can be used as synonym for capillary sprouting (Coman et al., 2010) as it is the formation of new vessels sprouting from pre-existing capillaries (Cai and Schaper, 2008). It is at this point the best understood form of neovascularisation (Keeley et al., 2008) taking place in ischemic areas, stimulated by ischemia and hypoxia (Deindl et al., 2001). Angiogenesis occurs in the growing embryo, but can also be observed in the adult during wound healing and reproduction (Coman et al., 2010). The genesis of new vessels is crucial in normal growth and development of any tissue but is also significant in the setting of tumour growth, metastasis, of chronic inflammatory and/or metabolic diseases. Capillaries have the function to locally supply tissue with oxygen and nutrients but not to transport blood through the body like arteries do. Therefore, angiogenesis is unable to restore the function of larger conducting blood vessels (Troidl and Schaper, 2012).

Arteriogenesis is a comparatively new term which was introduced to distinguish it from other mechanisms of vascular growth (Cai and Schaper, 2008). It can be defined as the development of collateral arteries from pre-existing arteriolar connections by growth

and proliferation finally resulting in the creation of a natural bypass (Deindl et al., 2001). Causative for such a type of vessel growth is the occlusion of a major conducting artery.

1.1.2 Arteriogenesis

The term "arteriogenesis" was proposed in 1997 by W. Schaper, R. Chapuli-Munoz and W. Risau to discriminate between arteriogenesis and true angiogenesis (van Oostrom et al., 2008). Research from past years showed that the stimulus that causes the growth of collateral arteries is fluid shear stress (FSS) (Schaper, 2009). FSS is proportional to blood flow velocity and inversely related to the collateral vessel radius, which means that increased blood flow also results in increased FSS (Cai and Schaper, 2008). Sudden arterial occlusion or a slow progressing stenosis can cause an increased pressure gradient in the anastomoses, leading to increased blood flow in pre-existing arteriolar connections. These small vessels respond quickly by actively proliferating and remodelling, resulting in increased lumen size and enhanced perfusion of the ischemic tissue (van Oostrom et al., 2008). FSS then drops with time, which may be the reason why arteriogenesis stops prematurely and restores only 35%-40% of the maximal conductance (Cai and Schaper, 2008).

Arteriogenesis is a complex process which starts with a structural dilation of the collateral artery wall, followed by vascular growth (Kampmann et al., 2009). Growth requires the proliferation of endothelial and smooth muscle cells and is temporally and spatially dissociated from ischemia in the peripheral model of arteriogenesis (Deindl et al., 2001). Also leukocyte recruitment, smooth muscle cell phenotypic shift, cell death and vascular matrix remodelling play an important role in the process of arteriogenesis. It is triggered by various growth factors and cytokines such as fibroblast growth factor 2 (FGF-2) or monocyte chemoattractant protein 1 (MCP-1) (Kampmann et al., 2009). Collaterals increase their diameter up to 20 times during arteriogenesis (Wolf et al., 1998). The expanding vessel arranges itself in loops and turns to accommodate extra length which gives it the typical corkscrew pattern (Figure 1) (van Oostrom et al., 2008).

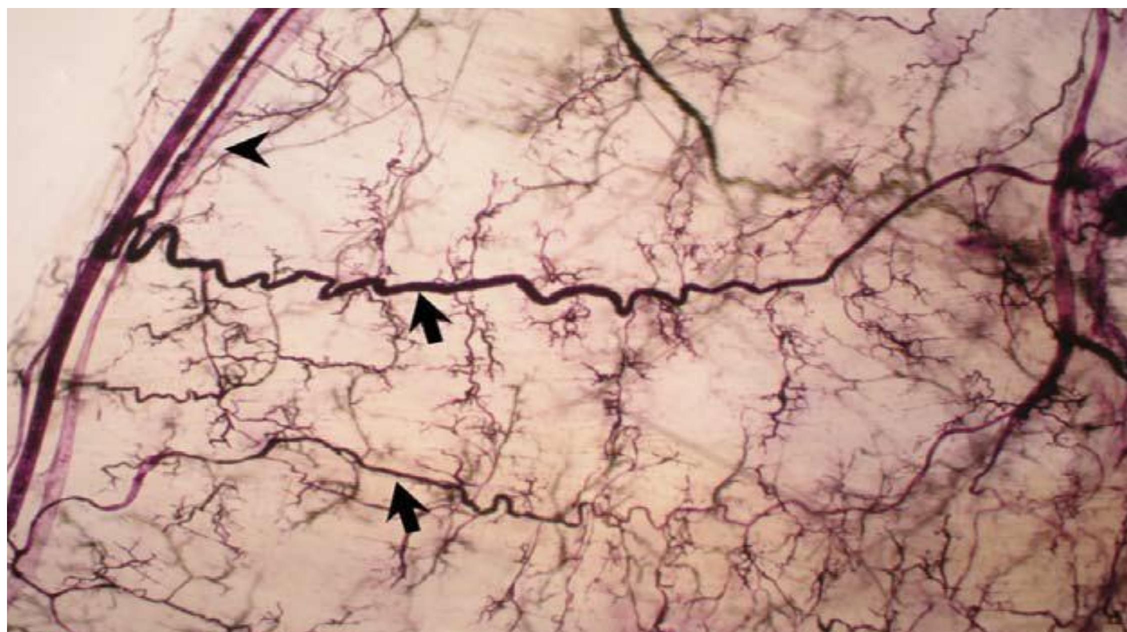


Figure 1: Collateral vessel formation after femoral artery ligation in mice. The vessels arrange themselves in these typical loops, which is called corkscrew pattern. Collaterals are indicated by the two arrows, where the proximal collateral is usually larger than the distal collateral (Limbourg et al., 2009).

To consider the role of the endothelium in arteriogenesis one has to recognise that after coronary or peripheral artery occlusion fluid shear stress causes a change in the cell structure of endothelial cells. This leads to an activation of the endothelium (Cai and Schaper, 2008), i.e. endothelial cells sense changes in FSS and transduce it into biochemical signals. The exact mechanism of this transduction pathway is unknown (van Oostrom et al., 2008).

Another step during the growth of collaterals is the activation, adhesion and migration of monocytes to and through the endothelium. The essential role of monocytes in arteriogenesis is supported by studies providing evidence that enhanced attraction of monocytes correlates directly with augmented collateral and peripheral conductance after femoral artery ligation (van Oostrom et al., 2008).

Another cell type involved in arteriogenesis, are smooth muscle cells. Smooth muscle cells are mostly affected by the arterioles' transformation into collateral vessels. They take on the most dramatic changes and increase their tissue mass highly depending on the species. This is due to their remarkable plasticity and particularly their ability to change phenotype from the contractile to the synthetic (Cai and Schaper, 2008).

It was observed that after artery occlusion, inflammatory cells such as monocytes and lymphocytes were recruited to the perivascular space of growing collaterals. It can be hypothesised that these inflammatory cells are needed for arteriogenesis (Cai and Schaper, 2008). To test this hypothesis, Schaper et al. applied anti-inflammatory

treatment and found that collateral vessel growth certainly depends on an inflammatory environment (Cai and Schaper, 2008).

Furthermore, it was observed that C57/Bl6 mice tolerated the occlusion much better than BALB/c mice (Scholz et al., 2002). The strains basically differ in their immune system (Cai and Schaper, 2008). It was also found that NK-cells (natural killer cells) and CD4 cells are important for collateral vessel growth and substitution of these cells in mice deficient for these cells accelerated collateral development (van Weel et al., 2007). The involvement and importance of lymphocytes in arteriogenesis was clearly shown by studies, however, their specific function in this process could not be specified. A probable function could be the secretion of chemokines (Schaper, 2007).

1.1.3 Future goals in arteriogenesis

Formerly the only options for treatment of occlusive arterial disease were revascularisation techniques such as percutaneous coronary intervention (PCI) (Poh et al., 2014) or bypass surgery. With the knowledge of the mechanism of arteriogenesis the emphasis in the treatment of these diseases could be placed on the growth stimulation of pre existing arteriolar connections to create natural bypasses (Deindl et al., 2003). One enormous goal for arteriogenesis studies will be to establish a treatment of human patients with arteriogenic agents (Cai and Schaper, 2008).

In cases of interventions such as bypass surgery or percutaneous transluminal angioplasty high rates of re-occlusion are observed. Therefore there is a need for alternative preferentially pharmacological strategies (VanRoyen et al., 2005). Attempts to stimulate arteriogenesis in clinical trials using various growth factors have largely failed because of impaired signalling of vascular endothelial growth factor (VEGF) in vascular tissues of patients with advanced vascular diseases (Ren et al., 2010). This was the reason why Ren et al. (2010) tried to establish a signalling pathway responsible for arterial growth that could be activated downstream of impaired growth factor signalling. A potential role of ERK in this process was examined as high levels of phosphorylated ERK1 and 2 were found in growing collateral arteries (Ren et al., 2010). Shear stress, a major contributor to arteriogenesis is capable of inducing ERK1 and 2 activation, and it was shown that ERK does restore arterial morphogenesis and branching in mice and zebrafish indicating a potential clinical relevance (Ren et al., 2010).

A controlled drug delivery system (DDS) for an integrative approach to therapeutic neovascularisation would be most favourable (Kubo et al., 2009). Kubo et al. (2009) developed a novel nanoparticle (NP)-mediated DDS, which was formulated from the bioabsorbable polylactide/glycolide copolymer (PLGA) which is effectively and rapidly taken up by vascular endothelial cells in vitro. Its advantages are a higher safety, delivery of the encapsulated drugs into the cellular cytoplasm, and slow cytoplasmic drug release (Ren et al., 2010). The drugs delivered in this approach were statins (3-Hydroxy-3-Methylglutaryl (HMG)-Coenzym-A (CoA)-redcuctase inhibitors) as they have a variety of pleiotropic vasculoprotective effects that are independent of their lipid-lowering activity and have little potential risk for tumour angiogenesis (Kubo et al., 2009). This study showed that the selective NP-mediated delivery system of statin to vascular endothelial cells increased neovascularisation and improved tissue perfusion in a murine model of hindlimb ischemia, indicating that this model would be a feasible approach for studies aiming to find a treatment for patients that suffer from ischemic diseases (Kubo et al., 2009).

1.1.4 Mast cells in arteriogenesis

Mast cells have been implicated in angiogenesis in models of injury (Wolf et al., 1998). Wolf et al. showed in 1998 that mast cells are present in the perivascular space of growing arterioles. Mast cells are a significant source of FGF-2, which is important for smooth muscle cell proliferation (Deindl, 2003). They also activate metalloproteinases such as tryptase; hence contribute to the lysis of the extracellular matrix enabling fibroblasts and macrophages to penetrate into the inflamed tissue (Wolf et al., 1998). Therefore, mast cells are likely to play an important role in the growth process of collateral vessels; however, their function has not been investigated so far.

1.2 Mast cells

1.2.1 Historical perspective

In 1876 Paul Ehrlich discovered a highly potent inflammatory cell type and named it "Mastzelle" (or "well fed cell" after the word "Mästung" – a root of the English word mastication) after the appearance caused by their basophilic storage granules (Bot et al., 2008; Galli and Tsai, 2008; Metcalfe, 2008). The granules stain red or violet, when treated with basic aniline dyes and were the reason for Paul Ehrlich to believe they were a product of overfeeding (Metcalfe, 2008). Ehrlich also observed the tendency of mast cells to be associated with blood vessels, nerves and glandular ducts which contributed to his Nobel Prize in Medicine in 1908 (Metcalfe, 2008). Mast cells have remarkable staining characteristics as a result of their proteoglycan and protease-rich cytoplasmic granules which led to their recognition by Paul Ehrlich in the late 1800s (Metcalfe et al., 1997). By the end of the 1800s mast cells had been recognised both in the normal state and associated with pathological disorders (Metcalfe, 2008).

In 1930´s mast cells were identified as carriers of heparine and histamine by Jorpes and Riley, respectively. In 1950 these observations lead to the conclusion that mast cells might play an important role in anaphylaxis; mastocytosis was known to cause death (Metcalfe, 2008). In 1977 Kitamura et al. recognised that mast cells originate from the bone marrow (Metcalfe, 2008). Newer research completed by Nabel et al. (1981) identified T-lymphocytes as crucial for stimulating mast cell proliferation by synthesising a certain factor. Ihle (1983) reported this factor to be IL-3 (Metcalfe, 2008). Finally in 1991 Kirshenbaum et al. determined that human mast cells arise from CD34+ human pluripotent stem cells. In 1992 SCF (stem cell factor) was described to be the principal growth factor for human mast cells (Metcalfe, 2008).

1.2.2 Cells with special features

Mast cells are bone marrow derived basophil cells with a small nucleus. They contain dense cytoplasmic granules, exhibiting metachromasia when stained with toluidin blue as shown in Figure 2 (Baumhoer et al., 2003).

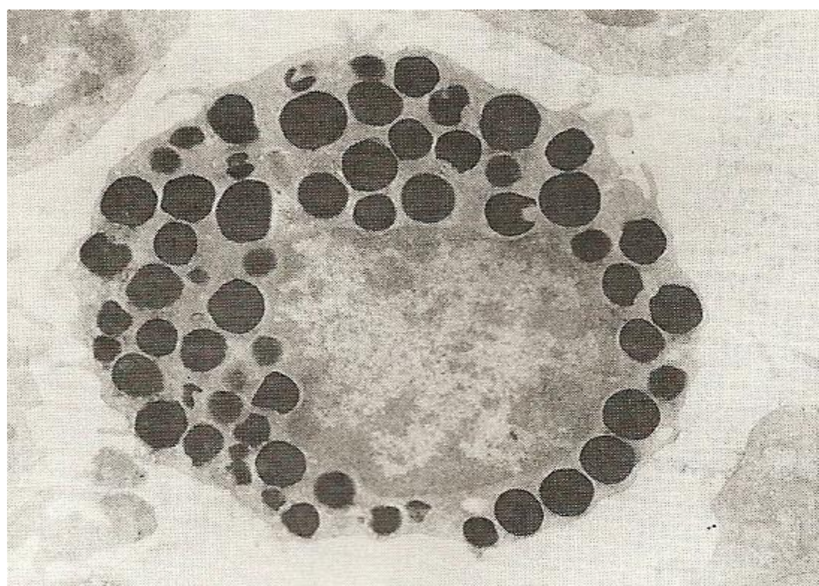


Figure 2: Electron microscopy picture showing a mast cell. The round, dark cytoplasmic structures are the granules, the light structure in the middle is the nucleus (Junqueira and Carneiro, 2004).

They are tissue-based inflammatory cells which respond to danger signals of the innate and acquired immunity (Beaven, 2009). Mast cells are approximately 10-20µm in diameter and are found in various organs such as lungs, thymus, lymphoid tissue and synovial (Prussin and Metcalfe, 2003). They reside in close proximity to blood vessels, nerves, smooth muscle cells and endothelial cells (Galli and Tsai, 2008).

In human tissues, mast cells are divided into two major subtypes: MC_{TC} and MC_{T} according to the presence of tryptase and chymase or tryptase only, respectively. MC_{TC} are mainly found in skin and small bowel submucosa. They are also called connective tissue mast cells (CTMC). MC_{T} cells predominate in normal airway and small bowel mucosa also called mucosal mast cells (MMC) (Prussin and Metcalfe, 2003).

Mast cells of all origins express a receptor for IgE (FcεRI) and for stem cell factor (c-kit) (Galli, 1990). They reside in the perivascular tissue of healthy arteries and during the progression of atherosclerosis, the cells accumulate in the adventitia and shoulder region of the atherosclerotic plaque. The heart is also one of the organs rich in mast cells (Bot et al., 2008). As already described above they develop from CD34+ pluripotent stem cells which are Kit^{+} (CD117) (Metcalfe 2008). Their precursor cells circulate in the blood and lymphatics and home to tissues. They survive and mature under the influence of SCF, produced locally by stromal cells such as fibroblasts and endothelial cells (Prussin and Metcalfe, 2003). Mature mast cells ordinarily do not circulate in the blood, instead they acquire their mature phenotype locally in the tissues

where they ultimately reside (Galli and Tsai, 2008; Kambayashi et al., 2009). Tissue mast cells´ survival, maturation and biologic expression is influenced by cytokines, such as IL (interleukin)-4, IL-5 and IFN (interferon)-γ (Prussin and Metcalfe, 2003). They respond to various exogenous signals from bacteria, viruses and parasites via recognition receptors such as toll-like receptors (TLRs) and immunoglobulins (Bot et al., 2008). This makes them critical effector cells in host defence against parasitic infections such as *Giardia lamblia* (Kambayashi et al., 2009). Activation of mast cells through TLRs led to the understanding that mast cells offer a protective function through their innate immune functions (Metcalfe, 2008). However, they also play a pathologic role in the development of T-cell mediated hypersensitivity disorders such as delayed-type contact hypersensitivity and asthma (Kambayashi et al., 2009; Sun et al., 2007).

Characteristics	Peritoneal mast cells	Mucosal mast cells
Size	10-20µm	5-10µm
Formaldehyde fixation	Resistant	Sensitive
T-cell dependence in development	No	Yes
Protease content	Chymase (RMCP I)	Chymase (RMCP Π)
Histamine	10-20pg/cell	1pg/cell
5-Hydroxytryptamine	1-2pg/cell	<0,5pg/cell
Proteoglycan´s molecular mass	Heparin 750-1000kDa	Chondroitin sulfate di B 100-150kDa
Activated by	FcεI aggregation Compound 48/80 Substance P	FcεI aggregation - -
Inhibited by sodium chromoglycerate	Yes	No

Table 1: Characteristics of peritoneal and mucosal type mast cells in rodents. They differ in granule content and type of activation. Peritoneal mast cells can be inhibited by sodium chromoglycerate whereas mucosal mast cells cannot (Metcalfe et al., 1997).

Mast cells phagocytose, process antigens, produce cytokines and release vasoactive substances and exhibit an array of adhesion molecules, immune response receptors and other surface molecules that enable mast cells to react to multiple nonspecific and specific stimuli (Metcalfe et al., 1997). Mast cell granules contain different substances called mediators which are released following a certain stimulus (Juncqueira and Carneiro, 2004) shown in Table 1. These mediators are divided into three categories:

preformed mediators, newly synthesised lipid mediators and cytokines (Prussin and Metcalfe, 2003).
Mast cells degranulate in response to a stimulus which is a substance binding to the type I Fcε-receptor (FcεRI) (Schweitzer-Stenner et al., 1997; Metcalfe, 2008). Cross linkage of IgE by the interaction of allergen specific determinants on the F_{ab} portion brings the receptor into juxtaposition and initiates mast cell activation and mediator generation and release (Metcalfe et al., 1997). They can also be activated by the complement components C3a and C5a through C3aR and C5aR (CD88) (Prussin and Metcalfe, 2003), adenosine, prostaglandine E_2 (PGE_2), sphingosine-1-phosphate (S-1P) and chemokines that bind G-protein coupled receptors (GPCRs) (Bansal et al., 2008).
Molecules that promote mast cell growth and proliferation are IL-3, IL-4, IL-9, IL-10, and nerve growth factor (NGF). The c-kit ligand also known as stem cell factor (SCF) induces proliferation of mouse mast cells *in vitro* and *in vivo* and promotes the survival of immature progenitor cells (Metcalfe et al., 1997; Galli and Tsai, 2008). The mast cell's proliferative potential is maintained even after degranulation (Metcalfe et al., 1997).

1.2.3 Peritoneal and bone marrow derived mast cells

Formerly there was a lack of adequate mast cell sources in terms of adequate numbers of primary mast cells for research. As opposed to neutrophils, monocytes and lymphocytes which are found in peripheral blood it was a lot harder to isolate mast cells. Scientists isolated rat peritoneal mast cells or mast cells from enzymatically or mechanically dispersed tissues (Metcalfe et al., 1997).
Mast cells are a minor population in tissues from which they cannot be purified. The biological properties of distinct mast cell populations that reside in different tissues are poorly known. Mast cells are also not identical in different tissues and different mast cells may not secrete the same mediators. They differentiate in the peripheral tissues so that mucosal-type mast cells develop in the mucosa of the gastrointestinal tract or the respiratory tract and serosal-type mast cells develop in the skin, submucosa of the respiratory tract, in joint synovia and in the peritoneum (Malbec et al., 2007). Another point is that the different types of mast cells are dependent on different growth factors

and can be distinguished by their morphology, histamine content and cell specific chymases and tryptases (Malbec et al., 2007).
Bone marrow derived mast cells (BMMCs) were often considered to be an *in vitro* equivalent to mucosal-type mast cells. They are immature cells whose physiological *in vivo* equivalent is not known (Malbec et al., 2007), however they possess the features needed for degranulation.
Peritoneal mast cells are mature serosal-type mast cells which represent more than 5% of cells recovered in peritoneal washings from normal mice (Malbec et al., 2007). They contain and release preformed vasoactive granular mediators and proteases but only small amounts of newly formed pro-inflammatory molecules such as eicosanoids, chemokines and cytokines. However, the cell count of a peritoneal lavage in a mouse model is insufficient for most experiments. The isolation of bone marrow derived mast cells (BMMCs) allows for higher numbers of cells, which by addition of SCF and IL-3 differentiate into mature mast cells (Laffargue et al., 2002).

1.2.4 Mediators released by mast cells

Mast cells release and generate a heterogeneous group of mediators that differ in their potency and biological activities as shown in Table 2. They are grouped into granule-associated mediators, lipid derived mediators and cytokines/chemokines (Metcalfe et al., 1997); Galli and Tsai, 2008). Many of these mediators are regarded as “pro-inflammatory” in that they can elicit vasodilation, plasma extravasation and the recruitment and activation of granulocytes, T cells, B cells, dendritic cells and monocytes or promote changes in tissues affected by an inflammatory response such as local degradation or remodelling of structural elements of the tissues (Galli and Tsai, 2008).

Mediators	Biological function
Biogenic amines:	
Histamine	Mucus and electrolyte secretions, muscle contractions, peristaltic vasodilation and plasma leakage, regulation of intestinal neurons, Granulocyte chemokinesis
Proteases:	
Tryptase	Degradation of fibrinogen, kininogen, neuropeptides and vasointestinal peptide (VIP) Regulation of endothelial cells
Chymase	Degradation of basal membrane substance and Substance P, Mucus secretion, Catalysation of the conversion of Angiotensin (AT)1 and AT2
Carboxypeptidase	Degradation of AT I
Proteoglycans:	
Heparin	Binding and stabilisation of histamine, proteases and cytokines Anticoagulation Inhibition of complement and kallikrein Regulation of endothelial cells
Chondroitinsulfate	Like heparin but less potent
Lipid mediators:	
Leukotriene (LT_4, D_4, E_4)	Mucus and electrolyte secretion Muscle contraction, peristaltic Vasodilation and plasma leakage
Prostaglandin D_2	Like Leukotrienes
Cytokines:	
TNF-α, IL-1, IL-6, IL-16, IL-18	Proinflammatory effects
IL-3, IL-4, IL-5, IL-13	T-helper 2 cells dominant immune responses
IL-10, TGF-β1	Regulatory effects on immune cells Wound healing, tissue remodelling
MIP-1α, MIP-1β, MCP-1, IL-8	Chemokines
b-FGF (=FGF2), VEGF	Growth factors for fibroblasts and endothelial cells

Table 2: All classes of mast cell mediators (Bischoff et al., 2000).

The most prevalent substance in the group of granule-associated mediators is histamine. Histamine is the only amine known to be stored in human mast cells, whereas rodent mast cells are known to store additional amines such as serotonin (Metcalfe et al., 1997; Metcalfe, 2008). Decarboxylation of the amino acid histidine to form histamine takes place in the Golgi apparatus of mast cells (Metcalfe et al., 1997). Histamine increases the capillary permeability by increasing their diameter and facilitates the contraction of smooth muscle (Juncqueira and Carneiro, 2004). Heparin, which is also contained in the mast cell granules, inhibits blood coagulation, and leukotrienes are inflammatory mediators, which also influence smooth muscle cells (Baumhoer et al., 2003).

Proteoglycans are also granule-associated mediators which are composed of a central protein core of repeating serine and glycine residues with extended, unbranched carbohydrate side chains called glycosaminoglycans of repeating disaccharide subunits containing uronic acid and hexosamine moietics (Metcalfe et al., 1997). Each disaccharide has up to three sulphate groups, which allows proteoglycans to act as extracellular mediators and as storage matrices for other preformed mediators. The proteoglycans heparin and chondroitin sulphate E have been associated with human mast cells. Both of these proteoglycans stabilise mast cell proteases and alter biological activity of many enzymes (Metcalfe et al., 1997).

The third group of granule-associated mediators are neutral proteases. Proteases of the serine class, as chymases which are specific for aromatic residues, and tryptase which has a high specificity for basic residues (Metcalfe et al., 1997). Chymase is stored in the active form as a protein of 26-29kDa. Carboxypeptidase A is also stored in mast cell granules complexed with proteoglycans. It acts as a hydrolytic enzyme at neutral pH to cleave the peptide and ester bonds of the amino end of the COOH-terminal aromatic amino acids. It is possible that chymase and carboxypeptidase A complement each other's action (Metcalfe et al., 1997).

The activation of mast cells not only causes the release of preformed granule-derived mediators but also initiates the de novo synthesis of lipid-derived substances such as cyclooxygenase- and lipoxygenase-metabolites of arachidonic acid because these substances acquire potent inflammatory activity (Metcalfe et al., 1997). Cyclooxygenase products include prostaglandins and thromboxanes, whereas lipoxygenase generates leukotrienes (LTs). Leukotriene B_4 has potent chemotactic activity for neutrophils and eosinophils, enhances lysosomal enzyme release and superoxide anion production (Metcalfe et al., 1997). Prostaglandin D_2 (PGD_2), generated after the immunologic activation of human mast cells, is a potent inhibitor of platelet aggregation, enhances histamine release, and has chemotactic activity (Metcalfe et al., 1997; Pussin and Metcalfe, 2003).

Cytokines released by mast cells have a broad spectrum of bioactivities and are important in cell growth, repair, inflammation and the immune response (Metcalfe et al., 1997). Cytokines and chemokines are protein or glycoprotein molecules. The interleukins, interferons and colony-stimulating factors were the first categories discovered (Metcalfe et al., 1997).

Tumour necrosis factor (TNF)-α is the major cytokine released by human mast cells. It up regulates endothelial and epithelial adhesion molecules, increases bronchial responsiveness and exerts antitumour effects (Prussin and Metcalfe, 2003). Other cytokines associated with mast cells are IL-4 which is associated with TH_2 (T-helper) cell differentiation, IgE synthesis, IL-3 and IL-5, which are critical for eosinophil development and survival, and IL-6, IL-8 and IL-16 (Prussin and Metcalfe, 2003).

1.2.5 Mechanism of mast cell degranulation by IgE activation

The major pathways of activating exocytosis in mast cells include the immunological trigger (aggregation of the high affinity FcεRI receptor for IgE) and the peptidergic pathway which is achieved by polycationic compounds such as the basic secretagogues of mast cells (Shefler et al., 1999; Bansal et al., 2008). These compounds act as receptor mimetic agents and thereby trigger mast cell exocytosis by directly activating pertussis toxin (Ptx)-sensitive G_i (G-inhibitory) proteins. Substances that act in this manner include the amine compound 48/80 (C48/80), ionomycin, positively charged peptides such as Substance P and bradykinin and naturally occurring polyamines (Sheflet et al., 1999; McLachlan et al., 2008). Cross-linking of the FcεRI receptors has been shown to induce phosphorylation on tyrosine residues of several proteins (Metcalfe et al., 1997).
None of the five cytoplasmic domains contains intrinsic protein tyrosine kinase (PTK) activity; aggregation stimulates previously bound or newly associated kinases (Metcalfe et al., 1997) as shown in Figure 3.
FcεRI mediated PIP_2 (phosphatidyl inositol diphosphate) hydrolysis requires external calcium ions. This dependence on Ca^{2+} is unique to the IgE receptor induced responses (Metcalfe et al., 1997). Phospholipase D (PLD) hydrolyses phosphatidylcholine, resulting in phosphatidic acid being converted to diacylglycerol (DAG). DAG activates protein kinase C (PKC) and IP3 leads to an increase of intracellular Ca^{2+} by binding to its receptor, leading to mast cell degranulation (Volná et al., 2004; Metcalfe et al., 1997; Lebduka et al., 2004).

In mast cells the rise of cytosolic free calcium concentration is a biphasic process: emptying of the intracellular stores and influx through the plasma membrane. Intracellular mobilisation is induced by the production of IP_3 and binding to its receptor, which leads to the opening of calcium selective channels on the endoplasmic reticulum membrane. On the other hand, it increases the probability of channel opening and ion flow into the cytoplasm from the extracellular space (Metcalfe et al., 1997; Volná et al., 2004; Lebduka et al., 2004; Bansal et al., 2008).

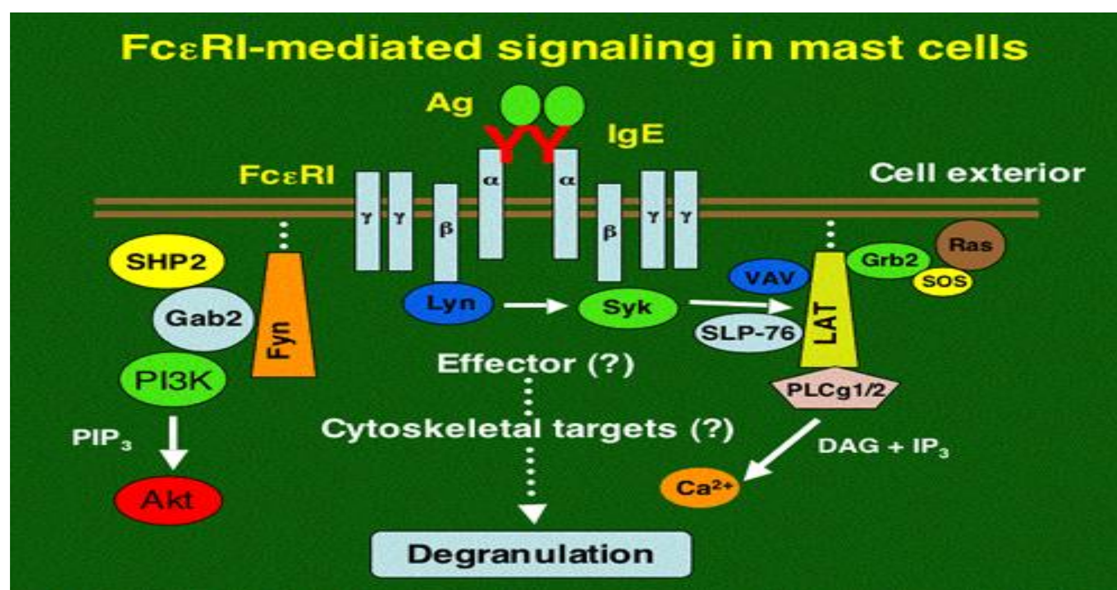


Figure 3: Molecular basis of mast cell degranulation. Signal transduction pathways in PI3Kα and β are initiated by engagement of protein tyrosine kinases and in PI3Kγ by G-protein coupled receptors. Lyn (tyrosine kinase) phosphorylates FcεRI β and γ (Volná et al., 2004). Aggregation of the receptors induces transphosphorylation of tyrosines on adjacent receptors. PI3 kinase is indirectly activated by Lyn-induced tyrosine phosphorylation (Metcalfeet al., 1997). It catalyses the synthesis of PI 3,4-bisphosphate and PI 3,4,5-triphosphate which contribute to the recruitment of the plasma membrane of Akt and PLCγ (Volná et al., 2004; Iyer and August, 2008). FcεRI mediated PIP_2 (Phosphatidyl Inositol Diphosphate) hydrolysis requires external calcium ions. This dependence on Ca^{2+} is unique to the IgE receptor induced responses (Metcalfe et al., 1997). Phospholipase D (PLD) hydrolyses phosphatidylcholine, resulting in phosphatidic acid being converted to diacylglycerol (DAG), which activates protein kinase C (PKC) and IP3 leads to an increase of intracellular Ca^{2+} by binding to its receptor. Concurrent activation of Ca^{2+} release by IP_3 and of PKC by DAG interact synergistically to elicit exocytosis in mast cells (Metcalfe et al., 1997; Volná et al., 2004). (Nishida, 2005)

1.2.6 Mast cell activation by compound 48/80 and ionomycin

C48/80 was discovered in 1939 and is a mixture of different-sized polymers synthesized by condensing N-methyl-p-methodyphenylethylamine with formaldehyde as shown in Figure 4 (Viaro et al., 2008). The molecule consists of 14 components; within these molecules, the nonamer and decamer compounds were found to induce histamine release independently of extracellular calcium (Metcalfe et al., 1997). Compound 48/80 interacts with membrane proteins. It penetrates the cell membrane but does not traverse

the membrane and directly activates G proteins. This activation involves insertion of the aromatic rings into the membrane and interaction of positively charged domains of the molecule with the COOH-terminal portion of G protein (Metcalfe et al., 1997).

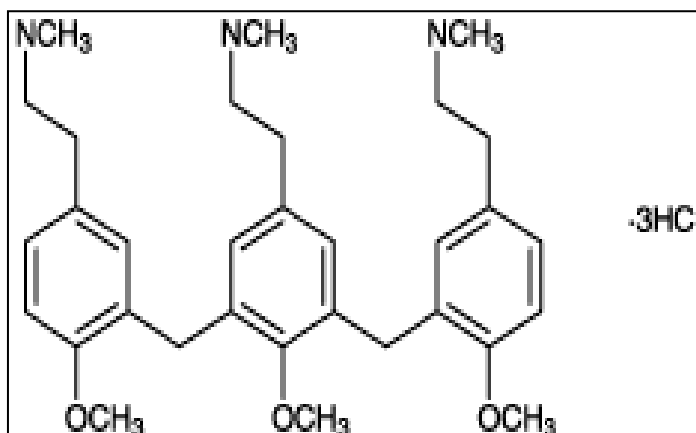


Figure 4: Chemical structure of C48/80 (Enzo life Sciences,2010).

As mast cell degranulation is a phosphoinositide 3-kinase (PI3K) mediated event, Byrne et al., (2007) showed that C48/80 is a PI3-kinase activator. As PI3Kγ is the only PI3K activated by GPCRs, C48/80 is an activator of the PI3Kγ. At higher (above 10µg/ml *in vitro*) concentrations the opposite effect with an inhibition of phosphorylation takes place (Byrne et al., 2007). C48/80 is a suboptimal stimulus for mast cell activation as the exocytosis of individual cells is partial as opposed to stimulation with ionomycin (Hide et al., 1993). Ionomycin leads to exocytosis of the mast cell granules by activation of Ca^{2+}-release both from intracellular stores and from the extracellular space (Morgan and Jacob, 1994). This follows an all or none principal and is a stronger stimulus than C48/80 (Hide et al., 1993).

1.3 The Phosphatidylinositol-3-Kinases (PI3K)

PI3K are an evolutionary conserved family of enzymes, which play a crucial role in regulation of numerous processes (Byrne et al., 2007; Madeddu et al., 2007), including cell growth, cell differentiation, proliferation, adhesion, protection from apoptosis, metabolism and immune function (Barberis and Hirsch, 2008; Crackower et al., 2002). PI3K posses lipid kinase activity and on activation they generate phosphatidylinositol-3,4,5-P_3 (PIP_3) that then activates Protein
kinase B (PKB/Akt) by phosphorylation as shown in Figure 5 (Madeddu et al., 2007). The activation of PI3K takes place by processes like receptor tyrosine kinases, Ras and heterotrimeric G-proteins (Byrne et al., 2007). PI3 kinases can be inhibited by wortmannin (Byrne et al., 2007), which disrupts C48/80 mediated MAP kinase activation and arachidonic acid from mast cells (Byrne et al., 2007). However, wortmannin is an unspecific PI3 kinase inhibitor, which is not able to distinguish between the different isoforms of PI3 kinases and structurally similar enzymes such as rapamycin (mTOR) or myosin light-chain kinase (Barberis and Hirsch, 2008).

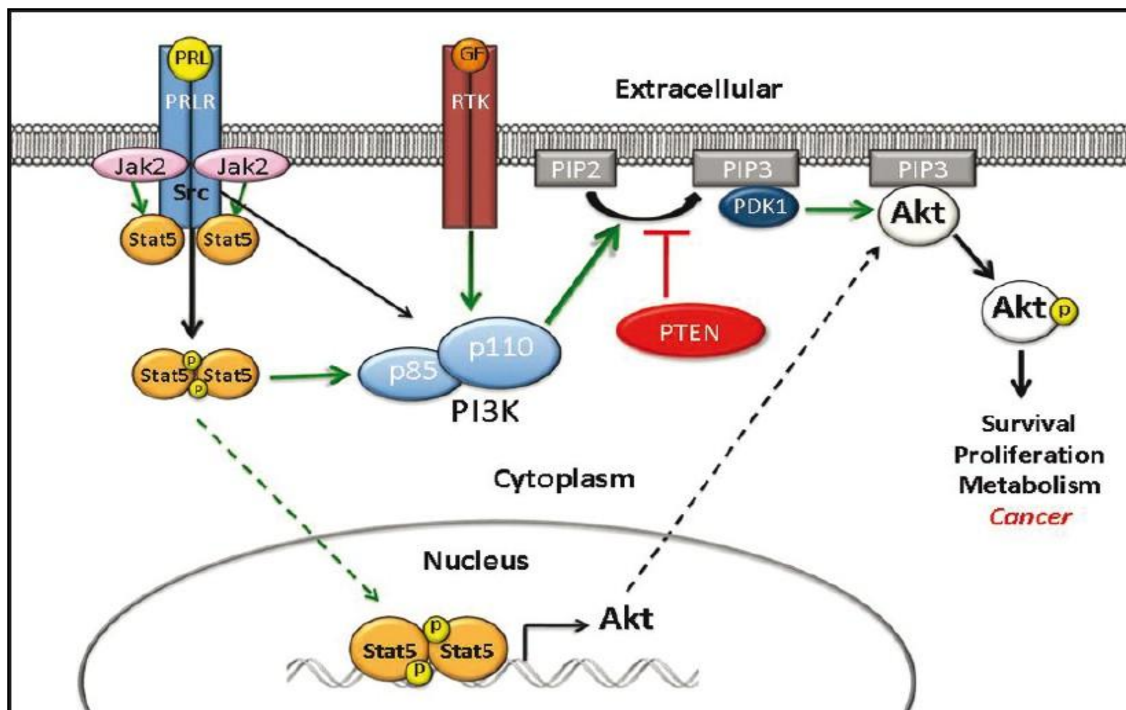


Figure 5: The PI3 kinase pathway: activation of PI3K leads to generation of PIP_3. Protein kinase B activation is followed by phosphorylation of PIP_2 of the phosphatydilinositol cascade. PI3Kα and β are activated by tyrosine kinase receptors, whereas PI3Kγ is activated by G-protein coupled receptors. (Wagner and Schmidt, 2011)

There are different classes of PI3 kinases as shown in Figure 6 (Barberis and Hirsch, 2008). Class I consists of 4 isoforms, which are divided into subclass IA and IB. The subclass IA include PI3 kinase α, β and δ, which are all activated by receptor tyrosine kinases. The only member of subclass IB is the PI3 kinase γ (PI3Kγ), which is activated by G-protein-coupled receptors (Madeddu et al., 2007). PI3Kγ has also scaffolding activity, by which it controls the activation of the phosphodiesterase 3B, linking the PI3Kγ to intracellular cAMP levels (Madeddu et al., 2007). The role of PI3Kγ in neovascularisation remains unknown; however, there is evidence that it might play a crucial role in chemokine-dependent neovascularisation (Madeddu et al., 2007). Madeddu et al., (2007) found that PI3Kγ modulates the capillarisation of ischemic muscles by controlling the balance between endothelial cell (EC) proliferation and death. PI3Kγ deficient mice had a delayed postischemic recovery and reduced arteriole density in their adductor muscles (Madeddu et al., 2007).

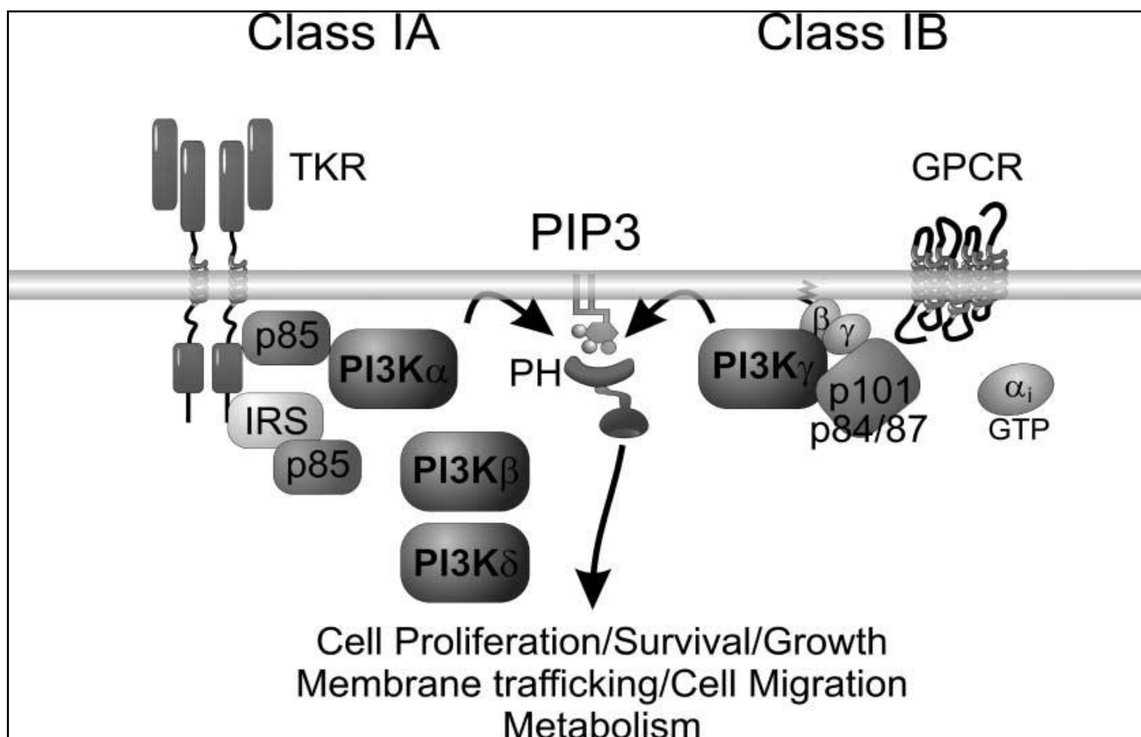


Figure 6: The different isoforms of the PI3K. Class IA are activated by tyrosine kinases. P110 is the catalytic subunit and p85 the regulatory subunit of Class IA. Class IB whose only member is PI3Kγ is activated by G-protein coupled receptor (Hirsch et al., 2007).

2.0 Aim

The aim of this work was to investigate the involvement of mast cells in arteriogenesis. *In vivo* and *in vitro* studies were planned in order to verify the involvement and importance of mast cells for arteriogenesis, which is described below.

1. There was evidence that inhibition of mast cell degranulation with cromolyn (Chillo, unpublished work) diminishes the process of arteriogenesis significantly. This led to the hypothesis that simulation of mast cells with compound 48/80 (C48/80) should improve arteriogenesis.
2. Further, when mast cells were involved in arteriogenesis, treatment of mice with a leukotriene synthesis inhibitor (MK-886) might have an impact on the outcome of arteriogenesis.
3. Another aim of this study was to quantify vascular cell proliferation following stimulation with supernatants derived from activated mast cells as arteriogenesis involves the growth and proliferation of vascular cells such as smooth muscle- and endothelial cells.
4. Additionally, the role of PI3Kγ-activation in mast cell degranulation was determined. The finding that arteriogenesis could be improved by mast cell stimulation in wt animals, but not in PI3Kγ knockout mice suggested that the PI3Kγ must play a crucial role in the process of mast cell degranulation. Therefore *in vivo* studies needed to include a comparison in arteriogenesis between wt and PI3K γ knockout mice and *in vit*ro, inhibition of PI3K by wortmannin should diminish mast cell degranulation in wt cells.

To determine the involvement of mast cell degranulation in arteriogenesis, especially its dependence on the PI3Kγ-pathway, the following *in vivo* and *in vitro* experiments were planned at the beginning of this study:

In vivo:

- Femoral artery ligation and treatment of wild type (wt) versus PI3Kγ deficient mice with C48/80 (induces mast cell degranulation).
- Femoral artery ligation and treatment of wt mice with MK-886 (induces leukotriene synthesis inhibition).
- Observation of revascularisation over 21 days by LDI measurements on day 0 (before and after surgery), at days 3, 7, 14 and 21.

<u>*In vitro*</u>:

- Degranulation of mast cells with ionomycin; collection of supernatants with subsequent measurement of the proliferation rate of vascular cells when treated with these supernatants.
- Inhibition of degranulation with wortmannin; collection of supernatants with subsequent measurement of the proliferation rate of vascular cells when treated with these supernatants.
- Measurement of RNA and in the mast cell supernatants as there was evidence that mast cells release angiogenin, hence mast cells might also release RNA.
- Measurement of RNase activity in the mast cell supernatants to determine whether or not mast cells release RNase upon stimulation.
- PCR analysis of RNA collected from smooth muscle and endothelial cells treated with mast cell supernatants to determine the expression of PDGF-BB in smooth muscle cells and MCP-1 and THBD in endothelial cells.

3.0 Material

3.1 Animals and cell lines

Male and female C57/Bl6 background mice (6-14 weeks old) were obtained from Charles River Germany. Male and female PI3Kγ knockout (-/-) mice were obtained from Emilio Hirsch (Hirsch et al., 2000) and SV129 background mice from Charles River.

The cell line Mouse Coronary Endothelial Cells (MCEC) derived from C57/Bl6 mice was purchased at CellBiologics (USA).

3.2 Apparatus and Materials

Apparatus/Material	Company
Benches	1. Holten LaminAir, Model 1.2: S.2010, Burladingen (Germany) 2. Heraeus Instruments, LaminAir, HBB 2448, Hanau (Germany)
Butterflies (Venofix® Safety)	B. Braun, Melsungen (Germany)
Chamber slides (8-well Permanox Slides)	Thermo Fisher Scientific, Rockford (USA)
Cell counter (Automated cell counter, Casy®1 TT)	Schärfe System GmbH, Reutlingen (Germany)
Cell strainer	BD Biosciences, San Jose (USA)
Centrifuges	1. Labofuge 400R, Heraeus Instruments, Hanau (Germany) 2. Centrifuge 5415R, Eppendorf, Hamburg (Germany) 3. Universal 30RF, Hettich Lab Technology, Tuttlingen (Germany) 4. EBA22, Hettich Lab Technology, Tuttlingen (Germany) 5. RC5C Sorvall SS34 Rotor, Thermo Fisher Scientific, Rockford (USA)
Combitips plus	Eppendorf, Hamburg (Germany)
Cryo Cooler (Refrigeration Device, Cryo -1°C Cooler)	VWR, Darmstadt (Germany)
Culture plates	Thermo Fisher Scientific, Rockford (USA)
6-well plates (Costar®)	Corning incorporated, Corning (USA)
96-well plates for ELISA (MaxiSorp)	Thermo Fisher Scientific (Nunc), Rockford (USA)
96-well plates for PCR (ThermoFast 96 PCR Detection Plate)	Thermo Fisher Scientific, Rockford (USA)
Electrophoresis chambers for SDS-gels	BioRad, München (Germany)
ELISA-reader (EL 808, Ultra microplate reader)	BioTek Instruments GmbH, Friedrichshall (Germany)
FACS (FACSCanto II flow cytometer)	BD Biosciences, San Jose (USA)
Freezer (-80°C)	Thermo Fisher Scientific, Rockford (USA)

Apparatus/Material	Company
Gel documentation computer with software	Intas, Göttingen (Germany)
Ice machine	Scotsman, Vernon Hills (USA)
Incubator (Sanyo CO_2 Incubator)	Sanyo Electric Co., Ltd, Osaka (Japan)
Isoflurane vapor	Drägerwerk AG, Lübeck (Germany)
KC4 Version 3.0 Power Reporter Software	BioTek Instruments, Bad Friedrichshall (Germany)
Laser-Doppler (Moor LDI Laser Doppler Imager)	Moor Instruments Limited, Devon (UK)
Laser-Doppler Software (MoorSoft Windows Software, MLDI Version 5.1, s/n: 530)	Moor Instruments Limited, Devon (UK)
Liquid nitrogen	Linde, Pullach (Germany)
Liquid nitrogen tank	Messer, Bad Soden (Germany)
Micropipettes	Gilson, Limburg (Germany)
Microscopes	1. T-Gradient, Biometra, Göttingen (Germany) 2. Hund , Typ Wilowert 30, Wetzlar (Germany) 3. Leica, DMR fluorescence microscopes, Leica, Wetzlar (Germany) 4. Zeiss Stemi DV4 Spot KL200
Nitrile gloves	Harmann, Oslo (Norway)
PCR-thermocycler	1. T3000, Biometra, Göttingen (Germany) 2. StepOnePlus, Life technologies, Thermo Fisher Scientific, Rockford (USA)
PCR tubes	Eppendorf, Hamburg (Germany)
PCR analysis software	StepOne software version 2.2.2, Life technologies, Thermo Fisher Scientific, StepOne software Rockford (USA)
pH-meter (Hi208)	HANNA, Kehl am Rhein (Germany)
Photometer	Eppendorf, Hamburg (Germany)
Refrigerators and freezers	Bosch, Gerlingen-Schillerhöhe (Germany)

Apparatus/Material	Company
Roller mixer (SRT9)	Stuart, Staffordshire (Great Britain)
Scales (SI-2002)	Denver Instruments, Bohemia (USA)
Shaking waterbath (1083 Julabo, SW22)	GFL Gesellschaft für Labortechnik mbH, Burgwedel (Germany)
Surgery instruments	FST fine science tools, Heidelberg (Germany)
Syringes	1. MicrolanceTM (30 G; 27 G) BD, Franklin Lakes (USA) 2. Sterican® (30 G; 23 G), B.Braun, Melsungen (Germany)
Thermo Shaker (TS1)	B.Braun, Melsungen (Germany)
Threads	1. Ligation thread: 6-0 Vicryl, Ethicon, Norderstedt (Germany) 2. Pearsalls Ltd. 10A05 1000, Silk Braided Suture, 5US 7/0, Somerset (England) 3. Catheter thread: 4-0, Ethicon, Norderstedt (Germany)
Ultrasound (Digital Sonifier® Cell Disrupter)	Branson, Germany
Waterbath	1. Memmert, Schwabach (Germany) 2. VWR International, Darmstadt (Germany)
Vortex (Charley2)	Süd-Laborbedarf GmbH, Gauting (Germany)

3.3 Buffers and solutions

Agarose	Sigma-Aldrich, Taufkirchen (Germany)
Albumin	Serva, Heidelberg (Germany)
b-FGF	Sigma-Aldrich, Taufkirchen (Germany)
BSA	Sigma-Aldrich, Taufkirchen (Germany)
$CaCl_2$	Merck, Darmstadt (Germany)
Collagenase	Sigma-Aldrich, Taufkirchen (Germany)
Collagen	BD-Biosciences, Heidelberg (Germany
Compound 48/80	Sigma-Aldrich, Taufkirchen (Germany)
	Santa Cruz Biotechnology, Heidelberg (Germany)
D-Glucose	Roth, Karlsruhe (Germany)

Dispase	Roche, Grenzach-Wyhlen (Germany)
DMEM	Dulbecco’s Modified Eagle’s Medium, Invitrogen, Karlsruhe (Germany)
DMSO	Dimethylsulfoxide, Merck, Darmstadt (Germany)
EBM-2	Endothelial cell basal medium, Lonza, Basel (Switzerland)
ECGS	Endothelial Cell Growth Supplement, PromoCell, Heidelberg (Germany)
EGM-2 Single Quot kit	Supplements and growth factors, endothelial cell growth medium, Lonza, Basel (Switzerland, Catalog)
Elastase	Sigma-Aldrich, Taufkirchen (Germany)
Endothelial Cell Medium	Medium and Supplements, Cell Biologics, Chicago (USA)
Ethanol	Roth, Karlsruhe (Germany)
fBSA	fetal Bovine Serum Albumin, Worthington, New Jersey (USA)
FCS	Fetal Cattle Serum, HyClone, Wien (Austria)
Gelatine	2% Gelatine Solution, Sigma-Aldrich, Taufkirchen (Germany)
Giemsa	Azur-Eosin-Methylblaulösung, Merck, Darmstadt (Germany)
Glutamax	Invitrogen,Karlsruhe (Germany)
Glycerine	Roth, Karlsruhe (Germany)
Glycine	Roth, Karlsruhe (Germany)
Hepes	Roth, Karlsruhe (Germany)
Histamine	Sigma-Aldrich, Taufkirchen (Germany)
H_2SO_4	Sulfuric Acid, Roth, Karlsruhe (Germany)
IL-1β	PeproTech, Hamburg (Germany)
IMDM	Iscove’s Modified Dulbecco’s Medium, Invitrogen, Karlsruhe (Germany)
Ionomycin	Ionomycin Calcium salt from Streptomyces, Sigma-Aldrich, Taufkirchen (Germany)
Isopropanol	Roth, Karlsruhe (Germany
KCl	Potassium chloride, Merck, Darmstadt (Germany)

KH_2PO_4	Potassium hydrogen phosphate, Merck, Darmstadt (Germany)
Lanthanum chloride	Roth, Karlsruhe (Germany)
Latex	Spartan Adhesives and Coastings Co. Inc., Crystal Lake (USA)
MEM	Non-essential-aminoacids, Invitrogen, Karslruhe (Germany)
Methanol	Roth, Karlsruhe (Germany)
$MgSO_4$	Magnesium sulphate, Merck, Darmstadt (Germany)
MK-886	Merck, Darmstadt (Germany)
Mouse IL-3	PeproTech, Hamburg (Germany)
NaCl	Sodium chloride, Roth, Karlsruhe (Germany)
$NaHCO_3$	Sodium bicarbonate, Roth, Karlsruhe (Germany)
Na_2HPO_4	Sodium phosphate, Merck, Darmstadt (Germany)
NaOH	Sodium hydroxide, Roth, Karlsruhe (Germany)
Na-Pyruvate	Invitrogen, Karlsruhe (Germany)
Neutral Red	Sigma-Aldrich, Taufkirchen (Germany)
Perchloric acid	Sigma-Aldrich, Taufkirchen (Germany)
PDGF-BB	Platelet derived growth factor, R&D Systems, Minneapolis (USA)
Penicillin/Streptomycin	Invitrogen, Karlsruhe (Germany)
Percoll	Amersham Biosciences, Freiburg (Germany)
Poly C	Polycytidylic acid potassium salt, Sigma-Aldrich, Taufkirchen (Germany)
PMA	Phorbol myriat acetate, Merck, Darmstadt (Germany)
Rat Serum	Sigma-Aldrich, Taufkirchen (Germany)
Rat T-STIM	BD, Heidelberg (Germany)
RNase 1	bovine, recombinant, Thermo Fisher Scientific (Fermentas), Rockford (USA)
RNase inhibitor	Ribo Lock RNase Inhibitor, Thermo Fisher Scientific, Rockford (USA)
	RNase free water B. Braun Melsungen AG, Melsungen (Germany),

RNA-loading dye	2× RNA Loading Dye, Thermo Fisher Scientific (Fermentas), Rockford (USA)
	RPMI Invitrogen, Karlsruhe (Germany)
SCF	Stem cell factor, Biomol, Hamburg (Germany)
SDS	Sodiumdodecyl sulphate, Roth, Karlsuhe (Germany)
SYBR Green	LightCycler FastStart DNA Master Plus set SYBR Green I, Roche, Grenzach-Wyhlen (Germany)
TEMED	Roth, Karlsruhe (Germany)
4-PNAG	4-Nitrophenyl N-acetyl β-D-glucosaminide, Sigma-Aldrich, Taufkirchen (Germany)
TNF-α	R&D Systems, Minneapolis (USA)
Trisodium-citrate	Roth, Karslruhe (Germany)
Tris	Roth, Karslruhe (Germany)
Triton	Roth, Karslruhe (Germany)
Tryple Express	Invitrogen, Karlsruhe (Germany)
2mercapto	Sigma-Aldrich, Taufkirchen (Germany)
Tween-20	Roth, Karslruhe (Germany)
Vectashield	Vectashield mounting medium with DAPI, Vector Laboratories, Peterborough (UK)
VEGF	PeproTech, Hamburg (Germany)
	Wortmannin Merck, Darmstadt (Germany)

3.3.1 Cell Culture media

Cells	Medium
Peritoneal mast cells	IMDM
	10%FCS (Fetal Cattle Serum), 1% Pen/Strep
Bone marrow derived mast cells (BMMC)	IMDM
	10% FCS
	1% Pen/Strep
	1% NEAA
	IL-3 (10ng/ml)
	SCF (25ng/ml)
Murine vascular smooth muscle cells (MVSMC)	IMDM
	10% FCS
	1% Pen/Strep
Mast cell line (MC9)	DMEM
	10% FCS
	1% Pen/Strep
	10% Rat-T-STIM
	$NaHCO_3$ (1,5g/L)
	Mercapto (0.05mM)
	L-glutamine (2mM)
Bovine aortic endothelial cells (BAEC)	DMEM
	10% FCS
	1% Pen/Strep
	1% Glutamine
	1% Na-Pyruvate
	1% non essential amino acids
Mouse coronary endothelial cells (MCEC)	Endothelial Cell Medium
	10% FCS
	1% Pen/Strep
	Supplements (Supplement kit)

3.4 Anesthetics

Domitor®	Active ingredient: Medetomidinhydrochloride, 10 ml injection solution for intravenous/intramuscular injection, Orion pharma, Hamburg (Germany)
Fentanyl®-Jannsen	0.5 mg, 5 ampullas with 10ml for intravenous/intramuscular injection, Janssen-Cilag, Neuss (Germany)
Flumazenil	Flumazenil-hameln, 0.5 mg, 10 ampullas with 1ml for intravenous injection, Hameln pharma plus GmbH, Hameln (Germany)
Ketamin	Ketavet®, 100 mg/ml, 5 ampullas with 10 ml for intravenous/intramuscular injection, Pharmacia GmbH, Karlsruhe (Germany)
Naloxon	Naloxon Inresa, 0.4 mg, 10 ampullas with 1 ml (0.4 mg Naloxonhydrochloride) for intravenous, intramuscular or subcutaneous injection, Inresa Arzneimittel GmbH, Freiburg (Germany)
Midazolam	Midazolam-ratiopharm, 15 mg/3 ml, 10 ampullas with 3 ml for intravenous/intramuscular injection, ratiopharm GmbH, Ulm (Germany)
Revertor®	Active ingredient: Atipamezolhydrochloride, 5 mg/ml, 10 ml injection solution for intravenous injection, CP-pharma GmbH, Burgdorf (Germany)
Rompun® 2%	Active ingredient: Xylazinhydrochloride, 25 ml injection solution for intravenous, intramuscular or subcutaneous injection, Bayer Vital GmbH, Leverkusen (Germany)

3.5 Antibodies

Anti-α-smooth-muscle-actin	α-smooth-muscle-actin-Cy3, Sigma-Aldrich, Taufkirchen (Germany)
Anti-CD31	Santa Cruz Biochtechnology, Santa-Cruz (USA)
Anti-CD45	Santa Cruz Biochtechnology, Santa-Cruz (USA)
Anti-CD117	CD117 (C-kit receptor) rat anti mouse (PE Alexa Fluor 647), Biolegend San Diego (USA)
	CD117 (C-kit receptor) rat anti mouse (PE Alexa Fluor 488), Biolegend, San Diego (USA)
DAPI	Diamine-2-Phenylindol, Vector Laboratories Inc., Peterborough (UK)
Donkey Anti Goat IgG	Alexa Fluor 488, Invitrogen, Karlsruhe (Germany)
	Alexa Fluor 568, Invitrogen, Karlsruhe (Germany)
Donkey Anti Mouse IgG	Alexa Fluor 488, Invitrogen, Karlsruhe (Germany)
Donkey Anti Rat IgG	Alexa Fluor 488, Invitrogen, Karlsruhe (Germany)
Anti-TNF-α	Santa Cruz Biotechnology, Heidelberg (Germany)
Anti-vWF	Santa Cruz Biotechnology, Heidelberg (Germany)
Anti-VEGF	R&D Systems, Minneapolis (USA)

3.6 Kits

cDNA synthesis kit	High Capacity cDNA Reverse Transcription kit, Applied Biosystems, Foster City (USA)
Proliferation Assay kit	Roche, Cell Proliferation ELISA BrdU (colorimetric), Grenzach Wyhlen (Germany)
Protein Assay	BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford (USA)
RNA isolation kit	GenElute Mammalian Total RNA Miniprep Kit, Sigma-Aldrich, Taufkirchen (Germany)

4.0 Methods

4.1 *In vivo* studies

Experimental groups contained 5 animals (n = 5), each value represents the mean ± SEM. Values of $p \leq 0.05$ were regarded as statistically significant.

4.1.1 Femoral artery ligation

Animal husbandry as well as all experiments were approved by the government of Oberbayern.
Surgical ligation of the femoral artery at a specific site induces arteriogenesis in femoral collateral arteries and angiogenesis in distal ischemic muscles (Limbourg et al., 2009). This method involves preparation, surgery and following the surgical procedure the measurement of hind-limb perfusion by Laser-Doppler-Imaging (LDI) (Limbourg et al., 2009).
Animals were anesthetised with 100µl of a solution containing 1.5ml Rompun, 3 ml Fentanyl and 3ml Midazolam, administered subcutaneously to the neck of the mice. After anaesthetisation, mice were placed on the surgery table under a Biometra microscope thoroughly and shaved to avoid contamination of the surgery field with animal hair. A small cut was applied to the area of surgery, on top of the site of ligation. By thorough preparation and separation of vein, artery and nerve, with fine preparation instruments, the ligation thread was placed directly distal of the origin of the deep femoral branch, so that the artery was completely closed over the whole length (Limbourg et al., 2009). A knot was tightened under microscope vision, not to injure other tissues or to provoke bleeding, but with the aim of inducing ischemia in the legs of the mice (Westvik et al., 2010).
After surgery, wounds were suturated with a thin thread and mice were allowed to recover (Westvik et al., 2010). The femoral artery ligation was performed on the right leg of the mice, the left leg was sham operated (Westvik et al., 2010).
The different steps of the femoral artery ligation (fal) can be viewed in Figure 7, 8 and 9, showing the area of surgery, the placement of the thread and the ligation with the knot, respectively.

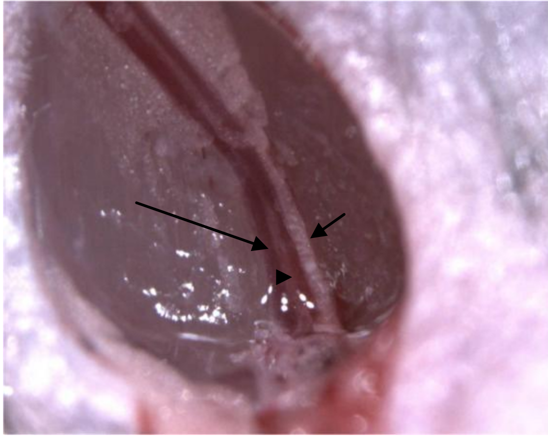


Figure 7: **Area of surgery:** Vein (long arrow), artery (arrow tip) and nerve (short arrow) need to be separated in order to place a thread underneath the artery.

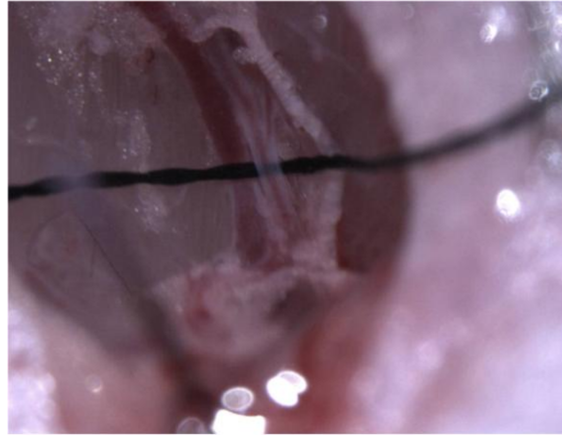


Figure 8: **Preparation for femoral artery ligation:** The thread is placed underneath the femoral artery. Ligation can be finalised by tying a knot around it.

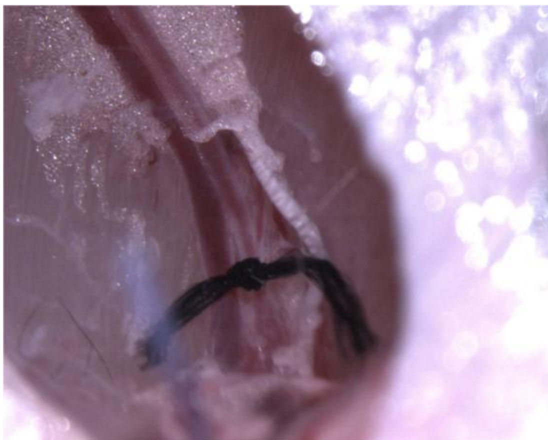


Figure 9: **Finalisation of fal:** Closure of the femoral artery by ligation.

4.1.2 Different treatments of animals for in vivo studies

Following femoral artery ligation, animals were subjected to different treatments in order to determine their effects on arteriogenesis.

C48/80 was dissolved in 0.9% saline solution and injected at 250µg/ml/leg in 100µl subcutaneously (s.c.) into the leg of the mice for the first 3 days of the week over 3 weeks (21 days).

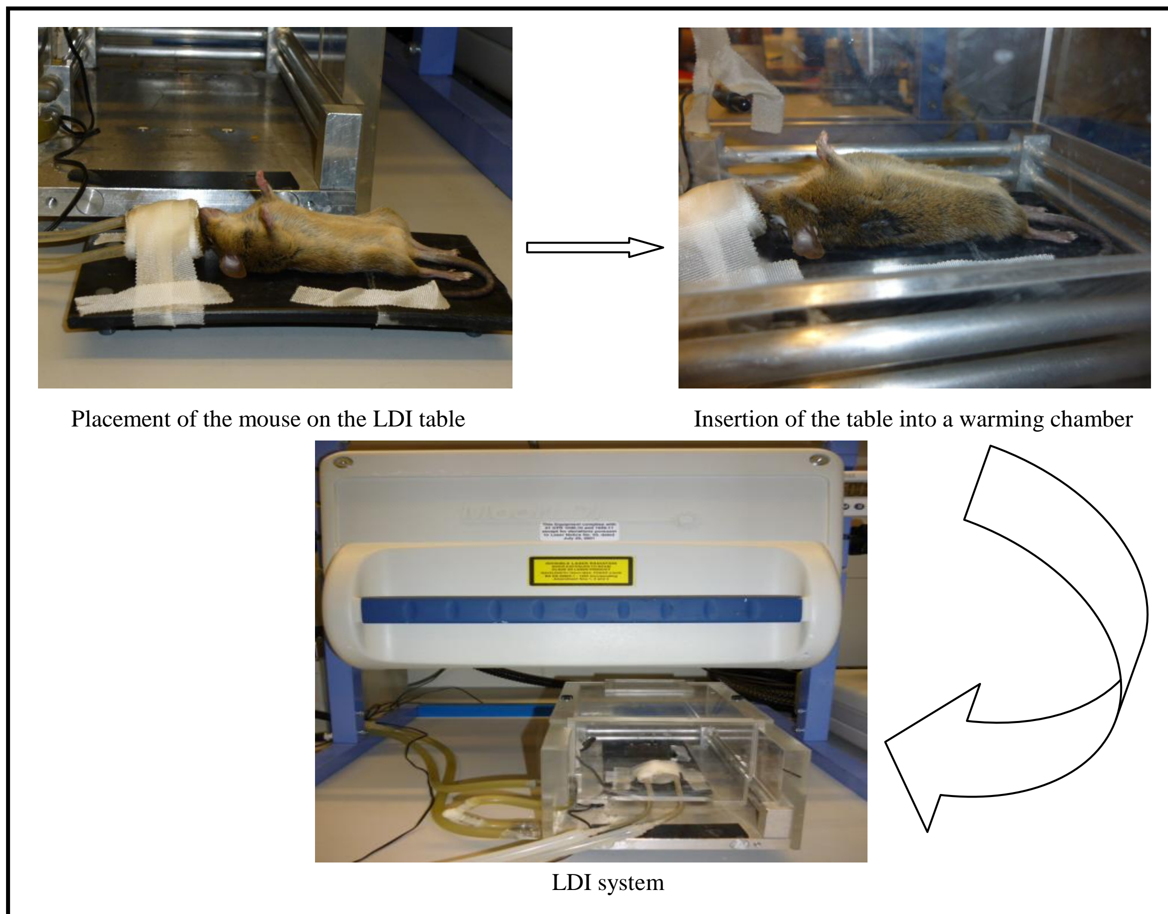
MK-886 was dissolved in 1% dimethylsulfoxide saline (DMSO) and injected at 3mg/kg/day intraperitoneally (i.p.) over 21 days (Uz et al., 2008).

Control groups were injected with NaCl 0.9% in the same manner.

All groups of animals were held under identical conditions and each group contained equal numbers of animals (n = 5).

4.1.3 Laser Doppler Imaging (LDI)

Blood flow recovery was monitored by LDI (Madeddu et al., 2007). LDI uses a low power He-Ne laser to scan animals; moving blood cells give rise to Doppler shifts in the reflected light that are then converted to perfusion values (Limbourg et al., 2009). The Doppler signal is proportional to perfusion of the upper 1mm of the skin, in regions of interests (ROI). The tissue perfusion was quantified by the ratio calculated by the perfusion of the ligated limb relative to the contralateral non-ligated side (Limbourg et al., 2009). This gives a ratio (%) of occluded versus sham operated limb. Figure 10 outlines the different steps of the LDI method



<u>Figure 10 :</u> Laser Doppler Imaging (LDI): Placement of the mouse on the LDI table, insertion of the table into the heating chamber. Measurement of limb perfusion after a defined time (10 minutes) and temperature (37°C) allows for comparable measurements.

Figure 11 demonstrates LDI measurements of hind limb perfusion in mice subjected to femoral artery ligation before and immediately after surgery, at day 3 and day 7 after surgery. The right occluded limb is compared to the left sham-operated limb. In the basal measurement, before surgery, the ratio of limb perfusion is 100%. After surgery this ratio is reduced to fewer than 10% and recovers with time. Improvement of blood

flow to the limbs after a few days following surgery is expressing the process of collateral artery growth, hence arteriogenesis.

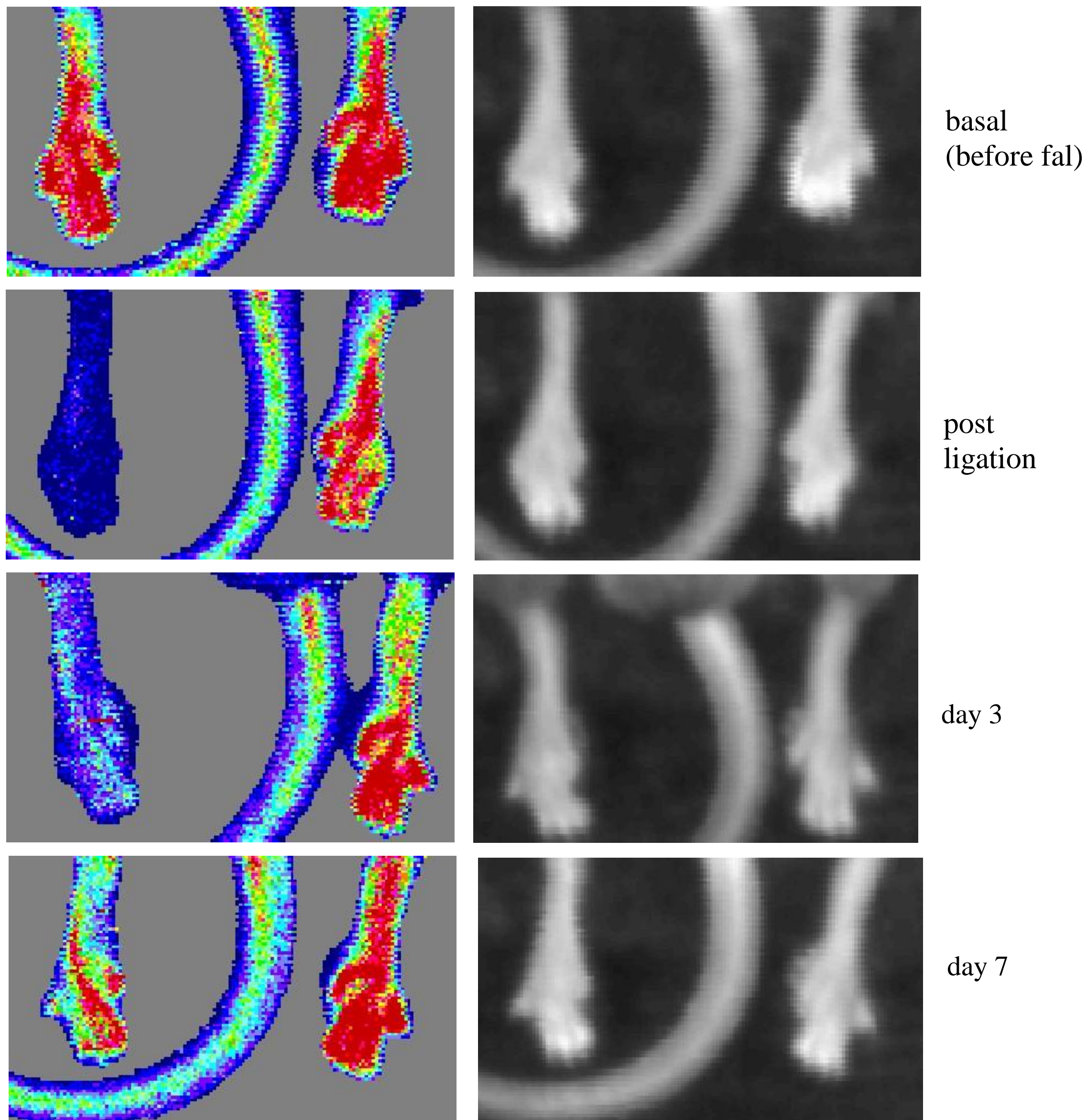


Figure 11: LDI measurements: In the basal measurement (before surgery) the ratio of hind limb perfusion in the right (occluded) limb compared to the left (sham-operated) limb is 100% which is visible by a comparable red colour on the left and right leg. After fal a reduction of limb perfusion to fewer than 10% was achieved, visible by a blue colour of th ligated limb compared to a red colour of the perfused sham operated limb. Recovery can be observed over 7 days as an improvement in limb perfusion leads to an increase in the percentage demonstrated by an increase in red colour between occluded versus sham-operated limb.

4.2 In vitro studies

In vitro experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

4.2.1 Cell culture

In this work primary cells as well as cell lines were used for experiments. Primary cells were peritoneal and bone marrow derived mast cells (BMMCs) and murine vascular smooth muscle cells (MVSMCs). MCEC (mouse coronary endothelial cells), which is a cell line extracted from mouse cardiac arteries was obtained from Cell Biologics Inc. All cell culture experiments were done under sterile conditions.

4.2.2 Cultivation and passaging of eukaryotic cells

Cells were cultured in a humidified incubator at 37°C and 5% CO_2 conditions. Every two to three days medium was changed to keep cells under best growing conditions. For medium change, consumed medium was removed and confluent adherent cells were washed with phosphate buffered saline (PBS). Cells were detached by incubation with Trypsin-EDTA for 30 seconds at 37°C. Fetal cattle serum (FCS) was added in order to stop the reaction. Cells were centrifuged at 1000 g for 3 minutes at room temperature. PBS was discarded; cells were resuspended in fresh medium and splitted 1:2 up to 1:4 depending on the cell type.

Culture plates were coated for cultivation of endothelial cells. Two millilitres of collagen (50µg/ml) or gelatine (0.2%) were added to the plate and incubated at 37°C for 30 seconds. After removal of the remaining collagen or gelatine, cells were resuspended in fresh medium and seeded onto the plate.

For mast cells, smooth muscle cells and endothelial cells IMDM-medium was used, cell specific supplements were added when required (see material).

4.2.3 Isolation of primary peritoneal mast cells

A Percoll gradient was prepared prior to the cell isolation in order to be able to process cells directly after gathering. For preparation of the gradient, 1ml gradient buffer (see table below) and 9ml Percoll were centrifuged in a Sorvall centrifuge at 11700rpm for 20 minutes at 4°C without brake to create a continuous gradient for the mast cell isolation (Aridor, Traub and Sagi-Eisenberg, 1990).

Gradient Buffer	
NaCl	1.37mM
KCl	27mM
Na_2HPO_4	50mM
KH_2PO_4	20mM
D-Glucose	56mM
Albumin	1%
pH=7.2	

8-12 week old mice were sacrificed via cervical dislocation. Peritoneal lavage was performed via the application of approximately 10ml of 4°C cold tyrodes buffer (see table below) to the peritoneal cavity of the mice via a syringe. The peritoneum was massaged for 30 seconds and the suspension containing the mast cells was gathered via the use of a pipette. Averages of 5-10% of all cells in the peritoneal cavity are mast cells (Jensen et al., 2006). Approximately 3-6×10^5 of these cells can be gathered in total in mice, and 1-3×10^6 cells in rats (Coleman et al., 2006; Eastmond et al., 1997).

Tyrodes Buffer	
NaCl	130mM
KCl	4.75mM
$CaCl_2$	2.54mM
KH_2PO_4	1.2mM
$MgSO_4$	1.2mM
D-Glucose	11mM
Hepes	10mM
Albumin	0.1%
pH = 7.2	

The cell suspension was centrifuged on the Percoll gradient at 1600rpm for 15 minutes; the last pellet was resuspended in 100µl of IMDM medium for cultivation (Jensen et al., 2006) or in PBS when used for experiments directly.

4.2.4 Isolation of primary bone marrow derived mast cells (BMMCs)

8-12 week old mice were sacrificed via cervical dislocation and the femurs extracted (Stopfer, P., Männel, D. and Hehlgans T., 2004).
Bone marrow cells were harvested by flushing the bone with a pipette containing warm IMDM medium (Abel et al., 2011; Stopfer, P., Männel, D. and Hehlgans T., 2004). The extracted cell suspension was further processed. Cells were separated by a cell strainer with 70µm in diameter, to get a single cell suspension of a pure mast cell colony.
Following this procedure, the cell suspension was centrifuged at 1000g for 10 minutes and the pellet was taken up in fresh IMDM medium at 37°C containing 10% FCS, 10ng/ml IL-3 and 25ng/ml mSCF (Sly et al., 2008).
Cells were cultured for 3 weeks to allow for differentiation, which was verified by FACS-analysis of the cells prior to the experiments. Splitting of the cells was achieved by centrifugation of the cell suspension, the used medium was discarded and fresh medium was added. This procedure was repeated every 2-3 days (Stopfer, P., Männel, D. and Hehlgans T., 2004).

4.2.5 Characterisation of mast cells by flow cytometry

Mast cells were isolated from the bone marrow of wt and knockout mice. Following, they were subjected to flow cytometry to analyse the expression of c-Kit (CD117). Kit is a tyrosine kinase receptor for SCF, which is essential for homing and differentiation of mast cells (Beaven, 2009).
The purity of the sample was determined via a Fluorescein isothiocyanate (FITC) labelled anti c-Kit IgG antibody (Madeddu et al., 2011). BMMCs (1×10^6 cells) were incubated with the antibody solution (100µl) for 1 hour. Before FACS analysis, cells were washed three times with PBS (Stopfer, P., Männel, D. and Hehlgans T., 2004). As

shown in Figure 12 and 13, the samples contained a pure mast cell sample, which was then used for further experiments.

4.2.6 Isolation of primary murine vascular smooth muscle cells (MVSMCs)

8-12 week old mice were sacrificed via cervical dislocation. The thorax was opened along the linea alba. One ml PBS with 1% Penicillin/Streptomycin (Pen/Strep) was injected over the left ventricle of the heart. The aorta was extracted with fine tools and pooled in a 50ml falcon containing sterile PBS with 1% Pen/Strep until further processing (Kobayashi et al., 2004).
The aortas were cleaned from connective tissue and debris under the microscope and pooled in a fresh 50ml falcon containing 1mg/ml collagenase and 1mg/ml elastase. The aortas were incubated in this solution for 90 minutes in a 37°C shaking water bath. Following this, the cell suspension was centrifuged at 200g for 10 minutes; cells were resuspended and cultured in IMDM, similar to the method Kobayashi et al. (2004) described previously. Cells were splitted when plates were confluent.
To determine the purity of the culture, cells were labelled with α-smooth-muscle-actin.

4.2.7 Characterisation of smooth muscle cells

MVSMCs were seeded onto chamber slides (100µl). Cells were incubated at 37°C over night to allow cell adhesion to the plate.
MVSMCs were fixed with PFA 4% (100µl) for 10 minutes at room temperature (RT). Cells were washed with PBS (200µl) once and were further treated with Triton 0.1% (100µl) for another 10 minutes at room temperature (RT). After washing with Tris-buffered saline (TBS) (200µl) three times, the samples were blocked with 3% BSA/TBST (Tris-Buffered Saline and Tween) (100µl) for 1 hour at RT.
Cells were incubated with the primary antibody (100µl) for one hour (α-smooth-muscle actin (Figure 14) or with CD31 to stain contaminating endothelial cells. Cells were washed three times with TBS (200µl and second antibodies coupled with a fluorescent dye were applied and incubated for one hour at RT in a dark box. After another three

washing steps with TBS (200µl) the chamber was taken off and remaining fluid was removed carefully. The Vectashield mounting medium (1 drop), containing 4',6-diamidino-2-phenylindole (DAPI) was applied to all samples.
All primary antibodies were diluted 1:100 and secondary antibodies 1:200 with 1% BSA/TBST.

4.2.8 Cultivation of the murine cardiac endothelial cell line

The murine cell line derived from coronary endothelial cells was cultured in endothelial cell medium, containing supplements. The cells were splitted 1:2 up to passage 3 until used for experiments. Medium was renewed every 2 days as described earlier.

4.2.9 Cryo-conservation, storage and thawing of the cells

In order to prepare cells for cryo-conservation, cells were washed with PBS and detached by addition of 2ml Trypsin-Ethylenediaminetetraacetic acid (EDTA). The reaction was stopped with FCS and cells were centrifuged at 200g for 3 minutes at room temperature.
The cell pellet was resuspended in 500µl of the respective culture medium containing 20% DMSO and equal amounts (500µl) of FCS.
Cells were frozen slowly in a 1°C cooler in the -80°C freezer which freezes the cells at a rate of 1°C per hour.
For longer storage primary cells were transferred into a liquid nitrogen tank until needed.
To thaw cells, tubes were transported to the laboratory in liquid nitrogen and heated quickly up to 37°C in a waterbath. The cells were taken up in fresh culture medium and centrifuged at 1000rpm for 3 minutes at room temperature. The cell pellet was taken up in culture medium and transferred onto a culture plate. Cells were incubated as explained earlier.

4.3 Biochemical and molecular biological methods

4.3.1 Protein Assay

Protein was determined using a protein assay kit from Pierce. After incubation of the samples with bicinchoninic acid (BCA) working solution for 30 minutes at 37°C, the absorption was measured in an enzyme linked immunosorbend assay (ELISA)-reader at 562nm. To calculate the amount of protein, all measurements were compared with a protein standard.

4.3.2 Proliferation Assay

MVSMCs and MCECs were seeded onto a 96 well plate at a density of 10,000 and 5,000 cells per well, respectively. The cells were allowed to adhere for 4-6 hours prior to stimulation.
Cells were washed with PBS and 100µl of different dilutions of mast cell supernatants were applied. Cells were incubated at 37°C. After 48 hours the proliferation of murine endothelial and smooth muscle cells was determined, using the BrdU cell proliferation kit. As cellular proliferation requires genomic DNA synthesis, monitoring DNA-synthesis is an indirect parameter of cell proliferation. 5-bromo-2´-deoxyuridine (BrdU) is a dye, developed to identify proliferating cells that can be detected in the ELISA-reader at 450nm.
Cells were labelled for 3 hours with BrdU labelling solution. Fixation of the cells was achieved by applying 200µl/well Fix Denat (bottle 2) for 30 minutes at RT. Fix Denat was removed and 100µl BrdU-POD antibody working solution was applied to the cells for 90 minutes at RT. After removing the antibody, the cells were washed 3 times and 100µl substrate solution was added for another 30 minutes at RT. The reaction was stopped by adding 25µl 1mM H_2SO_4 and the plates were read in the ELISA-reader at 450nm. The percentage of proliferation was calculated against the negative control represented the cells treated with culture medium without supplements. Substances used for the cell stimulation prior to the proliferation assay are listed in the table below.

substance	concentration
TNFα	5, 10 and 25ng/ml
VEGF	5, 10 and 25ng/ml
FGF	5, 10 and 25ng/ml
PDGF	5, 10 and 25ng/ml
Histamine	5, 10 and 25ng/ml
VEGF+FGF	10ng/ml
PDGF+FGF	10ng/ml

4.3.3 Mast cell degranulation

For mast cell degranulation, a standard number of 2×10^6 cells per ml culture medium were seeded onto multi-well plates. Mast cells were treated with C48/80 (10µg/ml), ionomycin (1µM) or TNF-α (10ng/ml) for one hour in a shaking water bath. Ionomycin induces a strong intracellular increase of Ca^{2+} ions, in response to which mast cells degranulate (Puri and Roche, 2007).
After one hour the cell suspension was centrifuged at 1000g for 3 minutes at room temperature. Supernatants were collected and frozen at -80°C until used for further experiments.
Cell pellets were resuspended in PBS and transferred into Eppendorf tubes. Cells were lysed via ultrasound by a cell disrupter. The suspension was centrifuged at 16,000g for 10 minutes at 4°C. The supernatants were collected and frozen at -80°C and the pellet was discarded.

4.3.4 Inhibition of mast cell degranulation

To inhibit mast cell degranulation, cells were pre-incubated with cromolyn (10µg/ml) for one hour or with a PI3 kinase inhibitor (wortmannin; 100µM) for half an hour prior to stimulation.
After stimulation, the cell suspension was centrifuged at 200g for 3 minutes at room temperature. Supernatants were collected and frozen at -80°C until used for further experiments.

Cell pellets were resuspended in PBS and transferred into Eppendorf tubes. Cells were lysed via ultrasound by a cell disrupter. The suspension was centrifuged at 16,000g for 10 minutes at 4°C. The supernatants were collected and frozen at -80°C, the pellet was discarded

4.3.5 Determining the β-hexosaminidase activity in mast cell supernatants

Following degranulation with ionomycin (1µM), the mast cell suspension was centrifuged and supernatants containing mast cell mediators were gathered (Abel et al., 2011; McCall-Culbreath et al., 2011). Cell pellets were lysed via the use of ultrasound. Supernatants and cell lysates were used for the β-hexosaminidase-assay to determine the amount of degranulation.

β-hexosaminidase-release is an indicator of mast cell degranulation as this enzyme is a component of mast cell granules (Kempuraj et al., 2010).

Supernatants (25µl) and lysates (2.5µl) were incubated with the substrate solution pNAG (50µl) for 90 minutes; the reaction was terminated by adding 0.2M NaOH-Glycine (150µl) buffer (McCall-Culbreath et al., 2011).

Absorbance was quantified in the ELISA reader at 405nm wavelength (Frank, 2010; Kempuraj et al., 2010). Measurement of the enzyme activity was based on the conversion of the 4-Nitrophenyl-2-acetamido-2deoxy-β-D-glucopyranosid (pNAG), which was contained in the substrate solution. The conversion was indicated by a yellow colour.

The degree of degranulation was determined as percentage β-hexosaminidase released into the culture supernatant (Abel et al., 2011) calculated by the following formula:

$$\text{Release [\%]} = (\text{test} - \text{spontaneous}) / (\text{total} - \text{spontaneous}) \times 100$$

Test represented the supernatants derived from mast cells treated with ionomycin (1µM), spontaneous corresponded to the mast cell supernatants treated with saline (negative control). Total was calculated from the amount of test plus spontaneous degranulation. Supernatants with a minimum of 70% degranulation in comparison to the control were used for further experiments.

Experiments were measured in triplets, each experiment was conducted 3 times (n=3).

pNAG Buffer (2mM)	
pNAG	100mg
Na-Citrate Buffer	146ml

Glycine-NAOH Buffer (0.2M)	
Glycine	0.2M
NaOH	0.2M
pH = 10.7	

Na-Citrate-Buffer (0.04M)	
Trisodium Citrate	2.94g
ddH_2O	250mL
pH = 4.7	

4.3.6 RNA isolation from mast cell supernatants

RNA was isolated from mast cell supernatants using the MasterPure RNA purification kit. Initially, 0.1ml cell supernatant was lysed in tissue and cell lysis solution, containing proteinase K. RNA was isolated according to the manufacturer's procedure. Shortly:

The probe was incubated at 65°C for 15 minutes; nucleic acids were denatured by addition of protein precipitation reagent and centrifugation with isopropanol. The last RNA pellet was washed twice with 75% ethanol and dried for 30 minutes. For further use the RNA pellet was taken up in RNase free water.

Quantification was achieved after adding a fluorescent dye specific for RNA and fluorometric measurements. RNA was measured in comparison to a 0ng and 10ng standard. Total RNA content in the supernatants was calculated using the following formula:

RNA concentration (probe) = RNA concentration (buffer) × (1000µl/Volume supernatant)

RNA was also isolated in this manner in the presence of RNase inhibitor (80U/ml) in order to detect differences between the two methods.

Experiments were measured in triplets, each experiment was conducted 3 times (n=3).

4.3.7 RNA isolation from cell lysates and photometric quantification

RNA isolation from smooth muscle cells and endothelial cells was achieved via the PeqGOLD Total RNA kit.

Cells were lysed and homogenised by a lysis buffer (500µl). Cell debris and DNA were removed through centrifugation on a DNA removing column (1 minute at 12,000g). Equal amounts of ethanol (70%) were added to the filtrate and mixed thoroughly; the solution was centrifuged in a PerfectBind RNA column to bind total RNA.

Up to 750µl of the filtrate were transferred to the PerfectBind RNA column and the tubes were centrifuged for 1 minute at 10,000g. 500µl of the wash buffer 1 were added to the column and spun for another 15 seconds at 10,000g. The filtrate was discarded and the column was transferred to a new collection tube. 600µl wash solution 2 were added to the column and spun for another 15 seconds at 10,000g. This step was repeated and the column spun for another 2 minutes to dry the column.

50-100µl RNase free water were added directly to the matrix and incubated for three minutes at room temperature. After spinning for 1 minute at 5,000g, the purified RNA could be eluted and used for further experiments, such as quantitative real-time polymerase chain reaction (qRT-PCR).

Experiments were measured in triplets, each experiment was conducted 3 times (n=3).

4.3.8 Determination of RNase activity in mast cell supernatants

RNase activity was measured in mast cell supernatants in comparison to a sample containing sterile water (negative control), and another sample containing 1µl RNase 1 (positive control) to determine the amount of RNase activity in the supernatants.

The sample (0.1ml) was incubated with Poly C (1mg/ml; 0.1ml) for 5 minutes at 37°C. Half of the probe (0.1ml) was transferred to another Eppendorff tube containing perchloric acid 6% with Lanthanum chloride (20mM; 0.25ml) and bovine serum albumin (fBSA) (10mg/ml; 0.1ml). The probe was vortexed thoroughly, incubated on ice for 15 minutes and centrifuged for another 15 minutes at 16000 g and 4°C.

The probe (0.1ml) was measured in a photometer at 260nm in comparison to a reference consisting of recombinant RNase1.

RNase buffer stock solution	
ddH_2O	200ml
Tris	121.4g/mol
NaCl	58.44g/mol
EDTA	372g/mol
pH = 8	

RNase buffer working solution	
RNase buffer stock	9.9ml
Acetyl. BSA (10mg/ml)	100µl

Lanthanum chloride + Perchloric acid	
Sterile ddH_2O	30ml
Lanthanum chloride	0.345g
Perchloric acid 70%	4.325ml

4.3.9 cDNA-synthesis

Complementary DNA (cDNA) is a DNA copy of an RNA template. cDNA-synthesis was performed via the use of the high-capacity cDNA reverse transcription kit. The enzyme reverse transcriptase was used to synthesise cDNA from RNA. Short random primers allow for binding of the enzyme to the RNA. Addition of RNase inhibitor protects the RNA from being degraded by ribonucleases during the reaction. In all reactions, the same amount of RNA (100ng) was used to be able to compare expression rates of the RNA of interest.

The mastermix (10µl) was added to the purified RNA (10µl). The sample was cycled in T3000 Cycler for 10 minutes at 25°C and for another 60 minutes at 37°C to reverse transcribe the cDNA. The cDNA was frozen at -20°C until further processing.

Master mix	
10×buffer	2µl
Random primer	1µl
dNTP	0.8µl
RNase inhibitor (40U/ml)	0.5µl
Reverse Transcriptase	0.5µl
H_2O	5.2µl
Final volume: 10µl	

4.3.10 Quantitative real-time Polymerase Chain Reaction (qRT-PCR)

In this present study qRT-PCR was used in order to detect the up- or down regulation of particular genes in endothelial- or smooth muscle cells in response to treatment with supernatants gained from mast cells stimulated with ionomycin.

In the presence of the intercalating dye *SYBR-green*® the amplification of nucleic acid sequences can be measured. *SYBR-green*® attaches to the newly formed DNA fragment and can be detected by fluorescence measurements. The fluorescence signal is hereby proportional to the amount of newly synthesised PCR product.

For quantification of different targets such as thrombomodulin (TBHBD), monocytes chemoattractant protein (MCP)-1 and platelet derived growth factor (PDGF)-BB, the generated cDNA (2-4µl) was diluted 1:5 with H_2O.

For amplification LightCycler FastStart DNA Master Plus set SYBR Green I was used which included the polymerase, deoxynucleotides (dNTPs) as well as the intercalating dye *SYBR-green*®.

The primer master mix and the pRT-PCR master mix were prepared as described in the following tables.

Primer master mix for qRT-PCR	
H_2O	180 µl
Forward primer (100 µM)	10 µl (end concentration 5 µM)
Reverse primer (100 µM)	10 µl (end concentration 5 µM)

qRT-PCR master mix	
Master SYBR green	2 µl × (number of samples + 10%)
Primer mix (forward + reverse) 5 µM	500 nM
H_2O	3- 5µl
= 8 µl/ sample	

A 96-well plate was put on ice; the qRT-PCR mastermix (8µl) was added. The diluted cDNA (2-4µl) was added to get a final volume of 10µl per well, ensuring to avoid bubbles when inserting the solutions into the wells.

The samples were inserted into a PCR machine; the reaction conditions for all amplified genes are demonstrated in the following table; the 64°C cycle was only run for MCP-1.

Quantitative real-time PCR conditions		
10 minutes	95°C	Denaturation
40 cycles		
15 seconds	95°C	Denaturation
1 minute	64°C (MCP-1)	Annealing
15 seconds	72°C	Elongation

Primers for the qRT-PCR are listed in the table below.

	Sequence (5'-3')	**Annealing-Temperature (°C)**	**Efficiency (%)**
MCP-1 forward	5`-CTCAAGAGAGAGGTCTGTGCTG-3`	64	97.4
MCP-1 reverse	5`-GTAGTGGATGCATTAGCTTCAG-3`	64	97.4
THBD forward	5`-TACTGGCGATAACCACACCA-3`	64	95.3
THBD reverse	5`-CAGCTTCAGTTGCTGTCGAG-3`	64	95.3
PDGF-BB forward	5`- ACCACTCCATCCGCTCCTT -3`	62	100
PDGF-BB reverse	5`-TGTGCTCGGGTCATGTTCAA -3`	62	100

For the analysis, the ΔΔCt-method with efficiency correction by Peirson was used (S. N. 2003).

4.4 Statistical analysis

Data analysis was performed by using Excel 2007 (Microsoft).
Each *in vitro* experiment was performed at least three times on independent occasions, within each experiment, samples were processed in triplicate. For *in vivo* experiments each experimental- and control group contained 5 animals which were held under identical conditions. Results for both *in vivo* and *in vitro* studies were expressed as means ± SEM. Experimental groups were compared with controls using the Student´s *t*-test. $P < 0.05$ was considered statistically significant.

5.0 Results

5.1 Characterisation of the cell types

5.1.1 Characterisation of mast cells by flow cytometry

Purity of mast cells was confirmed by FACS analysis.

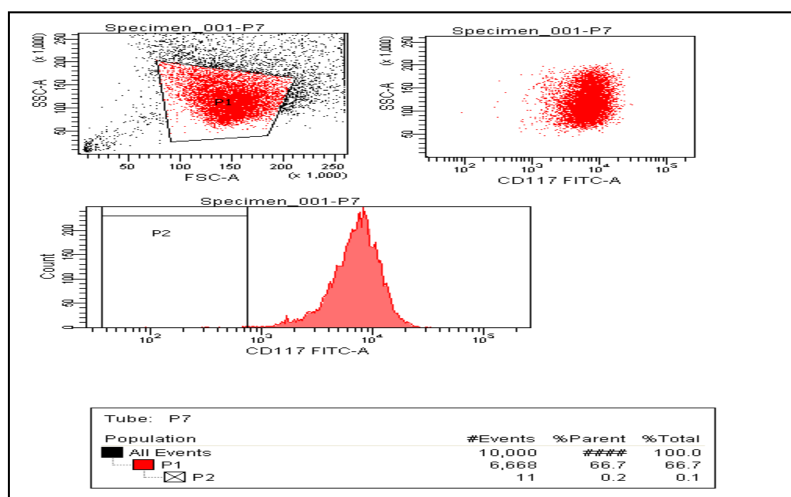


Figure 12: FACS analysis of SV129 background wt bone marrow derived mast cells (BMMCs). BMMCs isolated from SV129 background wt mice were analysed for purity of the isolated cell colony via FACS analysis with a mast cell specific FITC labelled antibody (CD117). The samples showed a purity of nearly 100%.

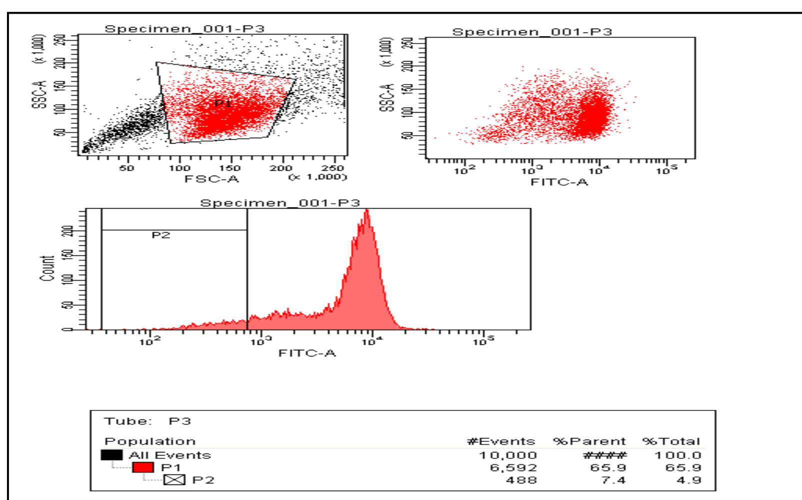


Figure 13: FACS analysis of SV129 background PI3Kγ -/- bone marrow derived mast cells (BMMCs). BMMCs from SV129 background PI3Kγ -/- mice were analysed for purity of the isolated cell colony via FACS analysis with a mast cell specific FITC labelled antibody (CD117). The samples showed a purity of nearly 100%

5.1.2 Characterisation of smooth muscle cells

Purity of MVSMCs was confirmed by staining for α-smooth-muscle actin, a typical protein of smooth muscle cells. Further staining for endothelial specific proteins for CD31 were negative demonstrating the purity of these cell cultures.

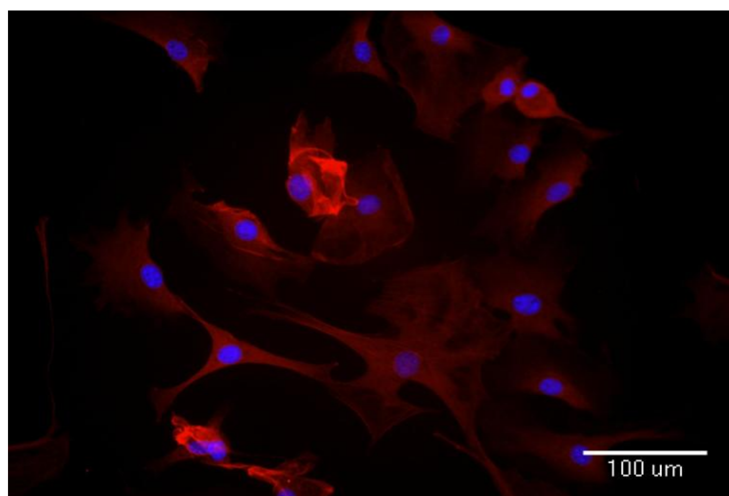


Figure 14: MVSMCs labelled with anti-α-smooth muscle actin. Blue represents cell nuclei and red the cell bodies of smooth muscle cells.

5.2 Involvement of PI3Kγ-dependent mast cell degranulation in arteriogenesis

Previous studies have shown that treatment of mice with cromolyn, an inhibitor mast cell degranulation, diminished arteriogenesis *in vivo* significantly (Chillo, unpublished data). This suggested an involvement of mast cells in the process of arteriogenesis; hence mast cell degranulation should improve vessel growth. This hypothesis became the background for this present study. Injection of a mast cell stimulating agent (C48/80) following femoral artery ligation (fal) was used to show an increased effectiveness of arteriogenesis *in vivo* following mast cell degranulation. When mast cells were involved in this process, mice should recover from fal faster compared to controls treated with saline. To investigate whether arteriogenesis is dependent on mast cell degranulation, in particular on a PI3Kγ-dependent mast cell degranulation, PI3Kγ deficient mice as well as wt mice were subjected to femoral artery ligation. In comparison uninfluenced arteriogenesis as well as arteriogenesis following mast cell degranulation with C48/80 were investigated. For reasons of control both types of mice were treated with 0.9% NaCl.

In the following first experiment, wild type (wt) mice were subjected to femoral artery ligation and were treated with C48/80 over 21 days. Hind limb perfusion was monitored via LDI. The results of this experiment are expressed in Figure 15.

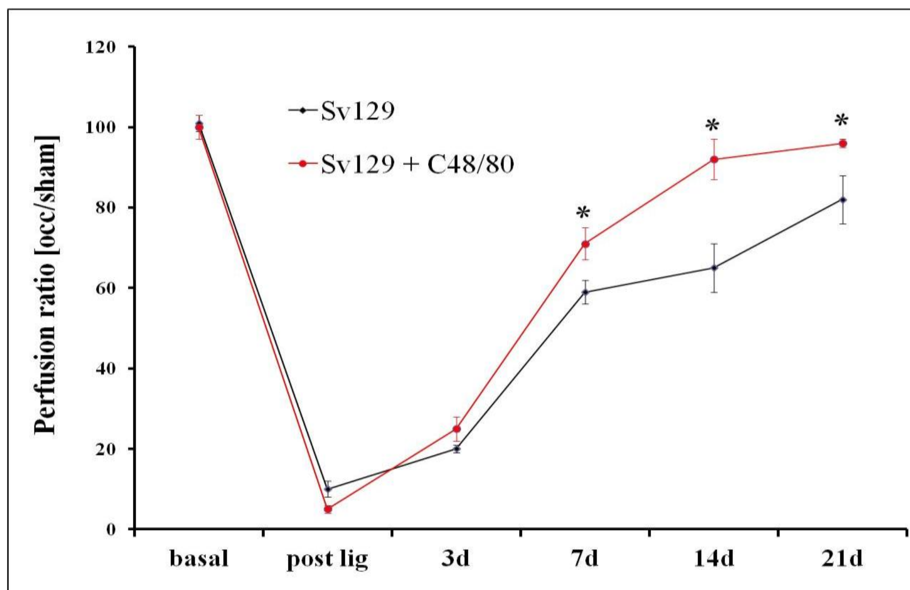


Figure 15: The involvement mast cell degranulation in arteriogenesis. SV129 wt mice were subjected to femoral artery ligation (fal) and perfusion was measured by LDI before, directly after and at days 3, 7, 14 and 21 after surgery. In this diagram, the perfusion ratio occluded (right leg) versus sham operated (left leg) (%blood flow) in animals before (basal) and after surgery (post lig), at day 3, 7, 14 and 21, is expressed. Animals were treated with C48/80 (red line) or NaCl (control; black line) in order to identify an involvement of mast cells in arteriogenesis. Each group contained 5 animals (n = 5), each value represents the mean ± SEM. Values of $p \leq 0.05$ were regarded as statistically significant.

Treatment with C48/80 improved hind limb perfusion in wt mice significantly compared to the control group, treated with NaCl in an equal manner (day3, R/L: 0.25±0.03 vs. 0.20±0.01, C48/80 vs. saline; day 7, R/L: 0.71±0.04 vs. 0.59±0.03, C48/80 vs. saline; day 14, R/L: 0.92±0.05 vs. 0.65±0.06, C48/80 vs. saline; day 21, R/L: 0.96±0.01 vs. 0.82±0.05, C48/80 vs. saline).

Together with the knowledge of the diminishing effects of treatment with cromolyn on arteriogenesis, this finding indicated an involvement of mast cells in arteriogenesis.

To further investigate whether arteriogenesis is dependent on mast cell degranulation, in particular on a PI3Kγ-dependent mast cell degranulation, PI3Kγ deficient mice as well as wt mice were subjected to femoral artery ligation. In comparison uninfluenced arteriogenesis was investigated. Hind limb perfusion was monitored via LDI.

As known from the literature, PI3Kγ -/- mast cells are less responsive to stimulation and release fewer granules than wild type cells (Laffargue et al., 2002). If mast cell degranulation had an impact on arteriogenesis, the mice lacking PI3Kγ should perform worse in arteriogenesis than their wt littermates.

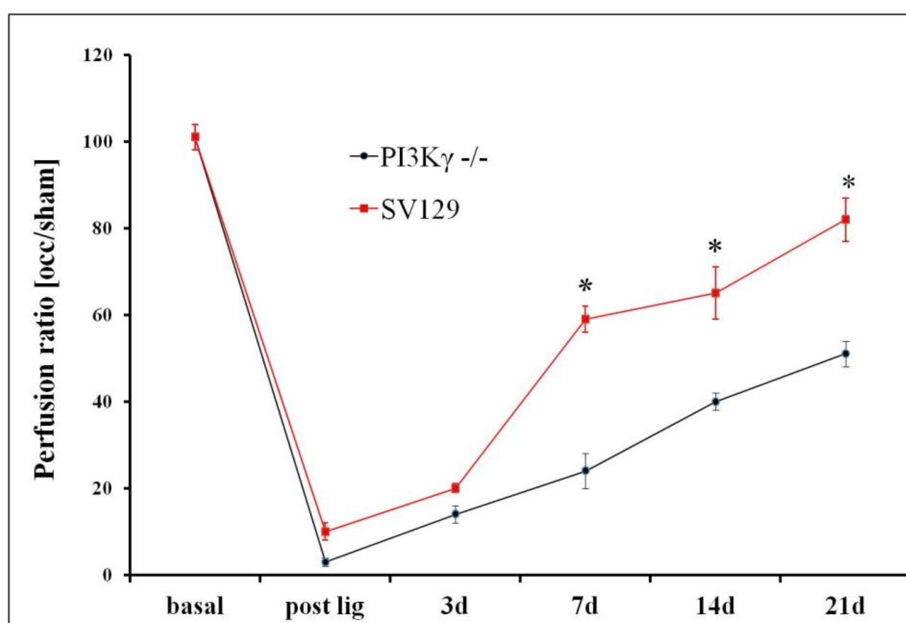


Figure 16: The involvement of PI3Kγ in arteriogenesis. Wt and knockout mice were subjected to femoral artery ligation (fal) and perfusion was measured by LDI before, directly after and at days 3, 7, 14 and 21 after surgery. In this diagram, the perfusion ratio occluded (right leg) versus sham operated (left leg) (%blood flow) in animals before (basal) and after surgery (post lig), at day 3, 7, 14 and 21, is expressed. Uninfluenced arteriogenesis is shown in order to express a dependency of arteriogenesis on PI3Kγ. Each group contained 5 animals (n = 5), each value represents the mean ± SEM. Values of $p \leq 0.05$ were regarded as statistically significant.

Indeed PI3Kγ -/- mice were performing significantly worse in arteriogenesis compared to their wt littermates (day3, R/L: 0.14±0.02 vs. 0.20±0.01, wt vs. k.o.; day 7, R/L: 0.24±0.04 vs. 0.59±0.03, wt vs. k.o.; day 14, R/L: 0.4±0.02 vs. 0.65±0.06, wt vs. k.o.;

day 21, R/L: 0.51±0.03 vs. 0.82±0.05, wt vs. k.o.). They recovered more slowly and even after three weeks, when the circulation of the wild type animals returned to more than 80%, the knockout animals´ perfusion was only about half of that, as demonstrated in Figure 16.

To confirm the relevance of PI3Kγ in mast cell degranulation during arteriogenesis, PI3Kγ -/- mice were subjected to femoral artery ligation and treated with C48/80 over 21 days. Hind limb perfusion was monitored via LDI

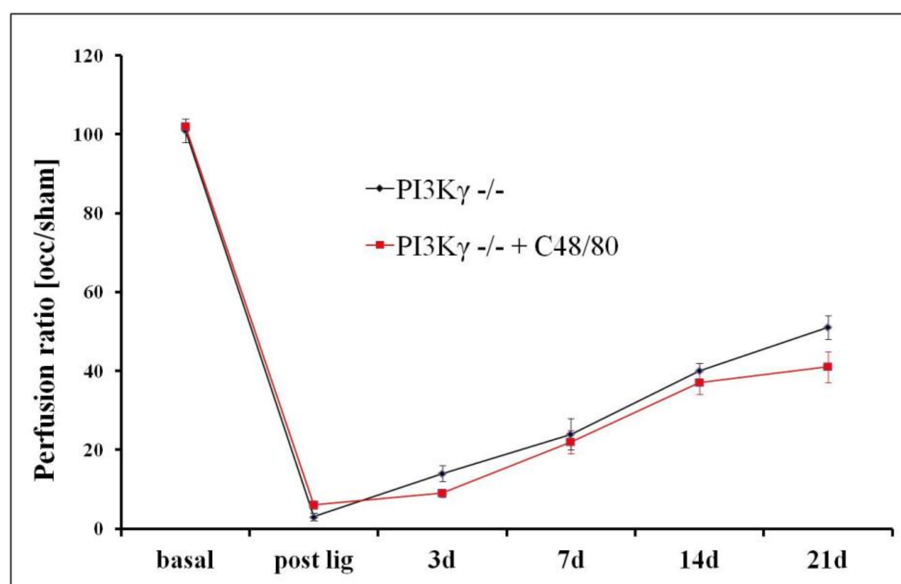


Figure 17: Arteriogenesis is dependent on PI3Kγ-mediated mast cell degranulation. PI3Kγ -/- mice were subjected to femoral artery ligation (fal) and perfusion was measured by LDI before, directly after and at days 3, 7, 14 and 21 after surgery. In this diagram, the perfusion ratio occluded (right leg) vs. sham operated (left leg) (%blood flow) in animals before (basal) and after surgery (post lig), at day 3, 7, 14 and 21, is expressed. Animals were treated with C48/80 (red line) or NaCl (control; black line) to further investigate the involvement of PI3Kγ in arteriogenesis. Each group contained 5 animals (n = 5), each value represents the mean ± SEM.

In fact, the results shown in Figure 17 expressed that there was no benefit for the outcome of arteriogenesis in PI3Kγ -/- mice when treated with C48/80, indicating that mast cell degranulation during arteriogenesis is dependent on PI3Kγ (day3, R/L: 0.09±0.01 vs. 0.14±0.02, C48/80 vs. saline; day 7, R/L: 0.22±0.03 vs. 0.24±0.04, C48/80 vs. saline; day 14, R/L: 0.37±0.03 vs. 0.4±0.02, C48/80 vs. saline; day 21, R/L: 0.41±0.04 vs. 0.51±0.03, C48/80 vs. saline).

5.3 The effect of Leukotriene synthesis inhibition on arteriogenesis

After showing the involvement of mast cells in arteriogenesis, a specific type of mast cell mediator primarily influencing arteriogenesis should be identified. In this context leukotrienes were discussed as they are known to be synthesised by mast cells upon stimulation.

SV129 wt mice were subjected to femoral artery ligation and treated with the leukotriene synthesis inhibitor MK-886. The control group was treated with 0.9% NaCl. Hind limb perfusion was monitored via LDI.

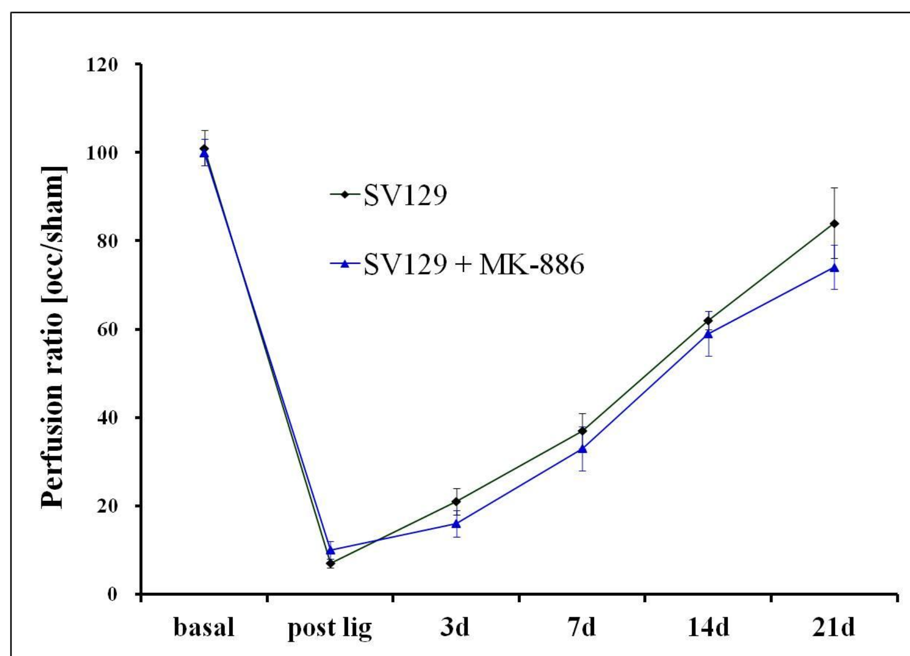


Figure 18: **The effect of leukotriene inhibition by MK-886 on arteriogenesis.** SV129 wt mice were subjected to femoral artery ligation (fal) and perfusion was measured by LDI before, directly after and at days 3, 7, 14 and 21 after surgery. In this diagram, the perfusion ratio occluded (right leg) vs. sham operated (left leg) (%blood flow) in animals before (basal) and after surgery (post lig), at day 3, 7, 14 and 21, is expressed. Animals were treated with MK-886 (blue line) or NaCl (control; black line) in order to identify an involvement of leukotrienes in arteriogenesis. Each group contained 5 animals (n = 5), each value represents the mean ± SEM.

Figure 18 outlines that leukotriene synthesis inhibition resulted in a slight and non significant reduction in perfusion recovery, indicating that leukotrienes play a minor role in arteriogenesis (day3, R/L: 0.16±0.03 vs. 0.21±0.03, MK-886 vs. saline; day 7, R/L: 0.33±0.05 vs. 0.37±0.04, MK-886 vs. saline; day 14, R/L: 0.59±0.05 vs. 0.62±0.02, MK-886 vs. saline; day 21, R/L: 0.74±0.05 vs. 0.84±0.08, MK-886 vs. saline).

5.4 Mast cell stimulation or inhibition *in vitro* in dependence of PI3Kγ

As an involvement of mast cells in arteriogenesis was proven *in vivo*, these findings now needed to be confirmed *in vitro*.

5.4.1 Determination of most efficient mast cell degranulation

In vivo, C48/80 was used for induction of mast cell degranulation. *In vitro*, C48/80 did not induce a stimulus strong enough to detect a significant difference between treatment and control group. Therefore, the potency of ionomycin was analysed and compared to that of C48/80. *In vitro*, ionomycin induced a much stronger stimulus for mast cell degranulation than C48/80.

Further investigations hinting towards other mast cell stimulating agents such as phorbol-12-myriate-13-acetate (PMA) and RNA, exceeding the potency of ionomycin in mast cell degranulation, did not induce comparable effects, expressed in Figure 19 below.

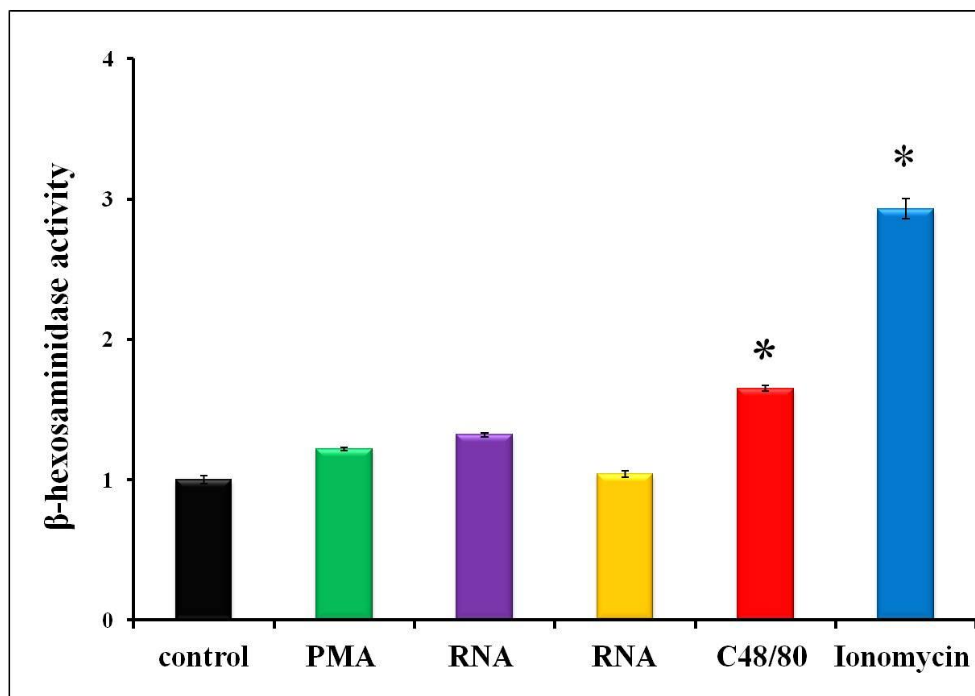


Figure 19: Mast cell degranulation induced by different stimuli. Cells were incubated with NaCl (control, black bar), C48/80 (10µg/ml, red bar), PMA (1µM, green bar) and RNA (25µg/ml: purple bar and 50µg/ml: yellow bar), for one hour. Mast cell degranulation is expressed as % β-hexosaminidase activity and a control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $P \leq 0.05$ was regarded as statistically significant.

Ionomycin was used for the following *in vitro* experiments to induce degranulation of mast cells.

5.4.2 *Dose dependency of mast cell degranulation induced by ionomycin*

Having identified the most efficient mast cell simulating agent, the most efficient ionomycin concentration was investigated.

The initial ionomycin concentration (1µM) was compared to a higher concentration (10µM) in order to investigate whether an increase in efficiency of mast cell degranulation was possible. The result of this experiment is shown in Figure 20 below.

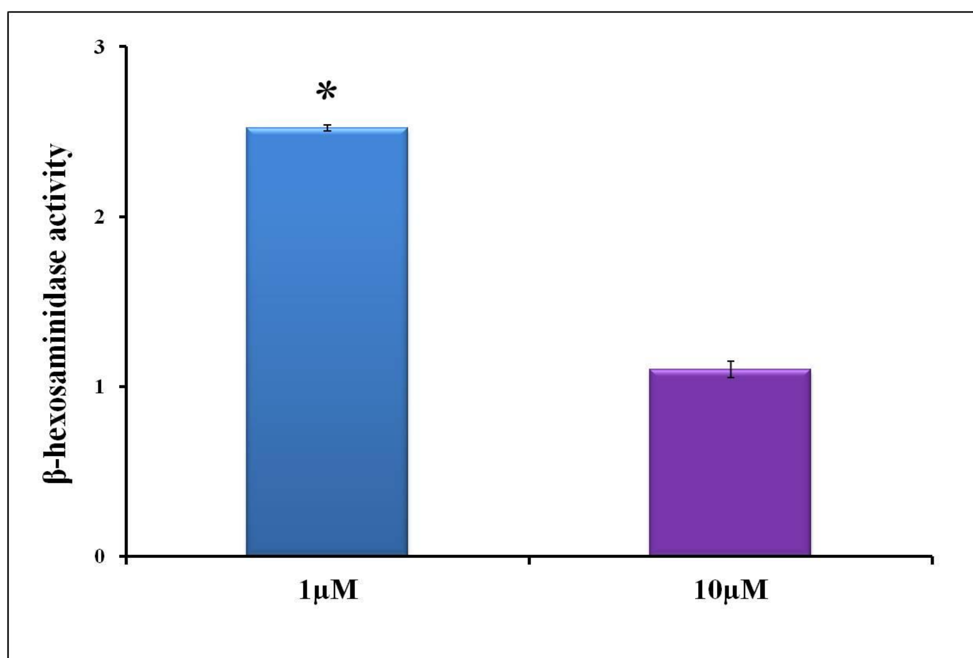


Figure 20: Concentration dependency of mast cell degranulation induced by ionomycin. Cells were treated with ionomycin (1µM and 10 µM) for one hour. Mast cell degranulation is expressed as % β-hexosaminidase activity and a control was set to 1.Experiments were measured in triplicate and each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $P \leq 0.05$ was regarded as statistically significant.

A higher ionomycin concentration did not improve mast cell degranulation in comparison to the initial dose (1 µM), it even showed diminishing effects.

5.4.3 Determination of the most appropriate incubation time

Next, incubation times were varied. Previously, cells were stimulated for one hour; however a longer stimulation might increase the degree of degranulation even further. The result of this experiment is expressed in Figure 21.

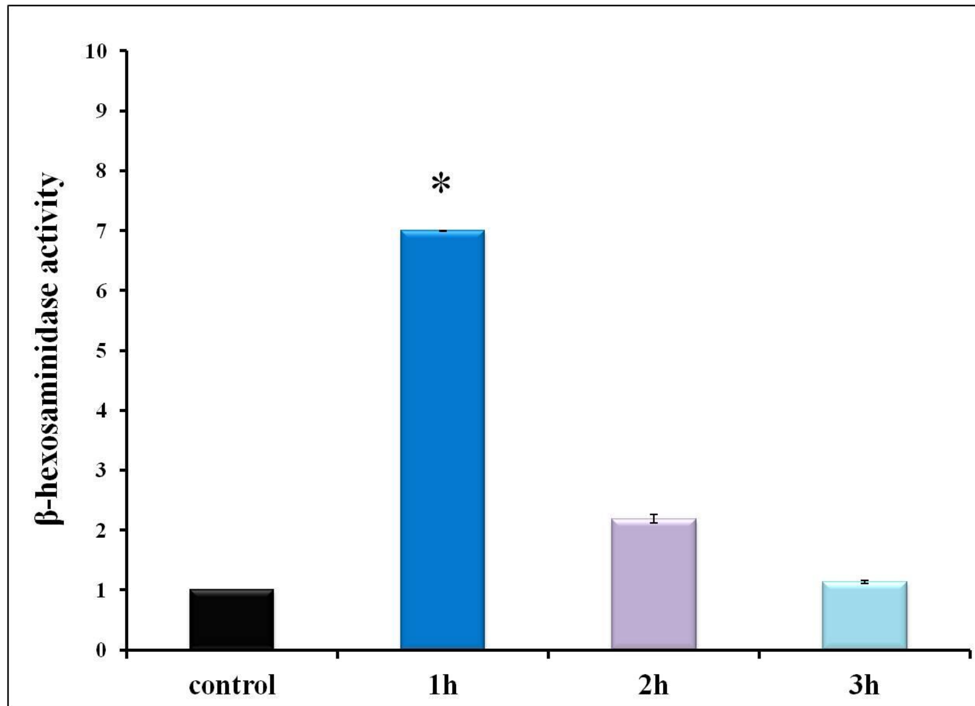


Figure 21: Time dependency of mast cell degranulation induced by ionomycin. Mast cells were incubated with ionomycin (1µM) for 1, 2 and 3 hours. Mast cell degranulation is expressed as % β-hexosaminidase activity and a control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times ($n = 3$). Each value represents the mean ± SEM. $P \leq 0.05$ was regarded as statistically significant.

A further improvement of mast cell degranulation could not be achieved by prolonged stimulation times. One hour was the most effective time to allow for maximal degranulation.

All following experiments were conducted with 1µM ionomycin and 1 hour stimulation time. Supernatants with a minimum of 70% degranulation were processed in further experiments.

5.4.4 The effect of cromolyn on mast cell degranulation

According to Omary Chillo's findings (Unpublished data, 2011) about the diminishing effects of on arteriogenesis *in vivo,* the inhibiting effects of cromolyn on BMMCs extracted from SV129 background wt mice were also analysed *in vitro*; the result of this experiment can be viewed in Figure 22.

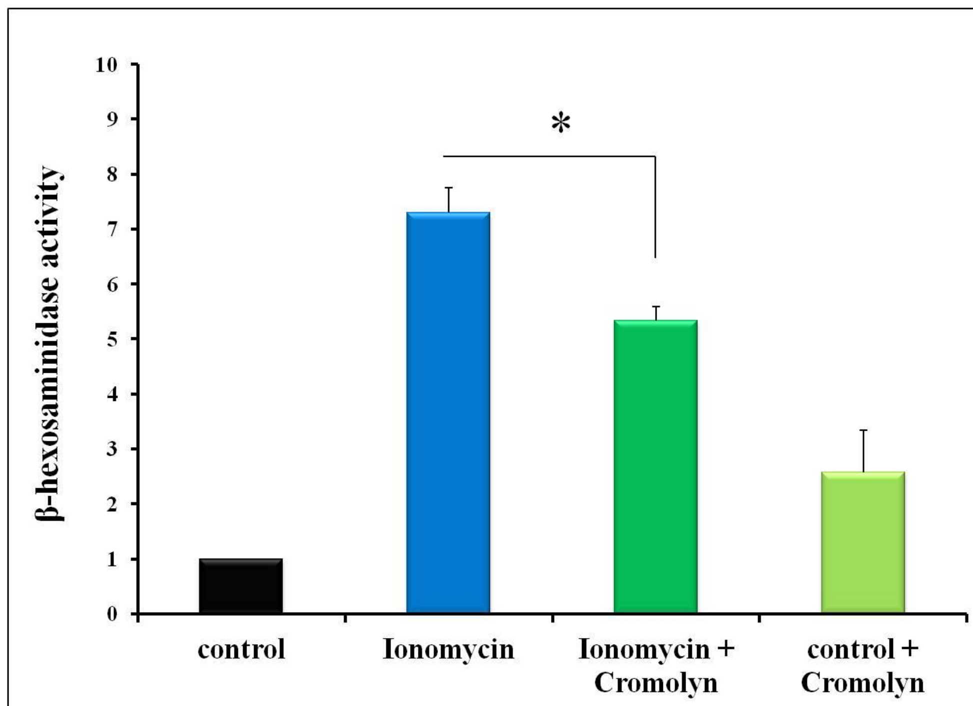


Figure 22: Mast cell degranulation induced by ionomycin in the presence of cromolyn. Cells were pre-treated with cromolyn (10µg/ml) for one hour before treatment with ionomycin (1µM). Mast cell degranulation is expressed as % β-hexosaminidase activity and a control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $P \leq 0.05$ was regarded as statistically significant.

Mast cells pre-treated with cromolyn for one hour prior to stimulation with ionomycin decreased ionomycin-induced degranulation significantly.

5.5 The involvement of the PI3Kγ in the process of mast cell degranulation

As was shown by the *in vivo* studies, the PI3 kinase γ might play a role in arteriogenesis. The difference in the outcome between wt and PI3Kγ -/- (knockout) mice lead to the assumption, that activation of PI3Kγ could be involved in mast cell degranulation.

5.5.1 Involvement of PI3Kγ activation in -/- mast cell degranulation

Mast cell degranulation of BMMCs from wt and PI3Kγ -/- mice was compared. Mast cells derived from SV129 wt and PI3Kγ -/- mice were incubated with ionomycin and C48/80 for one hour in comparison, which is demonstrated in Figure 23.

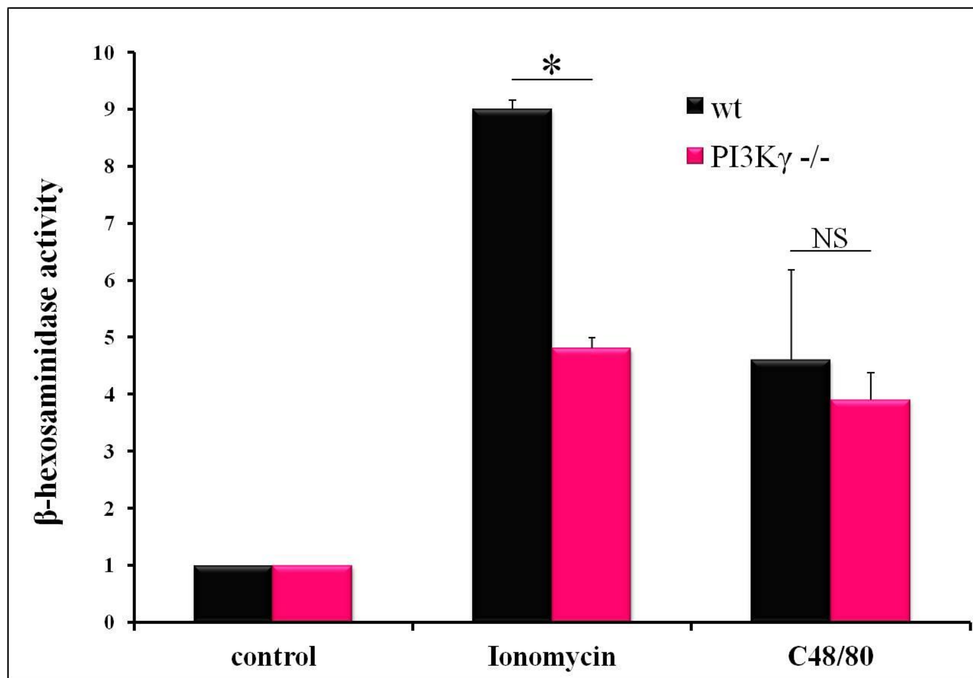


Figure 23: Degranulation of mast cells derived from SV129 wt versus PI3Kγ -/- mice. Mast cells derived from SV129 wt and PI3Kγ -/- mice were incubated with ionomycin (1µM) and C48/80 (10µg/ml) for one hour. Mast cell degranulation is expressed as % β-hexosaminidase activity and a control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $P \leq 0.05$ was regarded as statistically significant.

Mast cells derived from PI3Kγ -/- mice responded poorer to the mast cell degranulating agents compared to those isolated from wt mice. This finding suggested an involvement of PI3Kγ activation in mast cell degranulation.

Furthermore, inhibition of PI3 kinase by wortmannin significantly reduced ionomycin-induced mast cell degranulation, which can be observed in Figure 24.

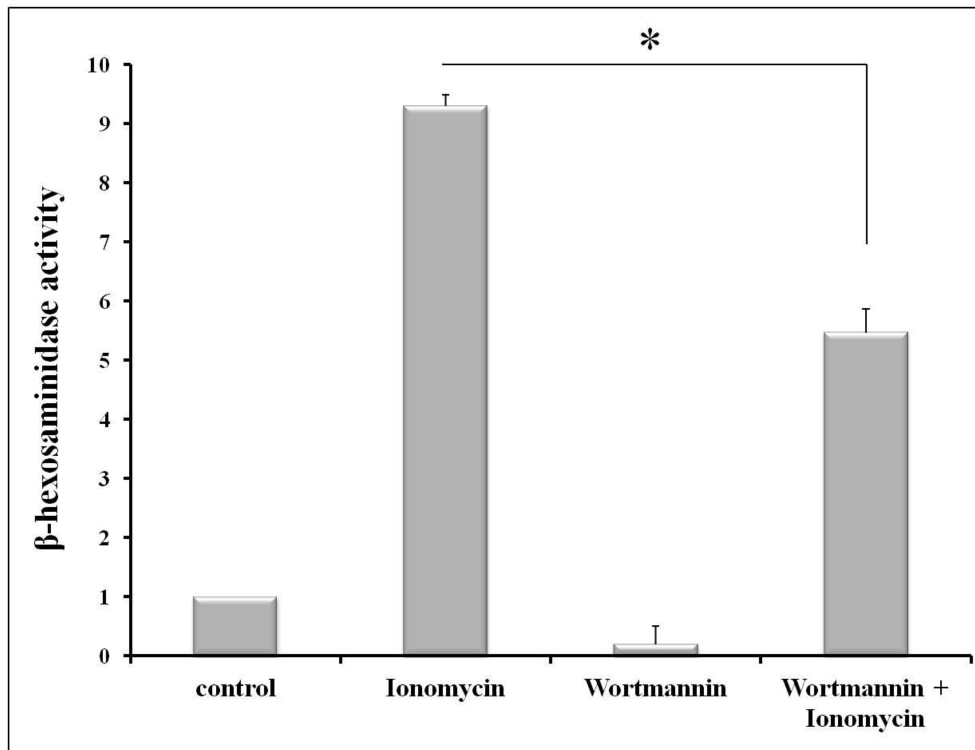


Figure 24: Degranulation of mast cells in the presence of wortmannin. Cells were either untreated (control) or pre-treated with wortmannin (100 µM) for 30 minutes before treatment with ionomycin (1 µM) or NaCl for one hour. Mast cell degranulation is expressed as % β-hexosaminidase activity and a control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

Inhibition of mast cell degranulation with wortmannin diminished mast cell degranulation to half of its actual potency. These findings suggested an involvement of PI3 Kinase in the process of mast cell degranulation.

5.6 Release of nucleic acids from mast cells

5.6.1 RNase activity released from activated mast cells

Mast cells have previously been found to release angiogenin (RNase 5), which is a member of the RNase A superfamily and which is one of the most potent inducers of neovascularisation in experimental models *in vivo* (Kulka, Fukuishi and Metcalfe, 2009). Here the amount of RNase activity in ionomycin-activated mast cell supernatants was measured, expressed in Figure 25.

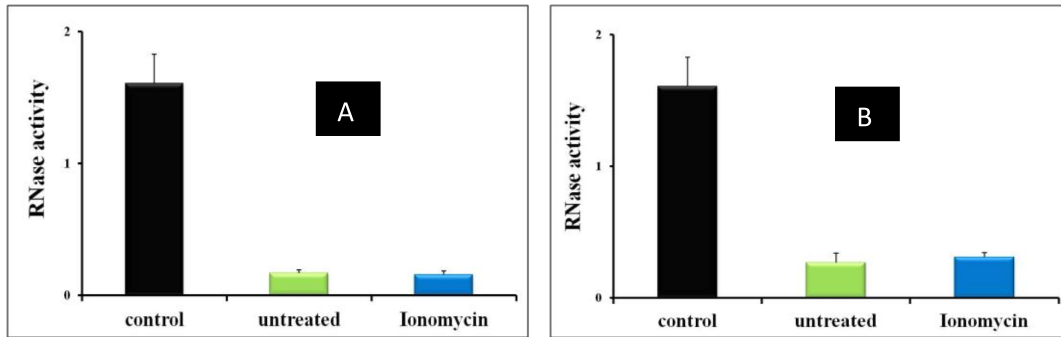


Figure 25: **RNase activity released from mast cells derived from wildtype- (A) and PI3Kγ -/- (B) mice**. Cells were either untreated (control) or treated with ionomycin (1 µM) for one hour, RNase activity in mast cell supernatants is expressed in relation to a positive control (sample containing RNase 1). Experiments were measured in triplicate, each experiment was conducted 3 times (n =3). Each value represents the mean ± SEM.

In this current study no increased RNase activity was found in ionomycin-activated mast cell supernatants derived from wild type and PI3Kγ -/- animals.

5.6.2 RNA-release by activated mast cells

Results obtained by our group in previous studies showed that extracellular RNA released during pathological conditions associated with cell necrosis or tissue damage has pro-coagulatory, permeability-increasing and pro-inflammatory activities (Fischer et al., 2014). As mast cells are one of the most important cells involved in inflammatory processes, we questioned whether mast cells might release extracellular RNA after activation.

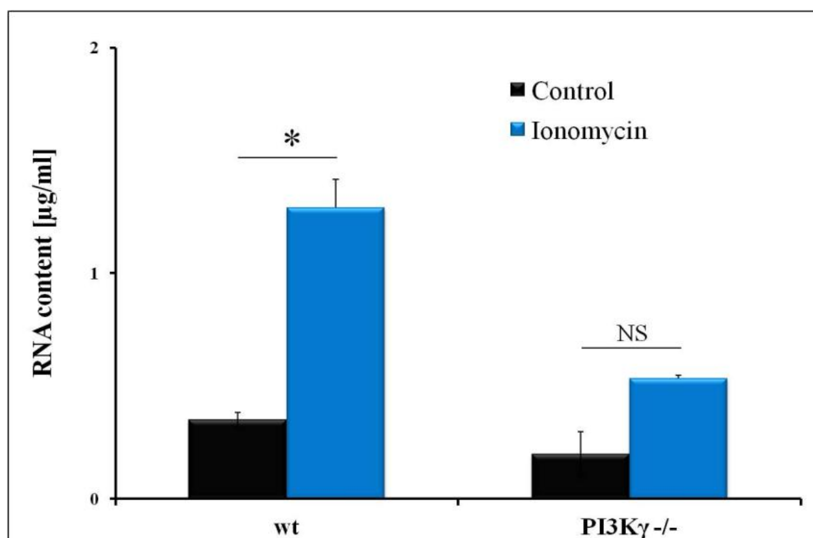


Figure 26: **Release of extracellular RNA from degranulating mast cells.** Mast cells derived from wild type (wt) or PI3K γ -/- mice were either untreated (control) or treated with ionomycin (1 µM) for one hour. RNA-content in supernatants of ionomycin-activated mast cells was determined. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

Supernatants derived from ionomycin-activated wild type mast cells contained higher amounts of RNA than those of ionomycin-activated mast cells derived from PI3K γ -/- mice, demonstrated in Figure 26.
Results suggested that mast cells release RNA upon stimulation, also dependent on PI3Kγ activation. An intriguing question then: “Could the RNA content in the mast cell supernatants be increased by treatment of the mast cell medium with RNAse inhibitor prior to stimulation or by adding RNase inhibitor into the supernatant immediately after mast cell stimulation?” In order to answer this question, RNase inhibitor was added to the mast cells before degranulation and to the supernatants after collection. The result of this experiment is demonstrated in Figure 27.

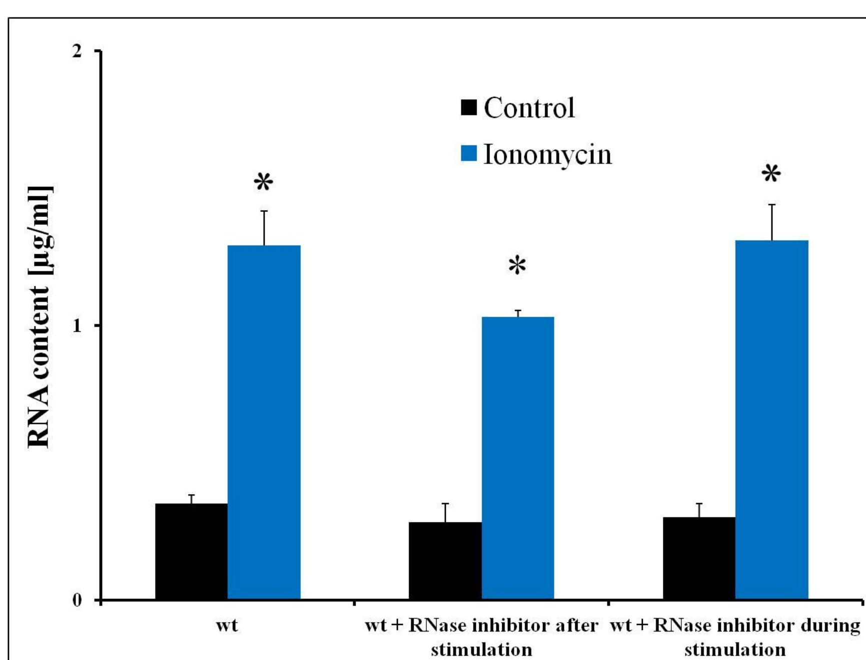


Figure 27: Release of extracellular RNA from degranulating mast cells in the presence of RNase inhibitor. Mast cells derived from wild type (wt) mice were either untreated (control) or treated with ionomycin (1 µM) for one hour in the absence and presence of RNase Inhibitor (RI, 80U/ml). RNA-content in mast cell supernatants was determined. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

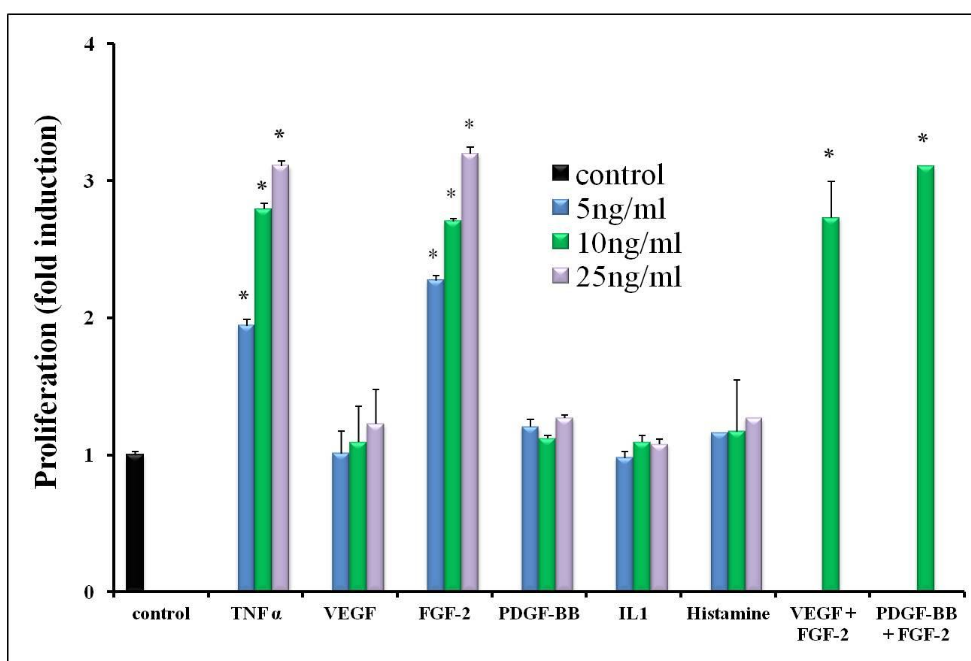
Addition of RNase inhibitor to the medium during mast cell stimulation or the addition of RNase inhibitor to the mast cell supernatants following stimulation, showed no effect on the RNA content measured in the supernatants derived from degranulated mast cells.

5.7 Smooth muscle- and endothelial cell proliferation in response to supernatants derived from activated mast cells

5.7.1 Influence of mast cell mediators on the proliferation of smooth muscle cells

Arteriogenesis is a complex process involving mechanisms of vascular growth (Kampmann et al., 2009). This growth requires the proliferation of endothelial cells and smooth muscle cells (Deindl et al., 2001).

Here, the proliferation rate of MVSMCs was analysed. Cells were treated with different mast cell mediators in order to determine a change in the proliferation rate of smooth muscle cells. The result of this experiment can be viewed in Figure 28.



<u>Figure 28:</u> Smooth muscle cell proliferation in response to different mast cell mediators. Cells were either unstimulated (control) or stimulated with different concentrations of mast cell mediators (TNF-α, VEGF, FGF-2, PDGF-BB, IL-1 and histamine). The proliferation rate of a control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

Different mast cell mediators either enhanced proliferation or showed no effect on the proliferation rate of MVSMCs.

TNF-α and FGF-2 seemed to possess proliferative potential, whereas other mast cell mediators including IL-1 did not have this proliferative ability. A combination of VEGF and PDGF-BB with FGF-2 did not further enhance cell proliferation.

5.7.2 Proliferation of smooth muscle cells in response to supernatants derived from activated peritoneal mast cells

MVSMC proliferation in response to stimulation with supernatants from ionomycin-treated peritoneal mast cells derived from wild type mice (C57/Bl6) was analysed. Cells were treated with different dilutions of the supernatants and proliferation of MVSMCs was analysed. The result of this experiment is demonstrated in Figure 29.

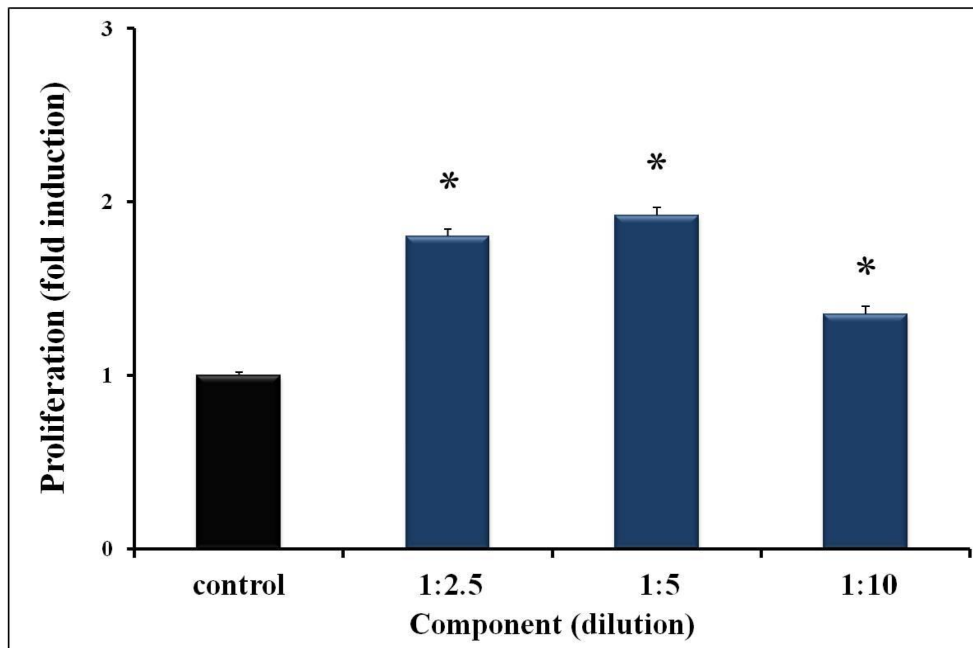


Figure 29: MVSMC proliferation in response to treatment with supernatants from ionomycin-treated peritoneal mast cells. MVSMC were either untreated (control) or treated with supernatants form ionomycin-treated peritoneal mast cells, diluted 1:2,5 (40 µl supernatant : 60 µl medium), 1:5 (20 µl supernatant : 80 µl medium) and 1:10 (10 µl supernatant : 90 µl medium). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

MVSMCs proliferated in response to supernatants derived from ionomycin-treated mast cells in a concentration-dependent manner.

This suggested that mast cell mediators are not only responsible for the improvement of arteriogenesis *in vivo*, but also enhance smooth muscle cell proliferation *in vitro.* In turn, this finding supported the hypothesis that mast cells are crucial cells for arteriogenesis.

5.7.3 MVSMC proliferation in response to supernatants derived from wild type bone marrow derived mast cells

Further proliferation experiments were performed with primary MVSMCs derived from C57/Bl6 mice and primary murine mast cells isolated from the bone marrow of SV129 background wt or PI3Kγ -/- mice.

Wt MVSMCs were treated with different dilutions of supernatants derived from ionomycin-activated mast cells; the result of this experiment can be viewed in Figure 30.

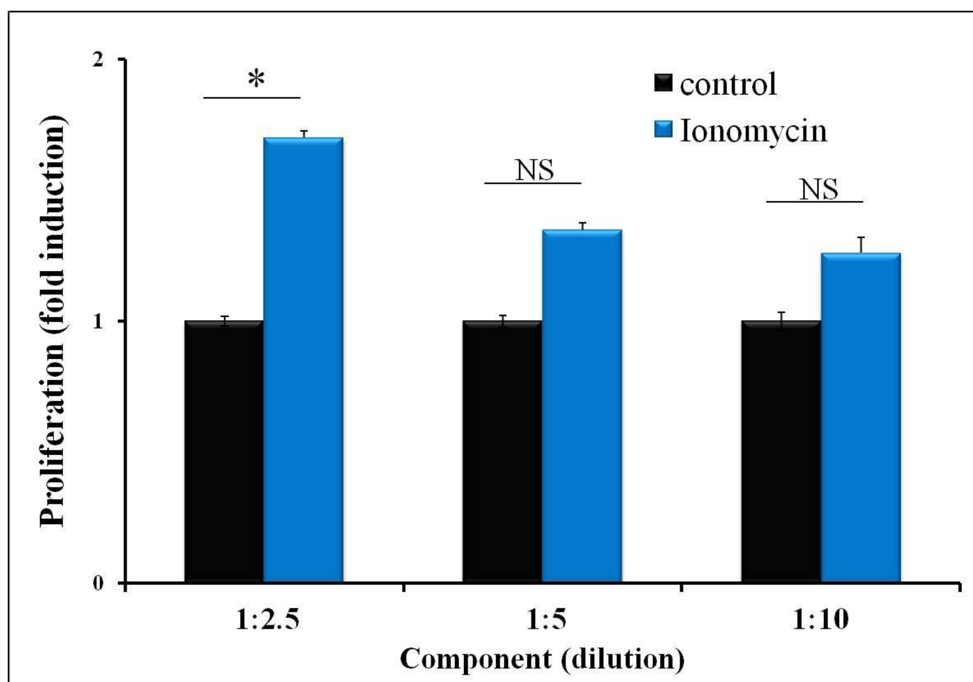
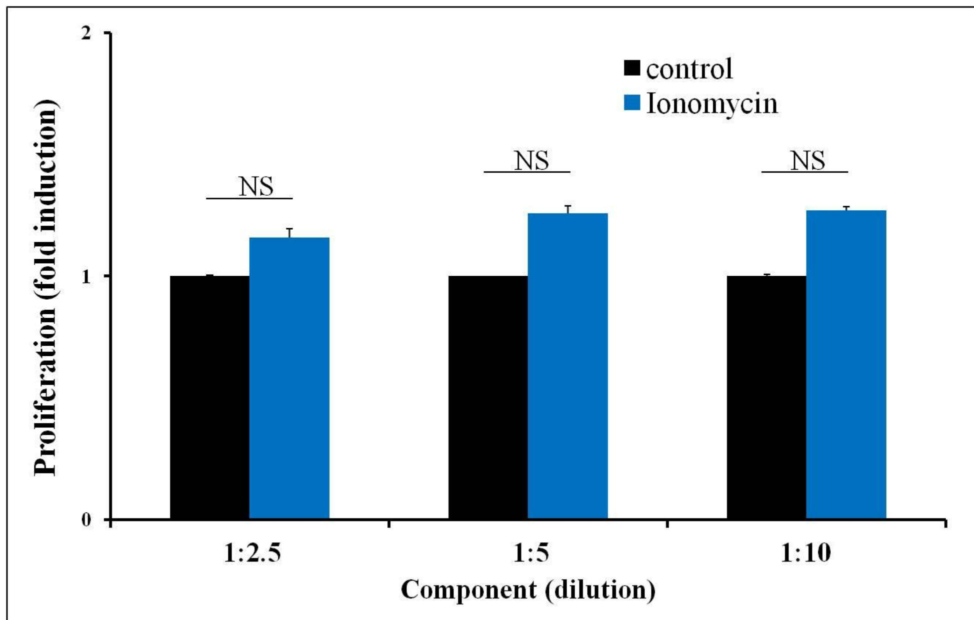


Figure 30: MVSMC proliferation in response to treatments with supernatants from ionomycin-treated wt bone marrow derived mast cells (BMMCs). MVSMCs were either untreated (control) or treated with supernatants form ionomycin-treated BMMCs, diluted 1:2,5 (40 µl supernatant : 60 µl medium), 1:5 (20 µl supernatant : 80 µl medium) and 1:10 (10 µl supernatant : 90 µl medium). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

MVSMCs proliferated in response to supernatants derived from wt ionomycin-treated bone BMMCs in a concentration-dependent manner. This suggested that mast cell mediators are not only responsible for the improvement of arteriogenesis *in vivo*, but also enhance smooth muscle cell proliferation *in vitro.* This finding further supported the hypothesis that mast cells are crucial cells for arteriogenesis.

5.7.4 Proliferation of smooth muscle cells in response to supernatants derived from PI3Kγ -/- mast cells

MVSMCs were treated with different concentrations of supernatants derived from ionomycin-treated mast cells from PI3Kγ -/- mice and the proliferation of MVSMCs was determined, which can be observed in Figure 31.



<u>Figure 31:</u> MVSMC proliferation in response to treatments with supernatants from ionomycin-treated mast cells derived from PI3Kγ -/- mice. MVSMCs were either untreated (control) or treated with supernatants form NaCl-treated (control) or ionomycin-treated mast cells derived from PI3Kγ -/- mice, diluted 1:2,5 (40 µl supernatant : 60 µl medium), 1:5 (20 µl supernatant : 80 µl medium) and 1:10 (10 µl supernatant : 90 µl medium). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM; results were not significant.

Stimulation of MVSMCs with mast cell supernatants derived from PI3Kγ -/- mice did not increase their proliferation rate.

5.7.5 Proliferation of smooth muscle cells in response to supernatants derived from PI3Kγ-inhibited mast cells

MVSMCs were treated with different concentrations of supernatants derived from - in the presence of wortmannin - ionomycin-treated mast cells from wild type mice (C57/Bl6) and the proliferation of MVSMCs was determined. The result of this experiment is shown in Figure 32.

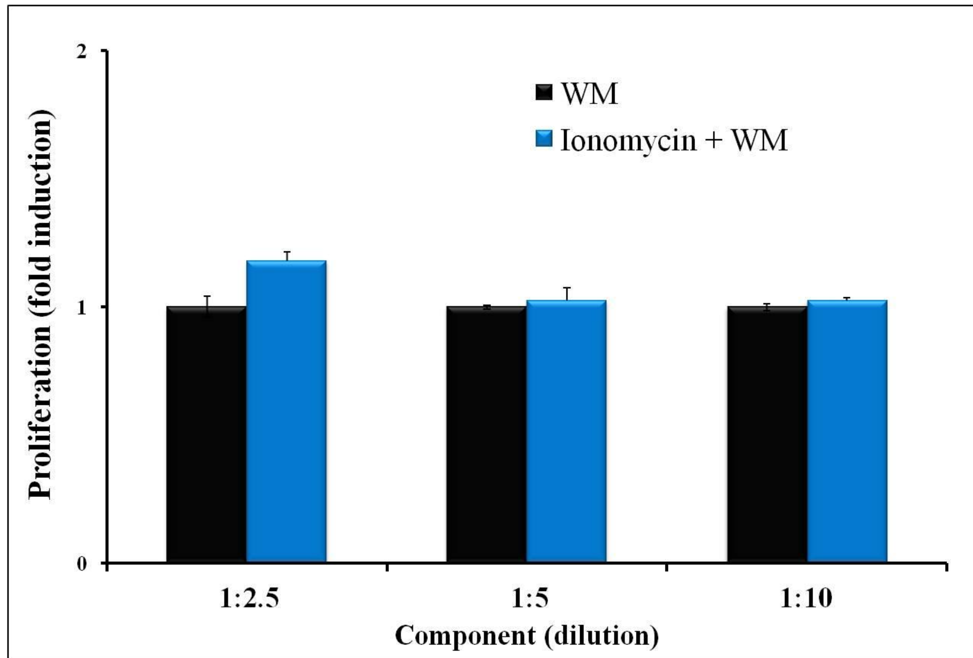


Figure 32: Proliferation rate of MVSMCs in response to treatment with supernatants derived from PI3Kγ-inhibited mast cell supernatants. MVSMCs were stimulated with different concentrations of supernatants from wortmannin inhibited BMMC supernatants, either with ionomycin (1 µM) activated or treated with wortmannin (100µM) alone. Supernatants were diluted 1:2,5 (40 µl supernatant : 60 µl medium), 1:5 (20 µl supernatant : 80 µl medium) and 1:10 (10 µl supernatant : 90 µl medium). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM.

Supernatants derived from mast cells treated with wortmannin prior to degranulation did not enhance MVSMC proliferation at all.

5.8 Effect of mast cell supernatants on endothelial cell proliferation

As previously mentioned, arteriogenesis is a complex process involving mechanisms of vascular growth (Kampmann et al., 2009) which requires the proliferation of endothelial cells (Deindl et al., 2001). Here, the proliferation rate of endothelial cells in response to stimulation with supernatants derived from degranulated mast cells was analysed.

5.8.1 Proliferation of bovine endothelial cells in response to supernatants derived from activated peritoneal mast cells

Primary BAECs were treated with different concentrations of supernatants derived from peritoneal derived mast cells from wt (C57/BL6) mice after treating with ionomycin. This experiment was conducted in order to determine the proliferative ability of these supernatants on endothelial cells. The result of this experiment is shown in Figure 33.

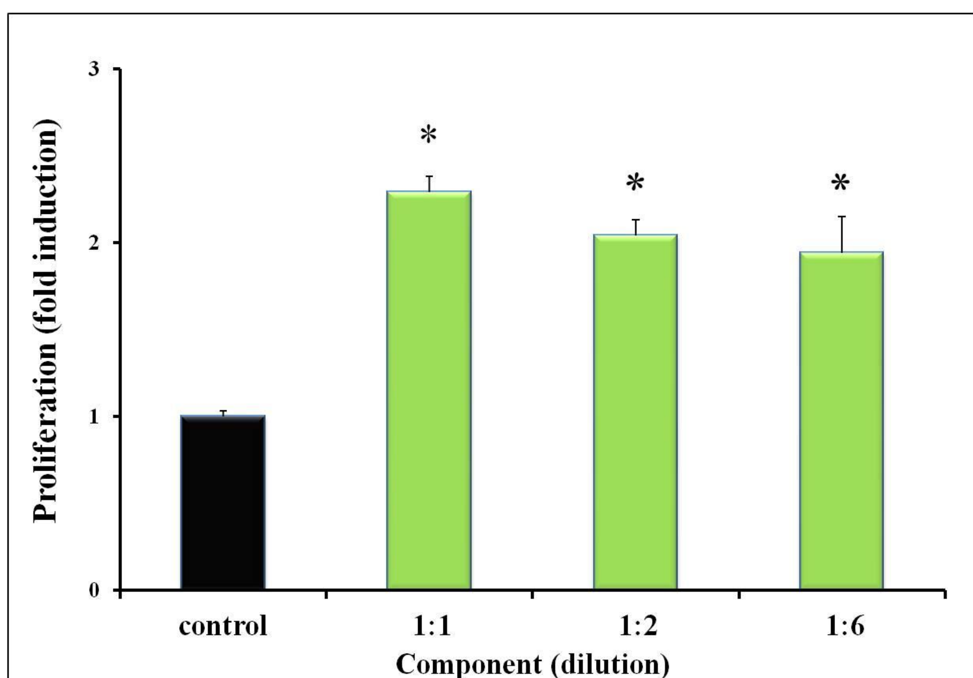


Figure 33: BAEC proliferation in response to treatment with peritoneal derived mast cell supernatants. Primary bovine endothelial cells (BAECs) were stimulated with different concentrations of degranulated murine peritoneal derived mast cell supernatants (green bars) against a control (black bar). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

BAECs showed an increased proliferation rate after treatment with supernatants derived from peritoneal mast cells.

5.8.2 Endothelial cell proliferation in response to supernatants derived from wild type bone marrow mast cells

Previous findings hinted towards the possible positive effects of BMMC supernatants on the proliferation of murine endothelial cells. MCECs were treated with different concentrations of the wt primary BMMC supernatants.

This experiment was conducted in order to determine the proliferative ability of these supernatants on murine endothelial cells, which can be observed in Figure 34.

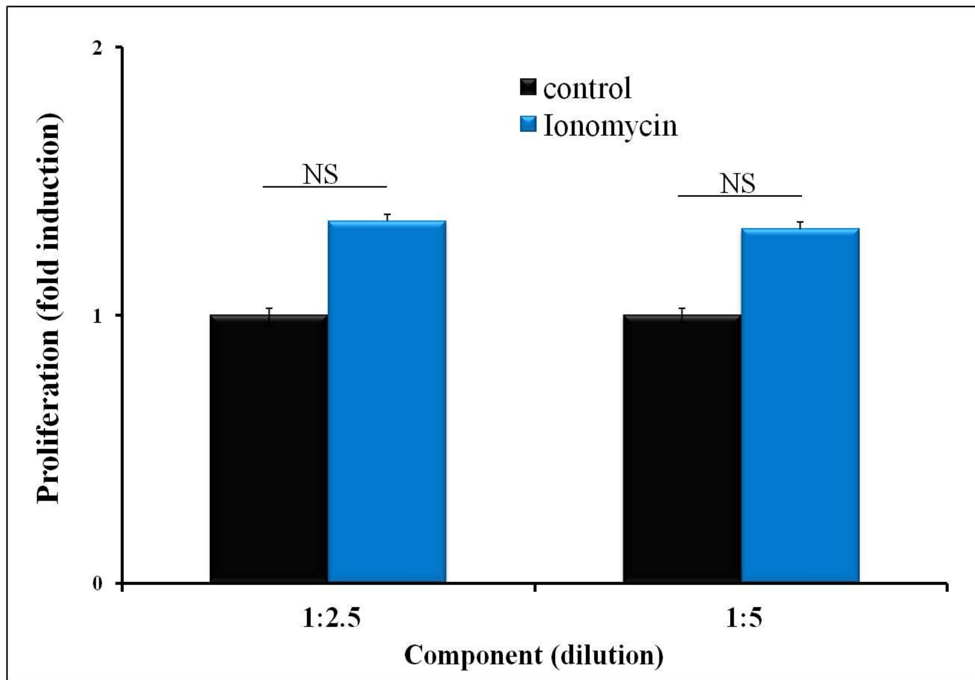


Figure 34: The effect of BMMC supernatants on MCEC proliferation. MCECs were stimulated with different concentrations of mast cell supernatants. The results from previously with ionomycin (1 µM) degranulated mast cell supernatants (blue bars), against a control (black bars). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM; results were not significant.

The proliferation of MCECs could be increased up to almost 50% by stimulation of the cells with supernatants derived from wt BMMCs.

5.8.3 Proliferation of endothelial cells in response to supernatants derived from PI3Kγ inhibited mast cells

As shown in previous proliferation experiments with smooth muscle cells, the treatment of these cells with supernatants derived from PI3Kγ -/-BMMCs or supernatants derived from wt BMMCs treated with a PI3K inhibitor (wortmannin) showed no effect on the proliferation rate of smooth muscle cells.

This experiment was also conducted on endothelial cells in order to determine differences between the two cell types. Endothelial cells were treated with mast cell supernatants derived from PI3Kγ -/- BMMCs or wortmannin-treated wt BMMCs; the results of these experiments are expressed in Figures 35 and 36.

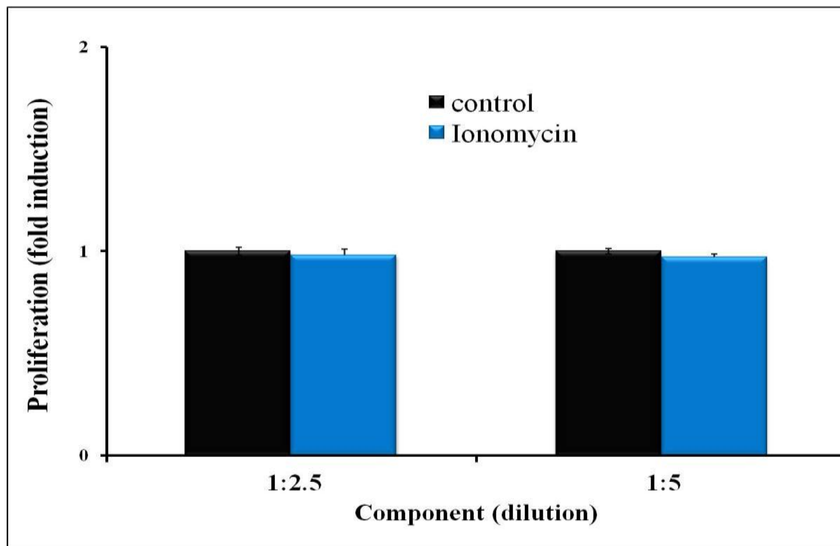


Figure 35: MCEC proliferation in response to treatment with PI3Kγ -/- BMMC supernatants. MCECs were stimulated with different concentrations of mast cell supernatants. ionomycin (1 µM) degranulated mast cell supernatants (blue bars) against the controls (black bars). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM.

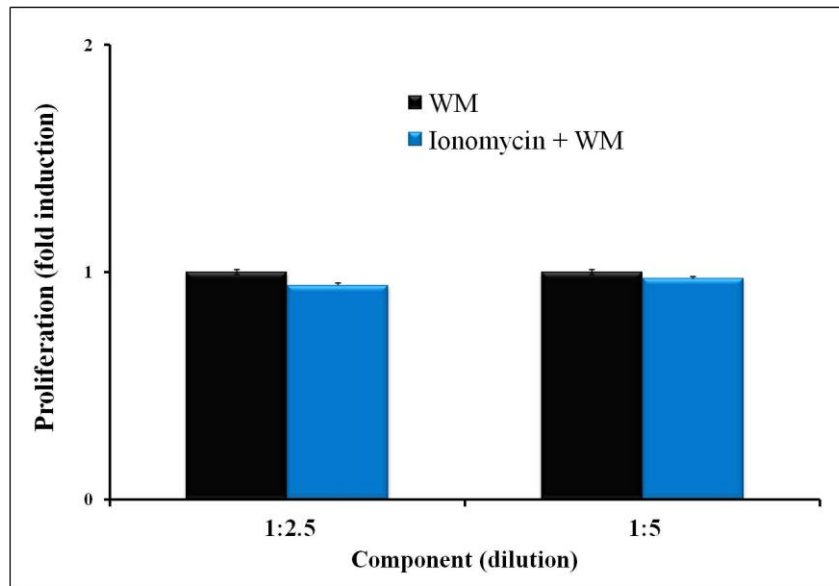


Figure 36: MCEC proliferation in response to treatment with supernatants derived from PI3K inhibited mast cells. MCECs were stimulated with different concentrations of mast cell supernatants. All mast cells were pre-incubated with wortmannin alone (100 µM; black bars) or prior to treatment with ionomycin (1 µM; blue bars). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM.

No increase in MCEC proliferation was observed after stimulation with supernatants derived from PI3Kγ -/- BMMCs or wortmannin-inhibited wt BMMC supernatants.

5.8.4 Influence of RNase 1 on the proliferation of smooth muscle and endothelial cells in response to supernatants derived from activated mast cells

Another interesting aspect was whether RNA released from activated mast cells might add to the proliferative effect of mast cell supernatants on smooth muscle and endothelial cells. The result of this experiment is shown in Figures 37 and 38.

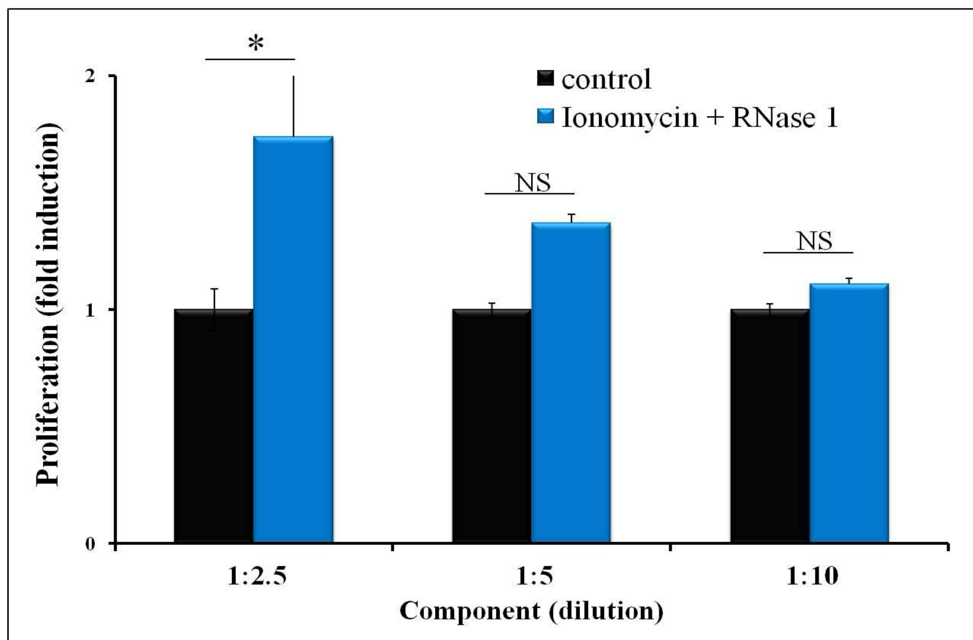


Figure 37: Influence of RNase 1 on the proliferation of MVSMCs in response to supernatants from activated mast cells. MVSMCs were either treated with supernatants form untreated (control) or ionomycin-treated mast cells derived from wild type mice, diluted 1:2,5 (40 µl supernatant : 60 µl medium), 1:5 (20 µl supernatant : 80 µl medium) and 1:10 (10 µl supernatant : 90 µl medium). Supernatants were pre-treated with RNase1 for 1 h. The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

As expected from previous proliferation experiments, addition of RNase 1 to mast cell supernatants did not influence MVSMC proliferation.

Next, this observation was examined in endothelial cells. Addition of RNase 1 to the mast cell supernatants prior to stimulation of MCECs showed no influence on the proliferation rate of MCECs.

These results demonstrated that RNA released from degranulated mast cells may not be involved in proliferative activities, demonstrated in Figure 38.

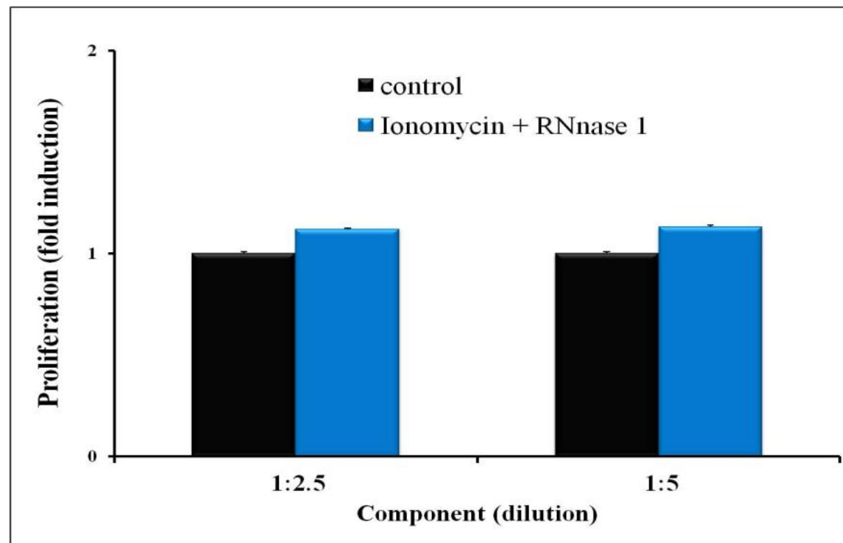


Figure 38: The impact of RNase 1 containing mast cell supernatants on MCEC proliferation. MCECCs were stimulated with different concentrations of mast cell supernatants. RNase 1 was added to all mast cell supernatants. ionomycin (1 µM) degranulated mast cell supernatants were examined against a control (black bars). The control was set to 1. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM.

5.9 Alteration of gene expression in vascular cells after treatment with activated mast cell supernatants

To investigate whether mediator-release from mast cells might influence gene expression in vascular cells, MVSMCs and MCECs were treated with supernatants of mast cells treated with ionomycin or for reasons of control with NaCl at different time points. RNA was isolated, reverse transcribed into cDNA and subjected to qRT-PCR.

5.9.1 PDGF-BB gene expression in smooth muscle cells

PDGF-BB gene expression in MVSMCs treated with supernatants derived from ionomycin degranulated mast cells or for reasons of control with NaCl treated mast cells was investigated. Cells were treated with the supernatants for one, three or 20 hours.

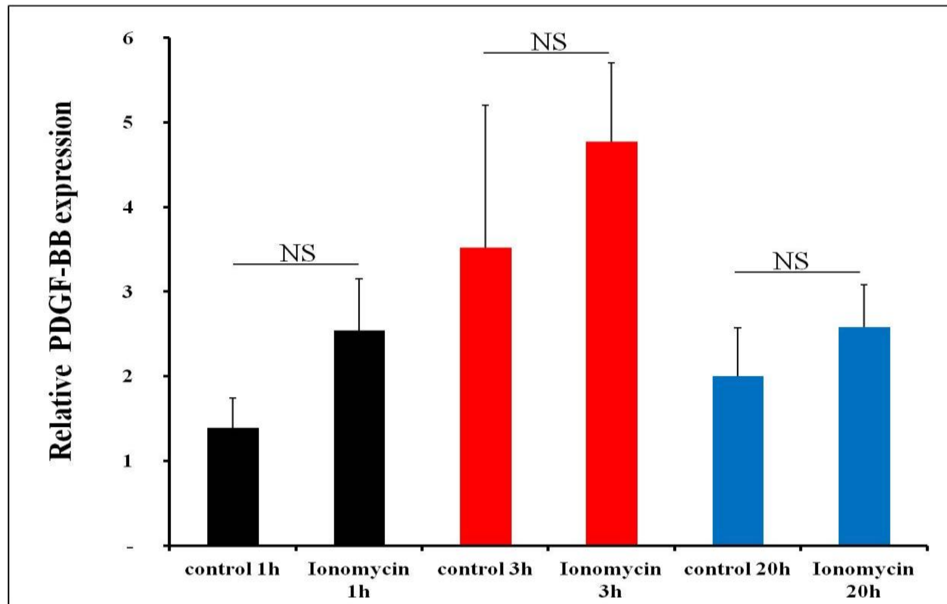


Figure 39: PDGF-BB gene expression in MVSMCs in response to treatment with supernatants derived from ionomycin-treated mast cells. Primary MVSMCs were stimulated with supernatants derived from mast cells treated with ionomycin (1µM) or NaCl (control) for 1 (black bars), 3 (red bars) and 20 hours (blue bars). MVSMCs were lysed and RNA was isolated. cDNA was synthesised from the isolated RNA and was amplified via qRT-PCR in order to detect up regulation of PDGF-BB gene expression. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM; results were not significant (NS).

Figure 39 outlines qRT-PCR results, which demonstrated that treatment of MVSMCs with conditioned medium (of mast cells degranulated with ionomycin) increased PDGF-BB gene expression - although not significantly - compared to MVSMCs treated with control medium (of mast cells treated with NaCl).

5.9.2 MCP-1 gene expression in endothelial cells

In order to investigate the effect of mediators released by ionomycin-treated mast cells on MCP-1 gene expression in MCECs, these cells were treated with supernatants derived from ionomycin-degranulated mast cells or for reasons of control with NaCl treated mast cells for one hour.

qRT-PCR results demonstrated in Figure 40 illustrate that treatment of MCECs with conditioned medium (of mast cells degranulated with ionomycin) increased MCP-1 gene expression - although not significantly - compared to MCECs treated with control medium (of mast cells treated with NaCl).

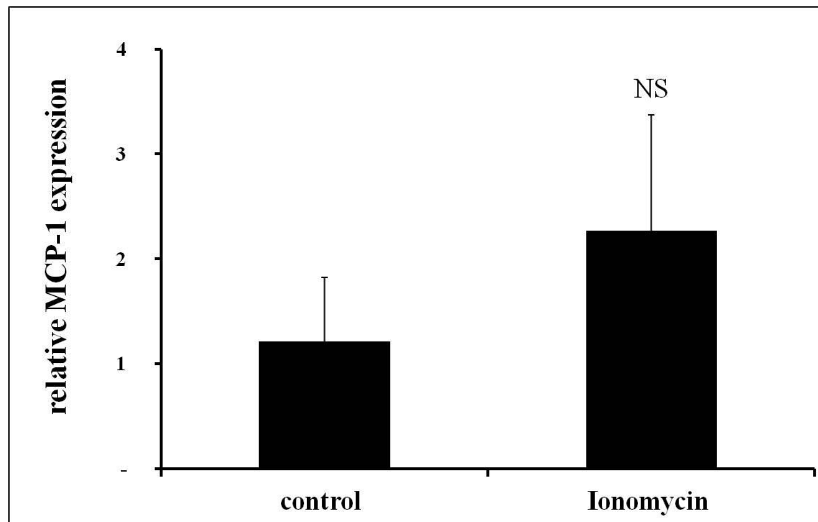


Figure 40: **MCP-1 gene expression in MCECs in response to supernatants derived from mast cells treated with ionomycin.** MCECs were treated with supernatants of mast cells treated with ionomycin (1µM) or NaCl (control) for one hour. MCECs were lysed and RNA was isolated. cDNA was synthesised from the isolated RNA and was amplified via qRT-PCR in order to detect up regulation of MCP-1 gene expression. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM; results were not significant (NS).

5.9.3 Thrombomodulin (TM) gene expression in endothelial cells

TM gene expression was measured after MCEC stimulation with ionomycin-treated mast cells versus a control, treated with NaCl.

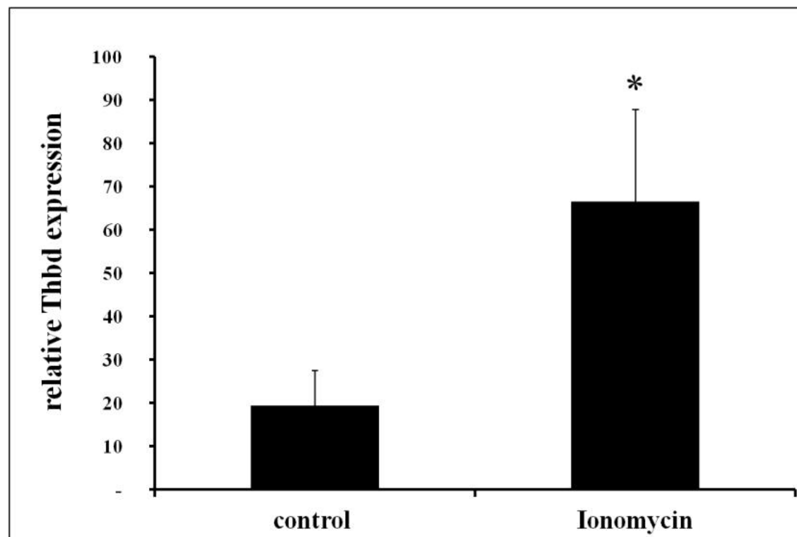


Figure 41: **TM gene expression in ECs in response to treatment with supernatants derived from ionomycin-treated mast cells.** MCECs were treated with supernatants of mast cells treated with ionomycin (1µM) or NaCl (control) for 3 hours. MCECs were lysed and RNA was isolated. cDNA was synthesised from the isolated RNA and was amplified via qRT-PCR in order to detect up regulation of MCP-1 gene expression. Experiments were measured in triplicate, each experiment was conducted 3 times (n = 3). Each value represents the mean ± SEM. $p \leq 0.05$ was regarded as statistically significant.

As expressed in Figure 41, incubation of endothelial cells with mast cell supernatants of ionomycin-treated mast cells resulted in an increased expression of TM compared to control endothelial cells stimulated with supernatants of mast cells treated with NaCl.

6.0 Discussion

6.1 *In vivo* studies

6.1.1 Mast cells and arteriogenesis

The background for this present study was Omary Chillo`s finding (unpublished work) that cromolyn, which is a mast cell stabiliser (Zi-Qing et al., 2007), diminishes arteriogenesis significantly when injected into mice after femoral artery ligation (fal).
Our hypothesis resulting from this finding was that *in vivo* simulation of mast cells with compound 48/80 (C48/80), which induces mast cell degranulation, would improve revascularisation if mast cells were important effector cells in arteriogenesis. Mast cells have been implicated in models of injury and Wolf et al. first described the presence of degranulated mast cells in the perivascular space of collaterals in 1998. Heissig et al. (2005) found that VEGF-release by mast cells promotes revascularisation following fal and that VEGF further improves mast cell migration by up-regulation of metalloproteinase-9 (MMP-9), further promoting revascularisation. These findings already hinted towards an involvement of mast cells in arteriogenesis. In fact, the present study showed that induction of mast cell degranulation by compound 48/80 following fal improves revascularisation significantly after seven days. Hence, mast cell degranulation does play a central role in the process of arteriogenesis. One possible cause for this improvement could be the release of a certain mediator which causes the positive effect directly or by recruitment of other effector cells.
Mast cells synthesise leukotrienes upon stimulation, therefore it was supposed that leukotrienes might be involved in the improvement of arteriogenesis caused by mast cell degranulation. MK-886, a 5-lipoxygenase, hence a leukotriene synthesis inhibitor (Uz et al., 2008), was injected into wt mice prior to femoral artery ligation to verify the leukotrienes´ involvement in arteriogenesis. Interestingly, inhibition of the leukotriene

synthesis had no major negative effect on the outcome of arteriogenesis, indicating that leukotrienes play a minor role in arteriogenesis.

6.1.2 PI3Kγ and mast cell degranulation in arteriogenesis

Laffargue et al. (2002) previously described that the lack of PI3Kγ disrupts mast cell degranulation. Mast cells from PI3Kγ deficient mice are less responsive to FcεRI cross-linking and release fewer granules upon stimulation. However, this is not due to a decreased mast cell number or poorer differentiation (Laffargue et al., 2002).
Accordingly, the present study showed that PI3Kγ deficient mice perform significantly worse in arteriogenesis compared to their wt littermates. They were much more prone to limb necrosis due to poor circulation because of inferior reperfusion and due to the fact that they presented abnormalities when their immune system is stressed which occurs when inducing arteriogenesis by femoral artery ligation (Barberis and Hirsch, 2008; Alcazar et al., 2004). Furthermore, no significant increase in arteriogenesis could be achieved by inducing mast cell degranulation by C48/80, suggesting that PI3Kγ is involved in mast cell degranulation. Therefore a lack in PI3Kγ does not result in sufficient mast cell degranulation needed for arteriogenesis.

6.2 *In vitro* studies

Cromolyn is known to cause a negative effect on arteriogenesis (Omary Chillo, unpublished work). We demonstrated that the reason for this diminishing effect is a disruption of mast cell degranulation which was shown to be necessary for collateral vessel growth. In contrast, ionomycin caused a strong effect on mast cell degranulation, and high β-hexosaminidase activities were measured in the mast cell supernatants after treatment with ionomycin.
In order to further confirm the involvement of PI3K in mast cell degranulation, mast cells were pre-incubated with wortmannin, which is a fungal metabolite that inhibits PI3K-activities specifically in that it blocks the release of granule associated mediators independent of the type of stimulus used (Marquardt, Alongi and Walker, 1996).

Mast cells from PI3Kγ -/- mice as well as mast cells from wt animals treated with the PI3K inhibitor wortmannin (Byrne et al., 2007) degranulated to a much lower degree than mast cells from their wt littermates or wt animals degranulated in the absence of wortmannin, respectively. This finding is supported by the results of Laffargue et al. (2002) that the lack of PI3Kγ prevents mast cell degranulation by inhibiting G-protein coupled signalling strongly, suggesting an involvement of the PI3Kγ in the process of mast cell degranulation. Additionally, the fact that mast cell degranulation enhances arteriogenesis whereas mast cell inhibition diminishes it, confirms that mast cells are key effector cells in the process of arteriogenesis.

6.2.1 Mast cell degranulation

During *in vitro* studies mast cell degranulation was induced by ionomycin treatment, as C48/80 previously used for mast cell degranulation *in vivo*, was less potent *in vitro*. The reason for this observation may have been that C48/80 is very dependent on the optimal concentration of the applied stimulants (Hide et al., 1993). Ionomycin on the other hand induces a strong intracellular increase of Ca^{2+} ions, which mast cells require for degranulation (Puri and Roche, 2007). Hide et al. (1993) found that stimulation with ionomycin follows an all or none principle, showing that even with a suboptimal stimulus, the culture showed fully degranulated cells. In contrast, a suboptimal dose of C48/80 results in only partial degranulated cells (Hide et al., 1993). As the stimulation with C48/80 did not induce a stimulus strong enough to detect a significant difference between treatment and control group, cells were stimulated with ionomycin for the following experiments.

6.2.2 Mast cell-related release of RNA and its supposed function

In the present study, analysis of supernatants from stimulated/degranulated mast cells revealed the presence of released RNA, termed extracellular RNA (eRNA). Previous studies also found that mast cell-derived exosomes contain RNA with the capacity to shuttle RNA between cells for cellular communication (Dvorak et al., 2010; Ekström et al., 2012).

The type of RNA released could not be identified at this point as the concentrations of the release products were too low for the analysis in a Bioanalyser. However, parallel studies from the Preissner group indicated that the majority of eRNA is composed of ribosomal RNA (Fischer et al 2014).

Mast cells were also found to express ribonuclease 5 (RNase5) or angiogenin, which they store in their granules and release it upon stimulation (Kulka, Fukuishi and Metcalfe, 2009). Angiogenin is expressed in many other cells and circulates as a plasma protein with potent angiogenic but very low ribonucleolytic activity (Kulka, Fukishi and Metcalfe, 2009). In the present study, no RNase-activity was found in mast cell supernatants, which may be beneficial for the release eRNA from the same cells. RNases like RNase 5 are highly basic proteins and may be bound to heparin and glycosaminglycans inside granules of mast cells, such that they are prevented to interact with eRNA during the degranulation process. Addition of RNase 1 to mast cell supernatants prior to vascular cell stimulation slightly diminished proliferation of endothelial (EC) but not smooth muscle cells (SMC). Likewise, RNase inhibitor had no effect on SMC proliferation, suggesting that mast cell-derived eRNA plays a minor role in vascular cell proliferation. Moreover, treatment of EC and SMC with eRNA did not influence the proliferation rate of these cells, suggesting that eRNA released by mast cells might have other functions, like inducing inflammatory activities as shown previously (Fischer et al., 2014).

As shown by Fischer et al. (2013), eRNA promotes vascular permeability (via VEGF) and inflammation (via cytokines) *in vivo* and *in vitro*, and eRNA may thereby act as a diffusible signalling molecule in order to stimulate release of substances, which enhance cell proliferation in the context of arteriogenesis as well. Moreover, eRNA promotes leukocyte emigration through endothelial monolayers, relevant for inflammatory in the recruitment of monocytes that are also part of the arteriogenesis mechanism (Fischer et al., 2013).

eRNA is also be associated with microparticles or exosomes, which may deliver their RNA content to target cells (Fischer et al, 2012), thus being capable of acting as signalling molecule. eRNA also promotes TNF-α release from macrophages by activation of TNF-α converting enzyme (TACE). Collectively, eRNA acts as alarm signal to provoke defence reactions, which may account in large part for the mast cell-dependent processes relevant for arteriogenesis.

6.2.3 Effect of mast cell mediators on vascular cell proliferation

Another interesting aspect in this study was to discover a specific mast cell mediator which may cause the positive effects of mast cell supernatants on vascular cell proliferation, hence on arteriogenesis (Heissig et al., 2005). SMCs were treated with mediators such as TNF-α, FGF-2, VEGF, PDGF, IL-1 and histamine. As a consequence, TNF-α and FGF-2 were found to have a positive proliferative effect on MVSMCs, other mediators did not affect MVSMC proliferation *in vitro*. Additionally, PDGF seemed to add onto the positive proliferative effect of FGF-2. As mentioned earlier, mast cells release FGF upon stimulation, hence mast cells may influence cell proliferation directly by mediator release. However, the effect that mast cells cause on cell proliferation does not necessarily need to be a direct one. Heissig et al. reported that mast cells may also cause a positive effect on arteriogenesis by recruitment of other inflammatory cells as well as bone marrow cells by enhancing vascular cell growth and differentiation through paracrine support cells. These cells could also release mediators that act on vascular cell proliferation.

6.2.4 Proliferation studies

In vivo, fluid shear stress (FSS) is the main stimulus for inducing arteriogenesis. However, FSS is only a weak force that acts on endothelial cells (ECs) (Schaper, 2009). Circumferential wall stress on the other hand acts on ECs and SMCs so this weak force may act as amplifier (Schaper, 2009). The endothelium has a leading role in arteriogenesis but SMC proliferation is also needed. However, there are no direct cell-cell contacts between the SMC layer and the endothelium (Schaper, 2009), how would this communication then work? There would be the need of diffusible factors of some kind (e.g. NO, oxygen radicals or endothelin) that would take over this communication between both cell types (Schaper, 2009). Identification of possible signalling pathways or specific factors realising this signalling needs to be investigated in future studies.

An improvement of vascular cell proliferation could be observed in response to treatment with murine peritoneal or bone marrow derived mast cell supernatants. Sathyakumar et al. (2012) reported that there is an increase in micro vessel density when the mast cell density rises in patients with oral epithelial dysplasia. This confirms

the finding of this study that mast cells secrete mediators that affect the proliferation of vessel wall cells. Cells treated with supernatants derived from PI3Kγ -/- animals or wt animals treated with wortmannin showed no increased proliferation rate. This supports the *in vivo* results, as an improvement in arteriogenesis requires cell proliferation.

6.2.5 Growth factor release by mast cells

Mast cells are a significant source of fibroblast growth factor (FGF) and platelet derived growth factor (PDGF), which are important for remodelling of the adventitia of growing collaterals (Bischoff et al., 2000). Further, extracellular proteases such as the MMPs are under control of several cytokines and contribute to the lysis of the extracellular matrix enabling fibroblasts and macrophages to penetrate into the inflamed tissue (Wolf et al., 1998; Namba et al., 2010). Growth factors and cytokines, including PDGF-BB are suggested to be the main stimulators for arteriogenic vessel growth (Wu et al., 2010). It is known that PDGF-BB and FGF are involved in the formation of vessel networks in hindlimb ischemia models (de Paula et al., 2009). Studies by Schierling at al. (2009) showed that growth factors and cytokines such as MCP-1, bFGF, PDGF, and VEGF also increase arteriogenesis; however the effect is much smaller than collateral vessel growth induced by fluid shear stress (Schierling et al., 2009).
PDGF is a dimeric protein, composed of A and B chains, which can form three different dimers: PDGF-AA, PDGF-AB and PDGF-BB. Both PDGF receptors (PDGFR-α and –β) have been shown to have tyrosine kinase activity (Namba et al., 2010). PDGFR-α is able to bind all PDGF isoforms and PDGFR- β binds PDGF-BB with high and PDGF-AB with low affinity; there is no binding to PDGF-AA (Namba et al., 2010). Wu et al. (2010) showed that PDGF is up regulated in collateral vessels of dog heart, suggesting that it plays a role during the process of arteriogenesis. It is secreted by various cells; accelerates wound healing by stimulating cellular proliferation, migration and production of extracellular matrix (ECM) (Namba et al., 2010). In this study, PDGF-BB did not have a direct effect on cell proliferation but it is known that PDGF-BB as well as FGF increased collateral vessel formation in rats (de Paula et al., 2009). In the present study, the combination of PDGF-BB with FGF-2 increased the proliferative effect of FGF-2 alone, suggesting a positive effect on cell proliferation. If mast cells were involved in arteriogenesis and cell proliferation, an up-regulation of PDGF-BB

expression in these cells would be expected following MVSMC treatment with stimulated mast cell supernatants, which, although not significant, could be confirmed by the present study.

Macrophages and monocytes are also known to be important effector cells in arteriogenesis; they are circulating cells that are recruited to sites of inflammation (Shireman, 2007). There is a large number of chemokines that can affect monocyte recruitment and thereby influence arteriogenesis. Increased blood flow and the resulting shear stress were found to increase MCP-1 levels, which attracts monocytes that produce a certain amount of growth factors, which in turn stimulate EC and SMC proliferation (Cannon et al., 2009; Shireman, 2007). MCP-1 also attracts macrophages, which produce VEGF, a growth factor, which next to MCP-1 is a critical mediator in arteriogenesis by contributing to growth signalling in endothelial cells (Cannon et al., 2009, Prior et al., 2004). The up-regulation of chemokines like MCP-1 leads to the recruitment of leukocytes (Dimicheva, Hecker and Korff, 2008). Increased MCP-1 levels were also found to lead to an increased collateral formation in the early phase of patients with myocardial infarction (Park et al., 2008). In a rabbit model, Schaper et al. showed that injection of MCP-1 into the proximal end of the ligated femoral artery increases arteriogenesis and an increase of monocyte accumulation in the collateral vessel walls can be observed in these MCP-1 treated animals and on the other hand MCP-1 deficient mice have a decrease in reperfusion after femoral artery ligation (Shireman, 2007). An *in vivo* hind limb ischemia model by Kuhlmann, Klocke and Nikol (2007) revealed that injection of MCP-1, which is also secreted by mast cells, improved collateral artery growth and restoration of limb perfusion. In this study an up regulation of MCP-1 gene expression was found in ECs – although not significant – following stimulation with mast cell supernatants. This strongly suggests an involvement of mast cell mediators in cell proliferation during arteriogenesis i.e. by recruitment of monocytes releasing growth factors stimulating cell proliferation.

Thrombomodulin (TM) is a membrane-bound glycoprotein, mostly expressed on endothelial cells (Morser, 2012) that acts as high affinity receptor for thrombin in the activation of protein C, relevant for the intrinsic control of the coagulation cascade and for vascular homeostasis in general (Esmon and Owen, 2004). Intracellular interactions of TM with cytoskeletal-associated ezrin may influence cell morphology or cell migration (Hsu et al., 2012), relevant for vascular remodelling. High expression of TM may account for intact and protected endothelium and to ensure a rapid and localised

inflammatory response to injury (Conway, 2011). In the present study, gene expression of TM was found to be significantly up-regulated in cultured EC that were treated with mast cell supernatants, indicative for a critical role of mast cells in endothelial morphogenesis, possible relevant for arteriogenesis. Since activated protein C (the product of the thrombin-TM-mediated substrate reaction) also promotes cytoprotective functions via protease-activated receptor-1 on EC (Ocak et al., 2014), together, the contribution of this system appears to be worthwhile to be followed in the context of arteriogenesis.

6.2.6 Future research

Lots of attempts to establish further ways to stimulate arteriogenesis by the use of various growth factors have failed, likely due to the inability of signalling in vascular tissues in patients suffering from vascular diseases (Ren et al., 2010). As it has been shown that mast cells are important cells in arteriogenesis and that the degranulation process is mediated by the PI3Kγ, this could be a potential target for future drug therapy for ischemic diseases. In fact PI3Ks are currently already an attractive drug target for therapeutic companies interested in the development of treatments for this type of disease such as myocardial infarction, stroke and peripheral arterial disease (Ghigo, Morello et al., 2012). However, stimulating PI3K solely in mast cells would be a difficult task. Another possible mechanism for future therapies could be the specific attraction of mast cells to the site of vessel closure to induce a natural stimulus by these cells. To achieve this, a local stimulus would be necessary, which attracts mast cells only to the desired area in order to achieve a specific stimulus.

The clinical START Trial (van Royen et al., 2005) has already made clear that patients suffering from claudication will benefit from the induction of arteriogenesis by GM-CSF (Granulocyte-macrophage colony stimulating factor). This therapy has initially been used to treat patients with leucopenia after chemotherapy (van Royen et al., 2005). Patients with claudication suffer from leg pain due to vessel closure, hence tissue hypoxia in the distal limbs. Enhancing collateral growth by inducing arteriogenesis would improve tissue vascularisation without the need of invasive intervention.

However, the induction of arteriogenesis by stimulation of the PI3K and thus degranulation of local residing mast cells may be a much more effective way of treating

those patients. The goal in this therapy would be to target the type of mast cells residing in the tissues of interest. However, induction of mast cell degranulation could result in a systemic mast cell activation, leading to severe systemic reactions and hence the patient`s death. Signalling molecules need to be identified that would enable a specific activation of mast cells, which would allow for a reduction of potential severe side effects caused by such drugs.

Future research needs to include pre-clinical studies to fully understand the mechanisms of inducing specific mast cell activation and possible induction of arteriogenesis without the risk of dangerous side effects such as tumour growth and systemic reactions due to mast cell activation.

7.0 Conclusion

In conclusion, this study confirmed that degranulation of mast cells does enhance collateral artery growth (arteriogenesis) in wt mice significantly.
Diminished arteriogenesis in PI3Kγ deficient mice compared to wild type (wt) mice could not be improved by mast cell stimulation, confirming that mast cell degranulation is critical for arteriogenesis. Additionally this finding confirms that mast cell degranulation is a PI3Kγ-depent mechanism crucial for arteriogenesis.
Leukotriene synthesis inhibition by MK-886 had only a minor effect on arteriogenesis in wt mice, suggesting that leukotrienes do not play an important role in arteriogenesis.
Additionally, *in vitro* studies revealed that the improvement in arteriogenesis is due to the induction of smooth muscle- and endothelial cell proliferation. An indicator for increased cell proliferation is the increase in the expression of MCP-1 and PDGF-BB in endothelial- and smooth muscle cells, respectively, following stimulation with activated mast cell supernatants. Mast cells therefore influence arteriogenesis by stimulation of cell proliferation. RNA could be involved as a signalling molecule as this present study revealed that mast cells release RNA upon stimulation. The explicit type of RNA and its function remains unknown but previous studies revealed that extraceullular RNA (eRNA) is recruiting monocytes, which are important for arteriogenesis and mediates TNF-α release from macrophages. eRNA can also be associated with microparticles or exosomes, which are capable of delivering their RNA content to target cells (Fischer et al, 2012) thus being capable of acting as signalling molecule. Different mast cell mediators may directly influence cell proliferation.
Taking all this into consideration, this study showed that hind limb perfusion following femoral artery ligation could be improved by mast cell degranulation *in vivo* and that mediators released by mast cells act on cell proliferation *in vitro*. It was established that mast cells play a crucial role in the induction of arteriogenesis.

8.0 Appendix

8.1 Abstract

Vascular occlusive disease is a major cause of death; however there is evidence that growth of collateral arteries developing from pre-existing anastomoses, known as arteriogenesis, could prevent patients from developing infarction. Mast cells, which are known to release vasoactive substances, seem to play an important role in this process. The aim of this work was to investigate the involvement of mast cells in the process of arteriogenesis.

To evaluate the role of mast cells, wild type (wt) mice and PI3 kinase (PI3K) γ knockout mice were subjected to right femoral artery ligation in order to induce arteriogenesis; the left side was sham operated. Relative perfusion recovery of hind limbs was monitored before and post ligation at days 3, 7, 14 and 21 by Laser Doppler Imaging. For *in vitro* experiments, mouse endothelial and smooth muscle cells were isolated from mouse aorta and mast cells from mouse bone marrow. After treating isolated mast cells with degranulating agents like ionomycin *in vitro*, supernatants were collected and used for measurements of proliferation of endothelial and smooth muscle cells as well as other biochemical investigations such as RNA isolation.

In vivo, treatment of wt-mice with mast cell degranulating compound 48/80 (C48/80) improved the outcome of arteriogenesis. Collateral growth in PI3Kγ -/- mice was reduced in comparison to wt-mice. Furthermore, treatment of PI3Kγ -/- mice with C48/80 had no effect on collateral growth indicating an important role of the PI3Kγ on mast cell degranulation. Furthermore, treatment of mice with a leukotriene synthesis inhibitor, MK-886, had no effect on the outcome of arteriogenesis in comparison to the controls indicating that leukotrienes might not be essential for arteriogenesis.

In vitro studies demonstrated that the proliferation of endothelial cells and smooth muscle cells was enhanced 1.2- and 1.7-fold, respectively, by supernatants derived from degranulated mast cells. The mast cell supernatants were found to contain an average of 1.29 ± 0.13µg/ml RNA but no RNase activity, suggesting that mast cells release RNA upon stimulation, which might function to recruit leukocytes.

Together, these findings suggest that cell mediators released from activated mast cells might influence the process of arteriogenesis by enhancing the proliferation of vessel wall cells, dependent on PI3Kγ-related mast cell degranulation.

8.2 Zusammenfassung

Vaskuläre Verschlusskrankheiten sind eine der Haupttodesursachen; allerdings gibt es Hinweise, dass das Wachstum von Kollateralgefäßen, die aus präformierten Anastomosen entstehen (Arteriogenese), protektiv wirken kann. Mastzellen, welche vasoaktive Substanzen sezernieren, scheinen dabei eine wichtige Rolle zu spielen. Ziel dieser Arbeit war es, zu erforschen welche Rolle Mastzellen bei der Arteriogenese spielen. Wildtyp (wt) und PI3 kinase (PI3K) γ knockout Mäuse wurden dazu einer rechtsseitigen Femoralligatur unterzogen. Hierdurch wurde der Prozess der Arteriogenese angeregt; die linke Seite wurde Schein operiert. Die relative Reperfusion der Läufe wurde vor und nach Ligatur, an Tag 3,7, 14 und 21 mit Hilfe des Laser-Dopplers gemessen. Für *in vitro* Experimente wurden primäre Endothel- und glatte Gefäßmuskelzellen aus der Mausaorta und Mastzellen aus dem Knochenmark der Mäuse isoliert. Mastzellen wurden mit degranulierenden Substanzen wie ionomycin behandelt und die Überstände abgenommen. Diese wurden für die Stimulation der Proliferation von Edothel- oder glatter Gefäßmuskelzellen verwendet.

Die Behandlung der ligierten wt Mäuse mit C48/80 verbesserte die Arteriogenese *in vivo* signifikant auch verglichen mit den PI3Kγ -/- Mäusen, welche keine Verbesserung der Arteriogenese durch C48/80 erzielten. Dies weist darauf hin, dass Mastzelldegranulierung für die Arteriogenese entscheidend ist. Desweiteren hatte die Behandlung der Mäuse mit dem Leukotriensyntheseinhibitor MK-886 einen nur geringen Einfluss auf die Arteriogenese; diese scheinen also keine entscheidende Rolle zu spielen.

In vitro Studien demonstrierten, dass die Proliferation von Endothel- und glatten Gefäßmuskelzellen jeweils 1,2- und 1,7-fach erhöht war, nachdem die Zellen mit Mastzellüberständen stimuliert wurden. Dies ist ein weiterer Hinweis auf die Relevanz von Mastzellen bei der Arteriogenese. Desweiteren wurden in den Mastzellüberständen im Durchschnitt 1.29 ± 0.13µg/ml RNA, aber keine RNase-Aktivität nachgewiesen. Dies weist darauf hin, dass stimulierte Mastzellen RNA sezernieren, welche eine Rolle bei der Rekrutierung von Leukozyten spielen könnte.

Alles in allem zeigen diese Ergebnisse, dass Zellmediatoren, die von stimulierten Mastzellen sezerniert werden, den Prozess der Arteriogenese positiv beeinflussen. Dies geschieht unter Anderem durch die Anregung der Proliferation von Gefäßwandzellen durch PI3Kγ abhängige Mastzelldegranulierung.

8.3 List of abbreviations

A

ACTH – adenocorticotropic hormone
Ag – antigen
Akt = protein kinase B
APCs – antigen-presenting-cells
AT-1 – Angiotensin 1

B

BAEC – bovine aortic endothelial cells
BCA - bicinchoninic acid
BMMC – bone marrow derived mast cell
BRDU - 5-bromo-2´-deoxyuridine
BSA – bovine serum albumin

C

Ca^{2+} - Calcium
CABG – coronary artery bypass graft
C48/80 – compound 48/80
CD – cluster of differentiation
cDNA – complementary DNA
c-kit - SCF receptor
CTMC – connective tissue mast cell
CVD – cardiovascular disease

D

DAG – diacylglycerol
DAPI - 4',6-diamidino-2-phenylindole
DDS – drug delivery system
DMEM – Dulbecco’s Modified Eagle Medium
DMSO – Dimethylsulfoxide
DNA – deoxyribonucleic acid
dNTP – deoxyribonucleotide triphosphate

E

EBM-2 – endothelial cell basal medium-2
ECs – endothelial cells

	ECM – extracellular matrix
	EDTA - Ethylenediaminetetraacetic acid
	EGM-2 - Endothelial Cell Growth Medium 2
	ELISA – enzyme linked immunosorbent assay
	EPCs – endothelial progenitor cells
	ER – endoplasmic reticulum
	ERK – extracellular signal-regulated kinase
F	F_{ab} – fraction antigen-binding
	FACS – fluorescent-activated cell sorting
	fal – femoral artery ligation
	fBSA – bovine serum albumine
	F_c – fraction crystallisable
	$F_c\varepsilon$RI - $F_{c\varepsilon}$ receptor type I
	FCS – fetal cattle serum
	FGF – fibroblast growth factor
	FITC - Fluorescein isothiocyanate
	FSS – fluid shear stress
G	G_i-protein – inhibitory G-protein
	GM-CSF – Granulocyte-Macrophage Colony Stimulating Factor
	GPCRs – G-protein-coupled-receptors
H	HE – Hematoxylin and eosin stain
	HIF – hypoxia inducible factor
	HMG-CoA 3-Hydroxy-3-Methylglutaryl-Coenzym-A
I	IFN - interferon
	IgE – Immunoglobulin E
	IL – interleukin

IMDM – Iscove's Modified Dulbecco's Medium
i.p. – intraperitoneally
IP_3 – Inositol-tri-phosphate
i.v. – intravenously

J

K kDA – kilo Dalton
knockout -/-
KRH – Krebs-Ringer-Hepes buffer (=Tyrodes buffer)

L LAT – linker of activated T cells
LDI – Laser Doppler Imaging
LPS – lipopolysaccharides
LTs - leukotrienes

M MAEC - mouse aortic endothelial cells
MCP-1 – monocytes chemoattractant protein 1
MC_{TC} – mast cell tryptase and chymase
MC_T – mast cell tryptase
MC9 – mast cell line
MCEC – mouse coronary endothelial cells
MHC – major histocompatibility complex
mM – milli Molar
MMC – mucosal mast cell
mSCF – murine SCF
mTOR - rapamycin
MVSMC - mouse vascular smooth muscle cells

N NFAT – nuclear factor of activated T cells
NFκB – nuclear factor κ-light-chain-enhancer of activated B cells
NGF – nerve growth factor

NK-cells – natural killer cells
NP – nanopeptide
NS – non significant
NSTEMI – non ST elevation myocardial infarction

O

P

PBS – phosphate buffered saline
PCI – percutaneous coronary intervention
PCR – polymerase chain reaction
PDGF – platelet derived growth factor
Pen/Strep – Penicillin/Streptomycin
PGD_2 – Prostaglandin D_2
PGE_2 – Prostaglandin E_2
PI3 kinase – Inositol Triphosphate kinase
PIP_2 – phosphatidyl inositol diphosphate
PIP3 – phosphatidyl inositol triphosphate
PKC – protein kinase C
PLC/D – protein lipase C/D
PLGA – polylactide/glycolide
PMA - phorbol-12-myriatate-13-acetate
pNAG – tetra- Nitrophenyl-2-acetamido-2-deoxy-β-D-glucopyranosid
PTCA – percutaneous transluminal coronary angioplasty
PTK – protein tyrosine kinase
Ptx – pertussis toxin

Q

qRT-PCR – quantitative real-time polymerase chain reaction

R

RNA – ribonucleic acid
RNase – ribonuclease
ROI – region of interest

	RT- room temperature
S	S-1P – sphingosine-1-phosphate
	s.c. - subcutaneously
	SCF – stem cell factor
	SDS – sodium dodecyl sulfate
	SH-domain – Src-homology-domain
	SMCs – smooth muscle cells
	STEMI – ST elevation myocardial infaction
T	TBS – Tris buffered saline
	TBST – tris buffered saline and tween
	TH_2-cells – T helper cells
	TLRs – toll-like-receptors
	TNF-α – tumour necrosis factor
	TM – Thrombomodulin
U	UP – urticaria pigmentosa
V	VEGF – vascular endothelial growth factor
	VSMCs – vascular smooth muscle cells
	vWF – vonWillebrand factor
W	wt – wild type
	WHO – world health organisation

8.4 Figures and Tables

Figures:

Figure 1: Collateral vessel formation after femoral artery ligation in mice .... - 4 -
Figure 2: Electron microscopy picture showing a mast cell. The round, dark .... - 8 -
Figure 3: Molecular basis of mast cell degranulation. .... - 15 -
Figure 4: Chemical structure of C48/80. .... - 16 -
Figure 5: The PI3 kinase pathway .... - 17 -
Figure 6: The different isoforms of the PI3K .... - 18 -
Figure 7: Area of surgery ....- 29-
Figure 8: Preparation for femoral artery ligation - 31 -
Figure 9: Finalisation of fal .... - 31 -
Figure 10 : Laser Doppler Imaging (LDI) .... - 32 -
Figure 11: LDI measurements .... - 33 -
Figure 12: FACS analysis of wt bone marrow derived mast cells ....- 47 -
Figure 13: FACS analysis of PI3Kγ -/- bone marrow derived mast cells .... - 48 -
Figure 14: MVSMCs labelled with anti-α-smooth muscle actin. .... - 48 -
Figure 15: The involvement mast cell degranulation in arteriogenesis. .... - 49 -
Figure 16: The involvement of PI3Kγ in arteriogenesis .... - 50 -
Figure 17: Arteriogenesis is dependent on PI3Kγ-mediated mast cell degranulation- 51 -
Figure 18: The effect of leukotriene inhibition by MK-886. .... - 52 -
Figure 19: Mast cell degranulation induced by different stimuli. .... - 53 -
Figure 20: Concentration dependency of ionomycin in mast cell degranulation .... - 54 -
Figure 21: Time dependency of mast cell degranulation induced by ionomycin ...... - 55 -
Figure 22: Mast cell degranulation by ionomycin in the presence of cromolyn .... - 56 -
Figure 23: Degranulation of mast cells from SV129 wt versus PI3Kγ -/- mice .... - 57 -
Figure 24: Degranulation of mast cells in the presence of wortmannin .... - 58 -
Figure 25: RNase activity in mast cell supernatants .... - 59 -
Figure 26: Release of extracellular RNA from degranulating mast cells .... - 59 -
Figure 27: Release of extracellular RNA in the presence of RNase inhibitor .... - 60 -
Figure 28: SMC proliferation in response to different mast cell mediators .... - 61 -
Figure 29: MVSMC proliferation after treatment with supernatants from ionomycin-treated peritoneal mast cells .... - 62 -

Figure 30: MVSMC proliferation in response to treatments with supernatants from ionomycin-treated wt bone marrow derived mast cells (BMMCs) .... - 63 -
Figure 31: MVSMC proliferation in response to treatments with supernatants from ionomycin-treated mast cells derived from PI3Kγ -/- mice .... - 64 -
Figure 32: Proliferation rate of MVSMCs in response to treatment with supernatants derived from PI3Kγ-inhibited mast cell supernatants .... - 65 -
Figure 33: BAEC proliferation in response peritoneal derived mast cell supernatants .... - 66 -
Figure 34: The effect of BMMC supernatants on MCEC proliferation. MCECs were stimulated with different concentrations of mast cell supernatants .... - 67 -
Figure 35: MCEC proliferation in response to treatment with PI3Kγ -/- BMMC supernatants. .... - 68 -
Figure 36: MCEC proliferation in response to treatment with supernatants derived from PI3K inhibited mast cells .... - 68 -
Figure 37: Influence of RNase 1 on the proliferation of MVSMCs in response to supernatants from activated mast cells. .... - 69 -
Figure 38: The impact of RNase 1 containing mast cell supernatants on MCEC proliferation. .... - 70 -
Figure 39: PDGF-BB gene expression in MVSMCs in response to treatment with supernatants derived from ionomycin-treated mast cells. .... - 71 -
Figure 40: MCP-1 gene expression in MCECs in response to supernatants derived from mast cells treated with ionomycin .... - 72 -
Figure 41: THBD gene expression in ECs in response to treatment with supernatants derived from ionomycin-treated mast cells .... - 72 -

Tables:

Table 1: Characteristics of peritoneal and mucosal type mast cells in rodents .... - 9 -
Table 2: All classes of mast cell mediators .... - 12 -

8.5 References

1. Global Status Report on Noncommunicable Disaeses 2010. World Health Organisation, 2011.
2. Abel J. et al. (2011) Staphylococcus Aureus Evades the Extracellular Antimicrobial Activity of Mast Cells by Promoting Its Own Uptake. *J Innate Immun*.
3. Alcazar I. et al. (2004) Phosphoinositide 3-Kinase Γ Participates in T Cell Receptor-Induced T Cell Activation. *J Exp Med* 204 (12): 2977-2987.
4. Aridor M.L., M. Traub and Sagi-Eisenberg R. (1990) Exocytosis in Mast Cells by Basic Secretagogues: Evidence for Direct Activation of Gtp-Binding Proteins. *J Cell Biol* 111: 909-917.
5. Bansal G. et al. (2008) Rgs13 Controls G-Protein-Coupled Receptor-Evoked Responses of Human Mast Cells. *J Immunol* 181 (11): 7882–7890.
6. Barberis L. and E. Hirsch. (2008) Targeting Phosphoinositide 3-Kinase Γ to Fight Inflammation and More. J *Thromb and Haemost* 99: 279-285.
7. Baumhoer D., I. Steinbrück, and G.W. Kurzlehrbuch Histologie. München: Urban&Fischer, 2003.
8. Bauriedel G., D. Skowasch and B. Lüderitz. (2007) Operationsrisiko Aus Der Sicht Des Kardiologen: Teil 1 Der Serie Zur Präoperativen Risikoeinschätzung. *Deutsches Ärzteblatt* 104 (22).
9. Bax J. et al. (2008) Esc Stemi Guidelines - Akutes Koronarsyndrom Mit Persistierender St-Streckenhebung (Stemi) *Europ Heart Journal* 29: 2909-2945.
10. Bayley R. Blood Vessels. National Cancer Institute, 2014.
11. Beaven M.A. (2009) Our Perception of the Mast Cell from Paul Ehrlich to Now. *Europ J Immunol* 541-566 .
12. Bischoff S.C., L.M. Marone and S.J. Lichtenstein. (2000) Regulation and Function of Human Intestinal Mast Cells. In Mast Cells and Basophils. *Ac Press* 541-566.
13. Boengler K. et al. (2003) A Rteriogenesis Is Associated with an Induction of the Cardiac Ankyrin Repeat Protein (Carp). *Cardiovasc Res* 4: 170-178.
14. Bot I., T.J.C. vanBerkel and E.A.L. Biessen. (2008) Mast Cells: Pivotal Players in Cardiovascular Diseases. *Curr Cardiol Rev* 4: 170-178.

15. Byrne R.D. et al. (2007) Differential Activation of the Pi3-Kinase Effectors Akt/Pkb and P70 S6 Kinase by Compound 48/80 Is Mediated by Pkcα. *Cell Signaling* 19: 321-329.
16. Cai W. and W. Schaper. (2008) Mechanisms of Arteriogenesis. *Acta Biochim Biophys Sin* 681-692.
17. Cannon J.G. et al. (2009) Femoral Artery Diameter and Arteriogenic Cytokines in Healthy Women. *Vasc Pharmacol* 50 (3-4): 104-109.
18. Chen T. Maec Isolation. Los Angeles: Iruela-Arispe Laboratory, 2006.
19. Chillo O. Mast Cells in Arteriogenesis - a Pre-Clinical Study Employing a Murine Hindlimb Model of Collateral Artery Growth. Munich: Walter-Brendel-Zentrum, 2011.
20. Cleveland D.W. et al. (1976) Peptide Mapping by Limited Proteolysis in Sodium Dodecyl Sulfate and Analysis by Gel Electrophoresis. *J Biol Chem* 252 (3): 1102-6
21. Coleman J.W. et al. (1993) Regulation of Mouse Peritoneal Mast Cell Secretory Function by Stem Cell Factor, Il-3 or Il-4. *J Immunol* 150: 556-562.
22. Coman L. et al. (2010) Vegf-Induced Corneal Neovascularisation in a Rabbit Experimental Model. *Romanian J Morphol Embryol* 51 (2): 327–236.
23. Conway E.M. (2012) Thrombomodulin and Its Role in Inflammation. *Semin Immunopathol* 34 (1): 107-125.
24. Crackower M.A. et al. (2002) Regulation of Myocardial Contractility and Cell Size by Distinct Pi3k-Pten Signaling Pathways. *Cell* 110: 737-749.
25. de Paula E.V. et al. (2009) Dual Gene Transfer of Fibroblast Growth Factor-2 and Platelet Derived Growth Factor-Bb Using Plasmid Deoxyribonucleic Acid Promotes Effective Angiogenesis and Arteriogenesis in a Rodent Model of Hindlimb Ischemia. *Transl Res* 153 (5): 232-239.
26. Deindl E. et al. (2003) Receptor-Independent Role of the Urokinase-Type Plasminogen Activator During Arteriogenesis *FASEB J*.
27. Deindl E. et al. (2001) Role of Ischemia and of Hypoxia-Inducible Genes in Arteriogenesis after Femoral Artery Occlusion in the Rabbit. *Circ Res* 89: 779-786.
28. Deindl E. et al. (2003) Involvement of the Fibroblast Growth Factor System in Adaptive and Chemokine-Induced Arteriogenesis. *Circ Res* 92: 561-568.

29. Demicheva E., M. Hecker and T. Korff. (2008) Stretch-Induced Activation of the Transcription Factor Activator Protein-1 Controls Monocyte Chemoattractant Protein-1 Expression During Arteriogenesis. *Circ Res* 103: 477-484.
30. Dvorak A.M. et al. (2000) RNA Is Closely Associated with Human Mast Cell Secretory Granules, Suggesting a Role(S) for Granules in Synthetic Processes. *J Histochem Cytochem* 48 (1): 1-12.
31. Eastmond N.C., E.M.S. Banks, and J.W. Coleman. (1997) Nitric Oxide Inhibits Ige-Mediated Degranulation of Mast Cells and Is the Principal Intermediate in Ifn-Gamm-Induced Suppression of Exocytosis. *J Immunol* 159: 1444-1450.
32. Ekström K. et al. (2012) Characterization of Mrna and Microrna in Human Mast Cell-Derived Exosomes and Their Transfer to Other Mast Cells and Blood Cd34 Progenitor Cells. *J Extracell Vesicles* 1: 18389.
33. Esmon C. and Owen W. (2004) The discovery of thrombomodulin. *J Thromb Haemost* 2: 209-213
34. Fischer S. et al. (2012) Extracellular RNA liberates Tumor Necrosis Factor-α to promote tumor cell trafficking and progression. *Cancer Res* 355 (3): 635-645.
35. Fischer S. et al. (2013) Extracellular RNA promotes leukocyte recruitment in the vascular system by mobilising proinflammatory cytokines. *Thromb. and Haemost* 73: 5080-5089.
36. Fischer S. et al. (2014) Impact of Extracellular Rna on Endothelial Barrier Function. *Cell Tiss Res* 108 (4).
37. Frank S.P.C. Rolle Von Snare-Proteinen Bei Der Mediatorfreisetzung Humaner Mastzellen. Fakultät Naturwissenschaften Universität Hohenheim, 2010.
38. Galli S.J. (1990) New Insights into the Riddle of the Mast Cells: Microenvironmental Regulation of Mast Cell Development and Phenotypic Heterogeneity. *Lab Invest* 62: 5-33.
39. Galli S.J. and Mindy Tsai. (2008) Mast Cells: Versatile Regulators of Inflammation, Tissue Remodeling, Host Defense and Homeostasis. *J Dermatol Sci* 49 (1): 7-19.
40. Gansler J. Das Vaskuläre Rna/Rnase-System: Funktionelle Aktivität Synthetischer Nukleinsäuren Und Extrazelluläre Rnase 1 Als Neuer Vaskulärer Regulator. Justus Liebig Universität, 2012.
41. Ghigo A. et al. (2012) Phosphoinositide 3-Kinases in Health and Disease. *Subcell Biochem* 58: 183-213.

42. Gounni A.S. (2006) The High-Affinity Ige Receptor (Fc Ri): A Critical Regulator of Airway Smooth Muscle Cells? *AJP - Lung Cellular and Molecular Physiology* 291: L312–L321.
43. Grundmann S. et al. (2004) Monocytes, but Not Neutrophils or Lymphocytes Are Essential Mediators of Arteriogenesis. *J Amer Coll Cardiol* 1045 (193).
44. Hamm C.W. et al. (2011) Esc Guidelines for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent St-Segment Elevation. *Europ Heart J* 32: 2999-3054.
45. Hartwell L.H. et al. *Genetics: From Genes to Genomes*. McGraw-Hill, 2004.
46. 45. Heissig et al. (2005) Low-dose irradiation promotes tissue revascularization through VEGF release from mast cells and MMP-9–mediated progenitor cell mobilization. *J Exp Med* 202 (6): 739-750
47. Hide Izumi et al. (1993) Degranulation of Individual Mast Cells in Response to Ca 2+ and Guanine Nucleotides: An All-or-None Event. *J Cell Biol* 123 (3): 585-93.
48. Hirsch E., C. Costa and E. Ciraolo. (2007) Phosphoinositide 3-Kinases as a Common Platform for Multi-Hormone Signaling. *J Endocrinol* 194: 243-256.
49. Hirsch E. et al. (2000) Central Role for G Protein-Coupled Phosphoinositide 3-Kinase Γ in Inflammation. *Science* 287 (5455): 1049-1053.
50. Honda T. et al. (2013) A Novel Strategy for Hemolytic Uremic Syndrome: Successful Treatment with Thrombomodulin A. *Pediatrics* 131 (3): 928-933.
51. Hsu Yun-Yan et al. (2012) Thrombomodulin Is an Ezrin-Interacting Protein That Controls Epithelial Morphology and Promotes Collective Cell Migration *FASEB J* 26: 3440-3452.
52. Iyer A.S. and A. August. (2008) "The Tec Family Kinase Itk Differentially Controls Mast Cell Responses. *J Immunol* 180 (12): 7869–7877.
53. Jensen B.M. et al. (2006) Generation, Isolation and Maintenance of Rodent Mast Cells and Mast Cell Lines. *Curr Protoc Immunol* Supplement 74.
54. Juncqueira, L.C., and J. Carneiro. *Histologie*. Heidelberg: Springer, 2004.
55. Kambayashi T. et al. (2009) Inducible Mhc Class Ii Expression by Mast Cells Supports Effector and Regulatory T Cell Activation. *J Immunol* 182: 4686–4695.
56. Kampmann A. et al. (2009) The Proteoglycan Osteoglycin/Mimecan Is Correlated with Arteriogenesis. *Mol Cell Biochem* 322: 15-23.

57. Keeley E.C., B. Mehrad and R.M. Strieter. (2008) Chemokines as Mediators of Neovascularization. *Atheroscl Thromb Vasc Biol* 28 (11): 1928-1936.
58. Kempuraj D. et al. (2010) Mercury Induces Inflammatory Mediator Release from Human Mast Cells. *J Neuroinfl* 7 (20).
59. Kobayashi M. et al. (2004) A Simple Mehtod of Isolating Mouse Aortic Endothelial Cells. *J Atheroscl Thromb* 12 (3): 138-142.
60. Kubo M. et al. (2009) Therapeutic Neovascularization by Nanotechnology-Mediated Cell-Selective Delivery of Pitavastatin into the Vascular Endothelium. *Atheroscl Thromb Vasc Biol* 36 (4): 253-260.
61. Kuhlmann M.T., R. Klocke and S. Nikol. (2007) Therapeutic Angiogenesis for Peripheral Artery Disease: Cytokine Therapy. *Eur J Vasc Med* 36 (4): 253-60.
62. Kulka M., N. Fukuishi and D.D. Metcalfe. (2009) Human Mast Cells Synthesize and Release Angiogenin, a Member of the Ribonuclease a (Rnase a) Superfamily. *J Leukoc Biol* 86: 1217-1226.
63. Laffargue M. et al. (2002) Phosphoinositide 3-Kinase Γ Is an Essential Amplifier of Mast Cell Function. *Immunity* 16: 441-451.
64. Limbourg A. et al. (2009) Evaluation of Postnatal Arteriogenesis and Angiogenesis in a Mouse Model of Hind-Limb Ischemia. *Nat Protoc* 4 (12): 1737-1748.
65. Lorentz A. and S.C. Bischoff. (2001) Regulation of Human Intestinal Mast Cells by Stem Cell Factor and Il-4 *Immunol Rev* 179: 57-60.
66. Lowe H.C., S.N. Oesterle and L.M. Khachigian. (2012) Coronary in-Stent Restenosis: Current Status and Future Strategies. *J Amer Coll Cardiol* 39 (2): 183-193.
67. Madeddu P. et al. (2007) Phosphoinositide 3-Kinase Γ Gene Knockout Impairs Postischemic Neovascularization and Endothelial Progenitor Cell Functions. *Arterioscl Thromb Vasc Biol* 28:68-76.
68. Malbec O. et al. (2007) Peritoneal Cell-Derived Mast Cells: An in Vitro Model of Mature Serosal-Type Mouse Mast Cells. *J Immunol* 178: 6465–6475.
69. Marquardt D.L., J.L. Alongi and L.L. Walker. (1996) The Phosphatidylinositol 3-Kinase Inhibitor Blocks Mast Cell Exocytosis but Not Il-6 Production. *J Immunol* 156 (5): 1942-1945.
70. Mathers C.D. and D. Loncar. (2006) Projections of Global Mortality and Burden of Disease from 2002 to 2030. *PLOS ONE* 3 (11): e442.

71. McCall-Culbreath K. et al. (2011) Selective, A2β1 Integrin-Dependent Secretion of Il-6 by Connective Tissue Mast Cells. *J Innate Immun* 3: 459-470.

72. McLachlan J.B. et al. (2008) Mast Cell Activators: A New Class of Highly Effective Vaccine Adjuvants. *Nat Med* 14 (5): 536-541.

73. Metcalfe D.D. (2008) Mast Cells and Mastocytosis. *Blood* 112 (4): 946-956.

74. Metcalfe D.D., Dana Baram, and Yoseph A. Mekori. (1997) Mast Cells. *Physiol Rev* 77 (4): 1033-1065.

75. Möller H.-J., G. Laux and A. Deister eds. *Duale Reihe Psychiatrie Und Psychotherapie*. Thieme, 2009.

76. Morgan A.J. and R. Jacob. (1994) Lonomycin Enhances Ca2+ Influx by Stimulating Store-Regulated Cation Entry and Not by a Direct Action at the Plasma Membrane. *Biochem J* 380: 665-672.

77. Morser J. (2012) Thrombomodulin Links Coagulation to Inflammation and Immunity. *Curr Drug Targets* 13 (3): 421-431.

78. Nigrovic P.A. et al. (2008) Genetic Inversion in Mast Cell-Deficient W^{sh} Mice Interrupts *Corin* and Manifests as Hematopoietic and Cardiac Aberrancy. *Immunopathol Infect Dis* 173 (6): 1693-1701.

79. Nishida K.S. et al. (2005) Fceri-Mediated Mast Cell Degranulation Requires Calcium-Independent Microtubule-Dependent Translocation of Granules to the Plasma Membrane. *J Cell Biol* 170: 115-126.

80. Ocak G. et al. (2014) Single Nucleotide Variants in the Protein C Pathway and Mortality in Dialysis Patients. *PLOS ONE* 9 (5): e97251.

81. Park H.J. et al. (2008) Coronary Collaterals: The Role of Mcp-1 During the Early Phase of Acute Myocardial Infarction. *Int J Cardiol* 130 (3): 409-13.

82. Peterson E.D. et al. (2010) Contemporary Mortality Risk Prediction for Percutaneous Coronary Intervention. *J Amer Coll Cardiol* 55 (18): 1923-1932.

83. Poh K.K. et al. (2014) Safety of Combination Therapy with Milrinone and Esmolol for Heart Protection During Percutaneous Coronary Intervention in Acute Myocardial Infarction. *Eur J Clin Pharmacol*.

84. Prior B. M., Yang, H. T., & Terjung R. L. (2004) What makes vessels grow with exercise training? *J App Physiol*.

85. Prussin C. and D.D. Metcalfe. (2003) IgE, Mast Cells, Basophils, and Eosinophils. *J Allergy Clin Immunol* 111: 486-494.

86. Puri N. and P.A. Roche. (2007) Mast Cells Possess Distinct Secretory Granule Subsets Whose Exocytosis Is Regulated by Different Snare Isoforms. *Proc Natl Acad Sci* 105 (7): 2580-5.

87. Ren B. et al. (2010) Erk1/2-Akt1 Crosstalk Regulates Arteriogenesis in Mice and Zebrafish. *J Clin Invest* 120(4): 1217–1228.

88. Sathyakumar M. et al. (2012) Immunohistochemical Evaluation of Mast Cells and Vascular Endothelial Proliferation in Oral Precancerous Lesion-Leukoplakia. *J Oral Maxillofac Pathol* 16 (3): 343-8.

89. Schaper J. et al. *Collateral Circulation Heart*. Oxford: Oxford University Press, 1999.

90. Schaper W. (2009) Collateral Circulation - Past and Present. *Bas Res Cardiol* 104: 5-21.

91. Schaper W. (2007) Prevention of Tissue Death by Killer Cells? The Role of the Immune System in Arteriogenesis. *Atheroscl Thromb Vasc Biol* 27: 2273-2274.

92. Schaper W. and K. Troidl. (2012) Arteriogenesis Versus Angiogenesis in Peripheral Artery Disease. *Diab Metab Res Rev* 28(1): 27-29.

93. Schierling W. et al. (2009) The Role of Angiogenic Growth Factors in Arteriogenesis *J Vas Res* 46(4): 365-374.

94. Scholz D. et al. (2002) Contribution of Arteriogenesis and Angiogenesis to Postocclusive Hindlimb Perfusion in Mice. *J Mol Cell Cardiol* 34(7): 775-787.

95. Schweitzer-Stenner R., I. Tamir, and I. Pecht. (1997) Analysis of Fceri-Mediated Mast Cell Stimulation by Surface-Carried Antigens. *Biophysiol J* 72:2470-2478.

96. Enzo Life Sciences. Compound 48/80 Trihydrochloride. 2010. Enzo Life Sciences Inc.2011.

97. Serruys P.W. et al. (2009) Percutaneous Coronary Intervention Versus Coronary-Artery Bypass Grafting for Severe Coronary Artery Disease. *New Engl J Med* 360(10): 961-972.

98. Shefler I., R. Seger, and R. Sagi-Eisenberg. (1999) Gi-Mediated Activation of Mitogen-Activated Protein Kinase (Mapk) Pathway by Receptor Mimetic Basic Secretagogues of Connective Tissue-Type Mast Cells: Bifurcation of Arachidonic Acid-Induced Release Upstream of Mapk. *J Pharmacol* 289(3): 1654-1661.

99. Shireman P.K. (2007) The Chemokine System in Arteriogenesis and Hind Limb Ischemia. *J Vasc Surg* 45(Suppl. A): A48-A56.

100. Siragusa M. et al. (2010) Involvement of Phosphoinositide 3-Kinase Γ in Angiogenesis and Healing of Experimental Myocardial Infaction in Mice. *Circ Res* 106(4): 757-768.

101. Sly L.M. et al. (2008) Ige-Induced Mast Cell Survival Requires the Prolonged Generation of Reactive Oxygen Species. *J Immunol* 181(6): 3850-3860.

102. Snustard P.D. and M.J. Simmons. *Principles of Genetics*. John Wiley & Sons, 2003.

103. Stempelj M. and I. Ferjan (2005) Signaling Pathway in Nerve Growth Factor Induced Histamine Release from Rat Mast Cells. *Infl Res* 54: 344-349.

104. Stopfer P., D.N. Männel and T. Helgans (2004) Lymphotoxin-Β Receptor Activation by Activated T Cells Induces Cytokine Release from Mouse Bone Marrow-Derived Mast Cells. *J Immunol* 172: 7459-7465.

105. Sun J. et al. (2007) Mast Cells Modulate the Pathogenesis of Elastase-Induced Abdominal Aortic Aneurysms in Mice. *J Clin Invest* 117: 3359-3368.

106. Tressel S.L. et al. (2008) Angiopoietin-2 Stimulates Blood Flow Recovery after Femoral Artery Occlusion by Inducing Inflammation and Arteriogenesis. *Atheroscl Thromb Vasc Biol* 28(11): 1989–1995.

107. Troidl C. and W. Schaper (2012) Arteriogenesis Versus Angiogenesis in Peripheral Artery Disease. *Diab Metab Res Rev* 28 (1): 27-29.

108. Ullerås E. et al. (2008) Nfat but Not Nf-Kb Is Critical for Transcriptional Induction of the Prosurvival Gene A1 after Ige Receptor Activation in Mast Cells. *Blood* 116 (6): 3081-3089.

109. Uz T. et al. (2008) Effects of Mk-886, a 5-Lipoxygenase Activating Protein (Flap) Inhibitor, and 5-Lipoxygenase Deficiency on the Forced Swimming Behaviour of Mice. *Neuroscience Letters* 436 (6): 269-272.

110. van Oostrom M.C. et al. (2008) Insights into Mechanisms Behind Arteriogenesis: What Does the Future Hold? *J Leukoc Biol* 84: 1379-1391.

111. van Royen N. et al. (2005) Start Trial - a Pilot Study of Stimulation of Arteriogenesis Using Subcutaneous Application of Granulocyte-Macrophage Colony-Stimulating Factor as a New Treatment for Peripheral Vascular Disease. *Circ Res* 112: 1040-1046.

112. van Weel V. et al. (2007) Natural Killer Cells and Cd4+ T-Cells Modulate Collateral Artery Development *Atheroscl Thromb Vasc Biol* 27: 2310-2318.

113. Viaro F. et al. (2008) Compound 48/80 Induces Endothelium-Dependent and Histamine Release-Independent Relaxation in Rabbit Aorta. *Nitric Oxide: Biology and Chemistry* 18 (2): 87-92.
114. Volná P. et al. (2004) Negative Regulation of Mast Cell Signaling and Function by the Adaptor Lab/Ntal. *J Exp Med* 200 (8): 1001-1013.
115. Wagner K.U. and J.W. Schmidt (2011). The Two Faces of Janus Kinases and Their Respective Stats in Mammary Gland Development and Cancer. *J Carcinog* 10 (32): 50-59.
116. Westvik T.S. et al. (2009) Limb Ischemia after Iliac Ligation in Aged Mice Stimulates Angiogenesis without Arteriogenesis. *J Vasc Surg* 49 (2): 464-473.
117. Wolf C. et al. (1998) Vascular Remodeling and Altered Protein Expression During Growth of Coronary Collateral Arteries. *J Mol Cell Cardiol* 30: 2291-2305.
118. Wu S. et al. (2010) Immunohistochemical Study of the Growth Factors, Afgf, Bfgf, Pdgf-Ab, Vegf-a and Its Receptor (Flk-1) During Arteriogenesis. *Mol Cell Biochem* 343 (1-2): 223-229.
119. Yamahata H. et al. (2012) Immunohistochemical Expression of Thrombomodulin in Vestibular Schwannoma. *Brain Tumor Pathol* 30 (1): 28-33.
120. Zi-Qing H. et al. (2007) Pretreatment of Sodium Prior to Reperfusion Attenuates Early Reperfusion Injury after the Small Intestine Ischemia in Rats. *World J Gastroenterol* 13 (38).

8.6 List of publications

Abstracts:

Mast cells contribute to arteriogenesis in a PI3 Kinase γ-dependent manner – Joint meeting of the European Society for Microcirculation (ESM) and the German Society of Microcirculation and Vascular Biology (GFMVB), October 2011 at the Ludwig-Maximilians-University Munich, Germany
Mauer A, Chillo O, Pagel JI, Reichel CA, Preissner KT, Fischer S, Deindl E
Journal of Vascular Research. Volume: 48 Supplement: 1 Pages: 82-82

Mast cells in arteriogenesis - a pre-clinical study employing a murine hindlimb model of collateral artery growth – Conference: Joint Meeting of the European-Society-for-Microcirculation (ESM)/Society-of-Microcirculation-and-Vascular-Biology (GfMVB). Journal of Vascular Research. Volume: 48. Supplement: 1. Pages: 133-133 Published: 2011
Chillo O, Pagel Jl, Mauer A, Caballero-Martinez A, Kleinert EC, Trenkwalder T, Mueller-Hoecker J, Troidl K, Fischer S, Preissner KT, Reichel C, Deindl E

Mast cells: Key players in leukocyte recruitment during arteriogenesis – Aegean Conferences, 10th International Conference on Innate Immunity, June 2013, Kos (Greece)
Chillo O, Pagel Jl, Mauer A, Caballero-Martinez A, Kleinert EC, Trenkwalder T, Kurz A, Mueller-Hoecker J, Troidl K, Fischer S, Preissner KT, Reichel C, Deindl E

The functional role of mast cells in Arteriogenesis - Doktamed day of the Ludwig-Maximilian-University Munich and the Technical University Munich; May 2011, Munich.
Chillo O, Pagel Jl, Mauer A, Trenkwalder T, Reichel C, Deindl E

Oral presentations

Mast cells in arteriogenesis – Walter Brendel Centre doctoral student day, March 2012, Munich.
Chillo O, Pagel Jl, Mauer A, Trenkwalder T, Reichel C, Deindl E

Original articles:

Mast cell activation protects tissue from severe damage by promoting the growth of natural bypasses (original article in revision)
Omary Chillo, Judith Irina Pagel, Eike Christian Kleinert, Amelia Caballero, Silvia Fischer, Annika Mauer, Kerstin Troidl, Angela Kurz, Sue Chandraratne, Gerald Assmann, Josef Mueller-Hoecker, Steffen Massberg, Norbert Weissmann, Klaus T. Preissner, Christoph Reichel, Elisabeth Deindl.

Erklärung zur Dissertation

„Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig und ohne unzulässige Hilfe oder Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nichtveröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten sowie ethische, datenschutzrechtliche und tierschutzrechtliche Grundsätze befolgt. Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen, oder habe diese nachstehend spezifiziert. Die vorgelegte Arbeit wurde weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde zum Zweck einer Promotion oder eines anderen Prüfungsverfahrens vorgelegt. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Insbesondere wurden alle Personen genannt, die direkt und indirekt an der Entstehung der vorliegenden Arbeit beteiligt waren. Mit der Überprüfung meiner Arbeit durch eine Plagiatserkennungssoftware bzw. ein internetbasiertes Softwareprogramm erkläre ich mich einverstanden.“

________________________ __________________________________

Ort, Datum Annika Mauer