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MPO-mediated monocyte and macrophage activation upon myocardial infarction

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1 List of abbreviations

AMI	Acute myocardial infarction
ApoE	Apolipoprotein E
BMDM	Bone marrow derived macrophages
CCL-2	CC-chemokine ligand type 2
CCL-8	CC-chemokine ligand type 8
CCR-2	CC-chemokine receptor type 2
CD11b	Cluster of differentiation molecule 11b
CM	Classical monocytes
Csf1r/CD115	Colony stimulating factor 1 receptor
CX3CR1	C-X3-X motiv chemokine receptor 1
Cx43	Connexin 43
DAMP	Danger-associated molecular patterns
DC	Dendritic cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linke immunosorbent assay
F4/80	Epidermal growth factor-like module-containing mucin-like hormone receptor-like 1
FCS	Fetal calf serum
GAPDH	Glycerinaldehyde-3-phosphat-dehydrogenase
HOSCN	Hypothiocyanous acid
i.p.	Intraperitoneal
i.v.	Intravenous
I/R	Ischemia/reperfusion
IFN-γ	Interferon-γ

IL-10	Interleukin 10
IL-23	Interleukin-23
IL-4	Interleukin 4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthetase
LAD	Ramus interventricularis anterior, left anterior descending
LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz
LV	Left ventricle/ventricular
Ly6C	Lymphocyte antigen 6C
M-CSF	Macrophage-colony stimulating factor
MDP	Monocyte-marcrophage dendritic cell progenitor
MHC-II	Major histocompatibility complex type 2
MI	Myocardial infarction
MPO	Myeloperoxidase
MPS	Mononuclear phagocyte system
mRNA	Messenger ribonucleic acid
NCM	Non-classical monocytes
NFκB	Nuclear factor κ-light chain enhancer of activated B-cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Permanent ischemia
PMN	Polymorphonuclear leukocytes
qRT-PCR	Quantitative real-time polymerase chain reaction
ROS	Reactive oxygen species

RyR	Ryanodine receptor
sCD40L	Soluble cluster of differentiation 40 ligand
SEM	Standard error of the mean
TIE-2	Angiopoetin 1 receptor
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TnT	Troponin T
VEGF	Vascular epithelial growth factor
WT	Wild type

Table 1: List of abbreviations

2 Abstract

Acute myocardial infarction is one of the major health burdens in western hemisphere and has a high mortality[1–4]. Plasma levels of the polymorphonuclear neutrophil (PMN)-derived enzyme myeloperoxidase (MPO) have been shown to correlate with the prognosis and severity of myocardial infarction[5–7]. Apart from its capacity to catalyse the generation of highly reactive oxygen species (ROS) MPO has been shown to attract and activate PMN[8]. However, its effect on circulating monocytes and infiltrating macrophages, which are typically attracted by PMN and which are major effector cells of myocardial structural remodelling after infarction has never been tested. Herein we evaluate the role of MPO on monocyte and macrophage activation in vitro and in vivo and finally transfer our observations to a murine model of myocardial infarction.

Peritoneal macrophages were harvested after thioglycolate stimulation in wild-type (WT) and MPO-deficient (*Mpo*$^{-/-}$) mice. mRNA analysis by quantitative Realtime PCR revealed enhanced macrophage activation as assessed by increased expression of TNF-α in cells from WT - compared to *Mpo*$^{-/-}$ animals. Similarly, bone marrow derived macrophages (BMDM) harvested from WT mice showed increased mRNA expression of the proinflammatory cytokines iNOS, TNF-α, interleukin 6 and interleukin 23 upon 24 h of MPO treatment. In vivo, intraperitoneal injection of MPO in *Mpo*$^{-/-}$ mice resulted in a marked increase in peritoneal leukocyte recruitment compared to injection of saline. Flow cytometric differentiation of cells revealed an increased percentage of macrophages after MPO-injection.

To confirm our results in a disease model, hearts from WT-and *Mpo*$^{-/-}$ mice were harvested 3 days after permanent LAD ligation and macrophage infiltration was assessed by immunohistochemical F4/80 stainings. *Mpo*$^{-/-}$ mice showed reduced macrophage infiltration in the infarct- and peri-infarct area as compared to WT animals.

To figure out whether increased macrophage-infiltration in WT-mice is a result of increased infiltration or a result of eased mobilization of monocytes from the spleen, animals were ligated and spleens and blood were harvested 1d after LAD. Blood monocytes were analysed via flow cytometry and splenic monocytes were quantified via immunohistochemical CD11b stainings. Blood monocytes showed no significant difference in distribution of Ly6C^{hi}- and Ly6C^{lo}-monocytes between the groups, but splenic mobilisation was significantly decreased in *Mpo*$^{-/-}$ mice.

As a conclusion, our data identify monocyte and macrophage activation as a novel pathway by which the neutrophil-derived enzyme MPO aggravates adverse post-infarct ventricular remodelling.

3 Zusammenfassung

Der akute Myokardinfarkt ist eine der größten Gesundheitsbelastungen in der westlichen Hemisphäre und hat eine hohe Mortalität[1–4]. Es hat sich gezeigt, dass die Plasmaspiegel des von polymorphkernigen neutrophilen Granulozyten (PMN) sekretierten Enzyms Myeloperoxidase (MPO) mit der Prognose und dem Schweregrad eines Myokardinfarkts korrelieren[5–7]. Neben ihrer Fähigkeit, die Bildung hochreaktiver Sauerstoffspezies (ROS) zu katalysieren, hat sich gezeigt, dass MPO PMN anzieht und aktiviert[8]. Seine Wirkung auf zirkulierende Monozyten und infiltrierende Makrophagen, die typischerweise von PMN angezogen werden und wichtige Effektorzellen des strukturellen Myokardumbaus nach einem Infarkt sind, wurde jedoch nie untersucht. In dieser Arbeit untersuchen wir die Rolle von MPO bei der Aktivierung von Monozyten und Makrophagen in vitro und in vivo und übertragen unsere Beobachtungen schließlich auf ein Mausmodell des Myokardinfarkts.

Peritoneale Makrophagen wurden nach Thioglykolatstimulation in Wildtyp- (WT) und MPO-defizienten (*Mpo*$^{-/-}$) Mäusen isoliert. Eine mRNA-Analyse mittels quantitativer Echtzeit-PCR ergab eine verstärkte Makrophagenaktivierung, die durch eine erhöhte Expression von TNF-α in Zellen von WT- im Vergleich zu *Mpo*$^{-/-}$ -Tieren bestimmt wurde. Ebenso zeigten Makrophagen aus dem Knochenmark (BMDM) von WT-Mäusen nach 24 Stunden Exposition zu MPO eine erhöhte mRNA-Expression der proinflammatorischen Zytokine iNOS, TNF-α, Interleukin 6 und Interleukin 23. In vivo führte die intraperitoneale Injektion von MPO bei *Mpo*$^{-/-}$ -Mäusen zu einem deutlichen Anstieg der peritonealen Leukozytenrekrutierung im Vergleich zur Injektion von Kochsalzlösung. Die durchflusszytometrische Differenzierung der Zellen zeigte einen erhöhten Anteil an Makrophagen nach MPO-Injektion.

Um unsere Ergebnisse in einem Krankheitsmodell zu bestätigen, wurden Herzen von WT- und *Mpo*$^{-/-}$-Mäusen 3 Tage nach permanenter LAD-Ligatur entnommen und die Makrophageninfiltration durch immunhistochemische F4/80-Färbungen untersucht. *Mpo*$^{-/-}$-Mäuse zeigten im Vergleich zu WT-Tieren eine geringere Makrophageninfiltration im Infarkt- und Periinfarktbereich.

Um herauszufinden, ob die erhöhte Makrophagen-Infiltration bei WT-Mäusen auf eine erleichterte Infiltration oder auf eine erleichterte Mobilisierung von Monozyten aus der Milz zurückzuführen ist, wurden die Tiere ligiert und Milz und Blut 1d nach LAD entnommen.

Die Monozyten im Blut wurden mittels Durchflusszytometrie analysiert, und die Monozyten in der Milz wurden mittels immunhistochemischer CD11b-Färbungen quantifiziert. Bei den Blutmonozyten zeigte sich kein signifikanter Unterschied in der Verteilung zwischen $Ly6C^{hi}$- und $Ly6C^{lo}$-Monozyten. Jedoch war die Anzahl an Monozyten in der Milz bei *Mpo*$^{-/-}$-Mäusen signifikant verringert.

Unsere Daten zeigen, dass die Aktivierung von Monozyten und Makrophagen ein möglicher Weg ist, über den MPO die nachteiligen Folgen des ventrikulären Umbaus nach einem Myokardinfarkt verstärkt.

4 Introduction

4.1 Myocardial Infarction

4.1.1 Epidemiology

Acute myocardial infarction (AMI) is one of the global major health problems [1]. In 2010 AMI caused over 7,000,000 million deaths worldwide, which are 13.3 % of total deaths, and with a rate of 5.3 % was the second most important reason of death in Germany [9,10]. The overall prevalence between 2011 to 2014 for American inhabitants over 20 adds up to 3.0 % according to an annual incidence of 605.000 new and 200.000 recurrent AMI [11,12]. Consequently, every 40 seconds one AMI occurs in the USA [11]. Although studies have shown that the relative incidence and mortality of AMI has been declining in developed countries, due to an ageing population AMI will still be the leading cause of death worldwide by the year 2030 [2–4,13–15].

4.1.2 Pathophysiology

Following the third universal definition of myocardial infarction, AMI is defined as "myocardial cell death caused by sustained ischemia due to different reasons". In the majority of cases it is a result of advanced atherosclerosis and can either follow earlier symptoms like stable angina pectoris or be the first manifestation of the disease [16].

In rare cases AMI can also be induced by coronary dissection, arteritis, myocardial bridges, thromboembolism or coronary vasospasm without obvious coronary artery disease [17]. Atherosclerosis, which is characterized by lipid deposition and chronic vascular inflammation driven by activation of the immune system leads to formation of vascular plaques [18]. A thin fibrous cap protects the plaques' lipid-rich core from the blood compartment [19]. Circulating monocytes infiltrate the plaque and differentiate into highly activated macrophages, the so called foam cells, that further promote inflammation [20]. There are two typical plaque complications that are connected to AMI, plaque erosion and plaque rupture. Inflammation leads to erosion and denudation of the endothelium which exposes the subendothelial connective tissue to the blood. Furthermore, macrophage-driven inflammation is weakening the fibrous cap and finally leading to its disruption via inhibiting collagen synthesis and supporting smooth muscle cell death. Both processes finally lead to the exposure of the highly thrombogenic plaque surfaces to circulating platelets and the coagulation system resulting in the formation of an endoluminal thrombus. Consequently, this thrombus can cause ischemia via occlusion of the coronary vessel, distal embolization or provoking vasospasm at the site of the culprit lesion [17,19,21] .

After 10-15 seconds of ischemia the myocardium becomes cyanotic and cardiomyocytes loose contractility [22]. Studies in animal as well as in human have shown that survival after reperfusion is strongly dependent on the ischemic time. In animal models, after 18 minutes of ischemia all cardiomyocytes survive without damage, whereas after 20 minutes started to die, after 40 minutes half and after 60 min all cells become necrotic [22–25]. In humans an ischemic time of 2 to 4 hours leads to complete necrosis of the infarcted area, which depends on characteristics of the collateral circulation, persistent or intermittent coronary occlusion, the sensitivity of the myocytes to ischemia and individual demands on oxygen and nutrition [26].

4.1.3 Acute cardiac remodelling and post-infarct inflammation

The process of post infarction cardiac or ventricular remodelling was first defined by Tennant and Wiggers in 1935. According to their definition, cardiac structural remodelling includes dilatation of the ventricle, scar formation of infarcted tissue and a transformation of the ventricular geometry [27]. Later, in 1978, Hutchins and Bulkley added wall thinning to this definition [28].

Acute myocardial infarction can be considered as a sterile inflammation [29]. The release of so called “danger associated molecular patterns” (DAMPs) activates Toll-like receptors and triggers the complement system. This leads to invasion and activation of innate immune cells [30]. The first leukocytes, predominantly polymorphonuclear neutrophile granulocytes (PMN), invade the infarcted area within minutes. They peak on day three after ischemia and accumulate up to 14 days in the infarcted myocardium [29,31]. Infiltrating monocytes and macrophages outnumber the count of neutrophils shortly after, even within the first hours. During the early phase of post-infarct inflammation, proinflammatory subsets of monocytes migrate into the damaged heart, followed by regulatory subsets in later phases [32]. Lower counts of other leukocytes like dendritic cells, lymphocytes or mast cells can also be found in the infarcted myocardium, orchastrating the inflammatory reaction [33–36].

Cardiac tissue after injury can be divided into three phases. The first phase is the inflammatory phase, followed by the proliferative phase and the third phase is the maturation phase. During the inflammatory phase, the infarcted area is cleared of perished cells and debris by neutrophiles and monocytes/macrophages to be prepared for the proliferative phase. In this phase, the dominant cell subsets of monocytes and macrophages are more regulative and secrete growth factors. The third phase consists of maturation of the new formed tissue like cross-linking of collagen and ends in scar formation [37–39].

4.2 Polymorphonuclear neutrophile granulocytes

Polymorphonuclear neutrophile granulocytes (PMN), are one of the main cellular mediators of the innate immune system and a key player in host defence and in initiating inflammatory reactions, as they are the first immune cells to infiltrate inflammatory sites. After infiltration, PMN degranulate and release their highly reactive reagents and enzymes, e.g. myeloperoxidase (MPO), into the damaged myocardium. Another important function is the secretion of chemotactic cytokines to attract other cellular members of the immune system like monocytes/macrophages and dendritic cells [40–42]. As a consequence, recent studies have shown that depletion of neutrophiles in mice subjected to myocardial infarction result in impaired ventricular function, exaggerated fibrosis and increased progression of heart failure [43].

4.2.1 MPO

Myeloperoxidase is a homodimeric heme-containing enzyme with a molecular weight of 140 kDa and an intense green colouring. Each monomer is composed of a heavy chain (55-64 kDa) and a light chain (10-15 kDa) [44,45]. It is mainly expressed in neutrophile granulocytes where it is stored in the azurophilic granules and can reach up to 5 % of the total protein weight [40,41]. Other sources for MPO are monocytes, with a proportion up to 1 % of total protein weight, and macrophages [46,47]. Although it was thought that macrophages lose their ability to express MPO during maturation [48–50], more recent studies have shown that subsets of macrophages are capable of expressing MPO and play a crucial role in atherosclerosis [51,52].

4.2.1.1 Catalytic activity and pathogen defence

MPO reaction catalysis occurs in a so called "MPO-system" consisting of the enzyme itself, an oxidizable reactant, probably most often a halide, and hydrogen peroxide (H_2O_2), that is usually generated by dismutation of hyperoxid. The main function is to generate reactive oxidants [53]. In the first step, called catalytic cycle, ground state MPO is oxidized by H_2O_2 forming an active state called Compound I and H_2O. Compound I then oxidates chloride (Cl^-) to hypochlorous acid (HOCl) and is reduced to its ground state. This reaction is called halogenation cycle. In situations where H_2O_2 is abundant, for example during an oxidative burst in pathogen defence, Compound I can react with H_2O_2 to Compound II [54–56]. Compound II can be reduced to the ground state, active MPO ($MPO\text{-}Fe^{III}$) under consumption of hyperoxide (O_2^-), which is also generated from the oxidative burst. This reaction is called peroxidase cycle [57–59].

Furthermore hyperoxide has the capability to react directly with ground state MPO to the instable state Compound III, which decays back to the ground state enzyme spontaneously [60]. Not only halides are oxidated, of whom chloride is most likely because of its physiological high concentration, but also are tyrosin or nitric oxide ($NO^{\cdot}$). MPO, H_2O_2 and tyrosin directly react to form tyrosyl radicals [61] and the MPO-system consumes $NO^{\cdot}$ to react to nitric dioxide (NO_2) [62].

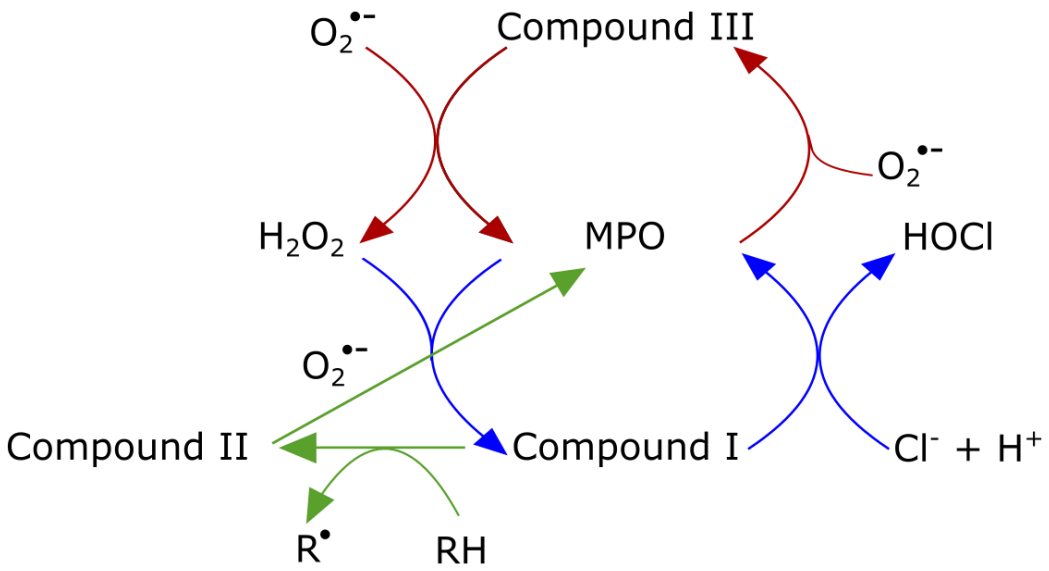

Figure 1: MPO reaction system (adapted from Klebanoff et al., 2013)[63]. MPO is oxidized by H_2O_2 to Compound I, under consumption of Cl^- and H^+ reduced to MPO and HOCl (halogenation cycle, blue arrows)[54–56]. Compound I can also react with H_2O_2 to Compound II, which can be reduced under consumption of O_2^- to ground state MPO (peroxidase cycle, green arrows)[57–59]. O_2^- can directly react with MPO and form Compound III, which decays back to the ground state spontaneously (red arrows)[60].

The catalytic products are highly reactive, especially HOCl. Due to its radical character, it is capable to oxidate organic groups and, due to its close location to the phagosome or on the surface of microbes, it leads to several antimicrobial processes, e.g. disfunction of microbial membrane transport and failure of the membrane electron transport chain. Subsequently this results in effective microbial cell death [64–66]. Although *in vitro* data suggest that the MPO-system is crucial for immune defence, clinical data are contrary. MPO-deficiency has a prevalence of one in 2000-4000 healthy individuals in north America and Europe, but the vast majority of concerned people are healthy [67–70]. There are signs that these persons might be more vulnerable for infections with *Candida albicans*, especially in interaction with diabetes mellitus, but the topic of the importance of the MPO-system for pathogen immune defence is controversial discussed in the literature [63,70–74].

4.2.1.2 MPO dependent PMN activation

Studies have shown, that MPO is a crucial protagonist in inflammation through its catalytical and non-catalytical properties. It shows involvement in multiple signalling cascades in different cell types involved in initiation and resolution of inflammation such as neutrophiles and monocytes [75].

MPO can interact with β_2-integrins (CD11b/CD18) which leads to activation of signal-transduction pathways in PMN like p38 mitogen-activated protein kinase (p38 MAPK) and nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB). Furthermore, it stimulates tyrosine phosphorylation, production of superoxide, degranulation and increases the expression of integrins (CD11b/CD18) [76–78]. MPO mediates neutrophil extravasation due to its strong cationic charge: MPO easily binds to the anionic wall of vessels and to surface of neutrophils which allows a closer contact and increased recruitment independent from catalytic activity [8]. In addition, MPO-binding raises intracellular Ca^{2+}-levels [79]. Taken together, MPO effects in neutrophils an enhanced activation, prolonged survival and amplified recruitment.

4.2.1.3 MPO and cardiac remodelling

The CAPTURE-Trial in 2003 showed that MPO is a powerful predictor of cardiovascular death in patients with acute coronary syndrome. Increased MPO-levels were specifically identified in patients with low TnT-levels but with high cardiovascular risk and had a reasonable prognostic value independent from TNT-levels or sCD40L-levels [5]. MPO promotes adverse cardiac remodelling. It is known that MPO-generated oxidants lead to increased LV-dilation and decreased LV-function in a post-AMI setting. Furthermore less collagen deposition can be found in mice genetically deficient for *Mpo* (*Mpo*$^{-/-}$) as compared to MPO competent (wildtype: WT) animals which correlated with a delayed early death caused by myocardial rupture [6,7]. MPO stimulates enzymatic degradation of Cx43 and induces fibroblast-to-myofibroblast trans-differentiation which leads to increased fibrosis after AMI and subsequently to augmented ventricular arrhythmias in terms of proarrhythmogenic myocardial remodelling [80].

On the other hand the application of PF-1355, a mechanism-based enzyme inhibitor for MPO, in mice with LAD-ligation resulted in decreased CD11b expression as well as in lower numbers of pro-inflammatory Ly-6C^{high}-monocytes and consequently attenuates cardiac remodelling and improves cardiac functional outcome via less inflammation and less oxidative stress [81].

4.3 Monocytes/Macrophages

4.3.1 Origin and maturation

Monocytes and macrophages are part of the mononuclear phagocyte system (MPS) together with dendritic cells (DC) and their common progenitor in the bone marrow, the monocyte-macrophage DC progenitor (MDP) [82,83]. The MPS is crucially involved in tissue homeostasis and reparation as well as in initiation and regulation of inflammatory reactions during innate immune responses. Monocytes are systemically circulating throughout the body but can also be stationary found within the bone marrow or in tissues such as the spleen. In steady state there is usually no proliferation of the circulating population, and they represent up to 10 % of total leukocytes in human blood und up to 4 % in mouse blood. In case of inflammation they become activated and infiltrate the affected tissue [84,85]. Monocytes can directly produce cytokines and antimicrobial reagents or they are able to differentiate into macrophages or DC [86]. The common progenitor cell, the MDP, is derived from myeloid stem cells and can give rise to monocytes. All these processes take place in the bone marrow [87–89]. Unlike myeloid stem cells the MDP is not able to differentiate into granulocytes [90]. The generation and maturation of the MPS is regulated via cytokines and their interactions with receptors. Critically for proliferation of monocytes is the expression of Csf1r (also known as CD115) on the surface of the cell and the stimulation through Csf1 (also known as M-CSF) and interleukin-34 [91–93]. After leaving the bone marrow, monocytes patrol the blood stream for about 3 days before migrating to specific tissues where they finally differentiate into macrophages or DC [94]. Mature murine monocytes divide into two subsets that are called classical monocytes (CM) and non-classical monocytes (NCM) with distinct functions in inflammation and tissue homeostasis [95]. The cells are categorized depending on their expression levels of lymphocyte antigen 6 complex (Ly6C) and fractalkine receptor (CX3CR1). CM express high levels of Ly6C, CCR-2 and CD62 but low levels of CX3CR1. The existence of CCR-2, which is a receptor for CCL-2 (MCP-1), allows them to infiltrate inflammatory tissues as CCL-2 is secreted at inflammatory sites, furthermore it is crucial for the cells for entering the blood stream after being produced in the spleen. Through either secretion of pro-inflammatory cytokines like TNF-alpha as well as phagocytosis and proteolysis CM contribute to the digestion of damaged tissue and support inflammation. NCM are characterized as $Ly6C^{lo}$, $CCR\text{-}2^{lo}$ and $CD62^{lo}$ but $CX3CR1^{hi}$. Via CX3CR1 they are able to infiltrate healthy tissue and seem to play a role in the resolution of inflammation and in promoting tissue healing by VEGF secretion to support angiogenesis and by phagocytosis[31,96–98]. Mature human monocytes are similarly classified on depending on their expression levels of CD14 and CD16 for CM ($CD14^{hi}$, $CD16^{lo}$) and NCM ($CD14^{lo}$, $CD16^{hi}$).

Furthermore they show comparable properties and fulfil comparable functions as compared to murine monocyte subsets [99]. In addition, there is a third subset in human monocytes called intermediate monocytes (IM) with high expression levels of CD14 and CD16. The function of is poorly understood, but it is known that they express high levels of the major histocompatibility complex class II (MHC-II) and angiopoietin 2-receptor (TIE-2) so they are thought to play a role in angiogenesis and inflammation [100].

Macrophages were orignially described as resident phagocytotic cells in lymphoid as well as in non-lymphoid tissue. In their physiological non inflammatory condition, they contribute to tissue homeostasis via clearence of apoptotic cell debris. In inflammation they support and coordinate the immune reaction through phagocytosis and the secretion of pro- or anti-inflammatory cytokines [101,102]. When first described as part of the MPS, macrophages were thought to be fully renewed and maintained through infiltrating blood monocytes and that there were no resident macrophages [94]. But today it is commonly known that in many tissues, like in the myocardium, there is a population of embryonic derived monocyte-independent resident tissue macrophages that are renewed by themselves which fulfil a wide range of specific tasks like tissue maintenance and regeneration [103,104]. Recent studies have suggested that monocytes contributing to tissue macrophage population may be a kind of emergency pathway to ensure tissue homeostasis [105].

Macrophages play a crucial role in host defence and in sterile inflammation like AMI. They can be activated through various stimuli and show different states of activation depending on the specific stimulus [106]. To characterize different macrophage polarizations, Mills et al. proposed the M1/M2 system that differentiates between inflammatory M1-macrophages and regulatory M2-macrophages [107]. M1-macrophages are activated by IFN-γ, TNF-α but also TLR-binding structures like LPS and express a wide panel of pro-inflammatory cytokines and chemokines like iNOS, TNF-α, IL-6, IL-23. M2-macrophages are activated by IL-4 and express more regulative cytokines and chemokines like Arginase-1, IL-10 or CCL8 [108]. Recent papers have stated that the M1/M2 dichotomy seems more like extremes of a wide range of either pro-inflammatory or regulatory properties of activated macrophages and that they should better be named as M1-like or M2-like macrophages [106,108–110]. M2-like macrophages can be further subdivided into M2a-M2d and differ in functions and cytokine production. M2a macrophages are more involved in anti-inflammation and wound healing, M2b macrophages in immune regulation and promote infection. M2c macrophages are responsible for phagocytosis and tissue remodelling and M2d support angiogenesis [111,112].

In addition, Erbel et al. stated yet another subset of macrophages induced by CXCL4 called M4 macrophages. They are thought to be pro-inflammatory and are associated with plaque-instability in atherosclerosis [113,114].

4.3.2 Role of monocytes in myocardial infarction and ventricular remodelling

Myocardial infarction and following ischemia of myocardium could be seen as a wound in the heart, and it triggers similar mechanisms like a trauma or surgery [32]. Shortly after ischemia, leukocytes start accumulating in the apoptotic tissue and infiltrating monocytes outnumber neutrophils within the first hour.

15-30 minutes after ischemic injury circulating monocytes start to invade the cardiac tissue, but after a short while, they are complemented from monocytes derived from the spleen. These splenic monocytes are cultivated in the subcapsular red pulp of the organ and easily excel the number of circulating monocytes. Studies with splenectomized mice revealed that increased monocyte production in the bone marrow contributes late to following monocytosis in blood after myocardial infarction. Swirski et al. have also shown that mobilization of splenic monocytes seems to be crucially dependent on Angiotensin-II – Angiotensin-II-subtype-I-receptor-(ATI)-signaling [85,115,116]. As mentioned above, cardiac remodeling after MI can be subdivided into three phases. In the early phase, $Ly6C^{hi}$ monocytes preferentially infiltrate the myocardium via CCR2-CCL2 interaction. CCL2, also known as MCP-1, is highly expressed in damaged tissue. The domination of $Ly6C^{hi}$ monocytes in the myocardium is not only driven by increased and eased infiltration, but also by selective amplified proliferation of $Ly6C^{hi}$ monocytes in the bone marrow and in the spleen. Infiltrated $Ly6C^{hi}$ monocytes secrete proinflammatory cytokines like TNFα to form an acute inflammatory environment. In the second phase after 4-7 days, $Ly6C^{hi}$ monocyte numbers increase and $Ly6C^{lo}$ monocytes begin to outline proinflammatory monocytes but do not reach comparable levels. These more "regulative" type of monocytes enter the infarction by CX3CR1 receptor interaction and show reparative properties like deposition of extracellular matrix and secretion of VEGF which leads to angiogenesis [31,117]. Although $Ly6C^{lo}$ monocytes were thought to be a distinct subgroup of monocytes that derive directly from the bone marrow, recent studies have shown that $Ly6C^{hi}$ monocytes can differentiate into $Ly6C^{lo}$ monocytes when required [118].

In steady state, resident tissue macrophages in the heart represent a distinct population compared to monocyte-derived macrophages. After myocardial infarction, monocyte-derived macrophages and their proliferation also contribute crucially to the expansion of cardiac macrophages in the infarcted tissue and even excel the number of resident tissue macrophages [103,104,119]. A recent study revealed evidence that resident tissue macrophages within the heart vanish after permanent coronary ligation and are later replenished by infiltrating blood monocytes [120]. Macrophages in the infarcted tissue show more proinflammatory M1-like but can later switch to a more intermediate type with M1 gene expression and some M2 reparative gene products [29]. In the early phase after MI, those proinflammatory M1-like macrophages are responsible for clearance of debris and phagocytosis of apoptotic myocardial cells whereas later M2-like macrophages mediate the resolution of inflammation, neovascularization and the formation of scar tissue [121,122].

In a murine model of induced atherosclerosis in $ApoE^{-/-}$ animals and following $Ly6C^{hi}$-monocytosis Panizzi et al. have shown that in an experiment with permanent coronary ligation, mice with monocytosis showed a significantly worse outcome than mice without monocytosis. Mice with stronger monocytosis showed higher inflammatory activity and an explicit deteriorated ejection fraction during the first 3 weeks after coronary ligation. This identifies $Ly6C^{hi}$-monocytosis as a marker for adverse cardiac remodeling [123]. Furthermore, several trials have investigated the relation between monocytosis following MI and outcome of MI heart function in human. Maekawa et al. found a very strong correlation between peak monocyte count after MI and heart failure as well as development of cardiac aneurysm. In addition, monocyte counts showed positive correlation to left ventricular end-diastolic volume and negative correlation to ejection fraction [124]. A retrospective trial by Mariani et al. showed that monocytosis post MI is a marker for worse contractile recovery after 6 months [125]. There is also evidence that low infiltration of inflammatory monocytes with a subsequently decreased level of monocyte derived macrophages in the infarcted myocardium show beneficial effect on structural remodeling especially within the early phase post ischemia. Mice with impaired CCL2/CCR2 interaction either by deletion of the CCL2 gene ($CCL2^{-/-}$) or by application of neutralizing antibodies against CCL2, show attenuated dilatation of the left ventricle post MI, although the long-term outcome remains unclear [117,126,127].

5 Aim of the study

MI is one of the leading causes of deaths in the western world and is known to be a major burden on the health system in industrial countries. Acute death after MI contributes to this high mortality.

Both MPO and MPS are known to play pivotal roles in post-infarct remodelling and, from a clinical point of view, level and/or activation of both are valuable predictors for adverse outcome in patients after MI.

The question if both are linked to each other, or if changes in the level of MPO might have additional effects on structural remodelling via MPS modulation, remains poorly investigated. Thus, the aim of this study is to uncover a possible role of MPO on the activation and migration of monocytes and macrophages in a setting of acute myocardial infarction.

6 Material and Methods

6.1 Material

6.1.1 Equipment

Name	Producer
BD FACS Canto II	Bioscience
BZ-9000	Keyence
C1000 Touch Thermal Cycler	BioRad
DMLB	Leica
Multiskan FC	Thermo Scientific
SterilGARD	Baker
SUB Aqua 26 plus	Grant
T3 Thermocycler	Biometrics
Tissuelyser	Quiagen
Titramax 100 T	Heidolph
Zentrifuge 5810 R	Eppendorf

Table 2: Equipment

6.1.2 Chemicals and Reagents

Name	Producer
Aceton	AppliChem Panreac
AEC (3-Amino-9-ethylcarbazol)	Sigma-Aldrich
Ampuwa	Fresenius Kabi
Bovine serum albumin (BSA)	PAN-Biotech
Dulbecco's modified eagle medium	Gibco
EDTA (Ethylenediaminotetraacetat)	AppliChem Panreac
FCS	Pan Biotech
H_2O_2	Merck
hMPO	Planta Natural products

O.C.T. Compound mounting medium	Tissue-Tek
PBS Tablets	Gibco
Penicillin/Streptomycin	Sigma-Aldrich
Triton X-100	Sigma-Aldrich

Table 3: Chemicals and Reagents

6.1.3 Kits

Name	Producer
Quantitect Reverse Transcription Set	Qiagen
RNeasy mini kit	Qiagen
SsoFast EvaGreen Supermix with low ROX	BioRad

Table 4: Kits

6.1.4 Antibodies

Name/Epitope	Serial number	Producer	Conjugation	Host
Anti-CD45	Ab10558	Abcam	-	Rabbit
Anti-Ly6C	130-102-341	Miltenyi	APC	Rat
Anti-CD115	12-1152-82	eBioscience	PE	Rat
Anti-F4/80	123120	Biolegend	Alexa Fluor 488	Rat
Anti-CD11b	53-0112-80	eBioscience	Alexa Fluor 488	Rat
Anti-rabbit IgG	Sc-3739	Santa Cruz	PE	Goat
Anti-rabbit IgG	A11034	Life Technologies	Alexa Fluor 488	Goat

Table 5: Antibodies

6.1.5 Primer

Name	Forward	Backward
iNOS	5´-CTC ACT GGG ACA GCA CAG AA-3´	5´-GGT CAA ACT CTT GGG GTT CA-3´
TNFα	5´-CCC CAA AGG GAT GAG AAG TT-3´	5´-CAC TTG GTG GTT TGC TAC GA-3´

IL6	5´-TACCACTTCACAAGTCGGAGGC-3´	5´-CTGCAAGTGCATCATCGTTGTTC-3´
IL23	5´-GCA CCT GCT TGA CTC TGA CA-3´	5´-TAG AAC TCA GGC TGG GCA TC-3´
Arginase	5´-CATTGGCTTGCGAGACGTAGAC-3´	5´-GCTGAAGGTCTCTTCCATCACC-3´
IL10	5´-CGGGAAGACAATAACTGCACCC-3´	5´-CGGTTAGCAGTATGTTGTCCAGC-3´
GAPDH	5´-CATCACTGCCACCCAGAAGACTG-3´	5´-ATGCCAGTGAGCTTCCCGTTCAG-3´

Table 6: Primer

6.1.6 Software

Name	Producer
Citavi 6	Swiss Academic Software
Excel 2013	Microsoft Corporation
Flowing Software	Turku Bioscience
GraphPad PRISM 6	Graphpad Software, Inc.
ImageJ	Wayne Rasband

Table 7: Software

6.1.7 Mice

Male, 8- to 14-week old *Mpo*$^{-/-}$-mice (K.O.) (C57bl/6J background, Jackson Laboratory, Bar Harbor, ME, USA), and wildtype littermates (WT) were used for all animal studies.

6.2 Methods

6.2.1 Working with cell culture

For extraction of peritoneal macrophages (PM) cell suspension obtained from peritoneal lavage, as described in 6.2.2.1, was centrifugated with 800 RCF for 8 min and supernatant was discarded. The remaining cell pellet was resuspended in 10 ml Dulbecco's modified eagle's medium (Dmem) plus penicillin/streptomycin plus 10 % fetal calf serum (FCS). Dishes were stored for 3 hours in incubators at 37°C, 95 % humidity and 5 % CO_2. Afterwards, medium was aspirated carefully and new medium with same properties was added. After 24 hours, medium was aspirated carefully again, washed gently once with Dulbecco's phosphate buffered saline (PBS) and cells were scretched with a cell scratcher and solved in 10 ml PBS.

Suspension was transferred in a 15 ml tube and centrifugated with 3600 RCF for 10 min. Supernatant was discarded and pellet was used for RNA isolation as described in 6.2.7.

For extraction of bone marrow derived macrophages (BMDM), detached femora were exempted from tissue and epiphysis were cut off. Bone marrow was rinsed into a 10 cm dish under application of PBS and a 26 G syringe. Solution was aspirated, transferred to a 15 ml tube and centrifugated with 800 RCF for 8 minutes. Supernatant was discarded and the remaining pellet was resuspended in 1 ml of DMEM plus penicillin/streptomycin and 10 % FCS. Cells were seeded in 6-well plates and each well was enriched with macrophage colony stimulating factor (M-CSF) to a concentration of 10 ng/ml. Plates were stored in an incubator with 37°C, 95 % humidity and 5 % CO_2 for 7 days. Medium change was performed on day 3 and 5 always with enrichment of M-CSF. 24 hours prior to harvesting the cells on day 7, 10 µg of MPO and 20 µM of H_2O_2 were added to each well. Cells were harvested on day 7 and used for RNA isolation as described in 6.2.7.

6.2.2 Working with mice

All experiments with mice were performed in accordance with current animal protection law and were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz, NRW (LANUV) under project number 2014.A234.

6.2.2.1 Model of peritoneal inflammation

For experiments with peritoneal inflammation described in figure 2, male 8-14 weeks old mice both WT and K.O. have been injected with 1 ml of either 3 % of thioglycolate medium with 0.9% NaCl, or pure 0.9% NaCl. Mice were sacrificed after 3 days via cervical dissection. Afterwards, the peritoneum was exposed, and 5 ml of ice-cooled PBS were injected with 26 G syringe into the peritoneal cavity without violate the intestines. The abdomen was gently massaged and PBS with solved cells was aspirated with a 20 G syringe and transferred into a 15 ml tube. The tube was immediately stored on ice. Bloody solution was discarded. Femora were cut of close to the hip joint a pair of scissors, dissected from muscle tissue and stored in PBS for further treatment. All collected samples were supplied to cell culture as described above.

For experiments with peritoneal inflammation described in figure 4 and 5, male 8-14 weeks old *Mpo*$^{-/-}$-deficient mice have been injected with 1 ml of either 3 % thioglycolate medium in 0.9 % NaCl or 10 µg/ml MPO in 0.9 % NaCl or pure 0.9 %. Further treatment took place as previously described above, except for no dissection of femora. Collected samples were supplied to cell counting and flow cytometry.

For assessment of MPO distribution, *Mpo*$^{-/-}$-deficient mice were injected with 1 ml of human MPO with 30 µg/ml and 0.9% NaCl into the peritoneum or with 30 µg of human MPO into the tail vain and sacrificed after 2h via final blood collection from the left ventricle. 15 minutes previously to blood collection, mice were objected to subcutaneous injection of 2500 I.U. heparin. Prior to sacrifice, peritoneal lavage was performed with 5 ml PBS plus 250 I.U heparin. All samples were immediately frozen in liquid nitrogen and supplied to ELISA for measurement of MPO.

6.2.2.2 Model of myocardial infarction

LAD-ligation was performed as previously described by Kolk et al. but with modifications. [128] WT and K.O. mice were anaesthetised with isoflurane and endotracheal intubated. Analgesia was applicated via i.p. injection of 250 µg buprenorphine 30 min prior to operation. LAD was ligated with 8.0 prolene thread through a small cut between the 3rd and 4th rib and either left ligated for permanent ischemia (PI) or reopened after 30 min for ischemia/reperfusion (I/R). Muscles and skin were sewed with 6.0 prolene thread. Anaesthesia with isoflurane was stopped and, when the mice showed sufficient breathing, extubated and placed back in the cage.

Depending on the experiment, mice were sacrificed on day 1,2 or 3 after ligation. For collection of blood cells, blood was directly aspirated from left ventricle and splenectomy was performed afterwards. Blood coagulation was immediately inhibited with application of 5 µl of EDTA to 100 µl of blood and storage on ice afterwards. Removed spleens were embedded in TissueTek O.C.T. mounting medium and freezed in liquid nitrogen as preparation for cryosections. Hearts were exposed for final extraction and embedded in TissueTek O.C.T. mounting medium and freezed in liquid nitrogen.

6.2.3 Immunhistochemical stainings

Stainings were performed with myocardium and splenic tissue from both WT and K.O. mice. For both stainings, organs were mounted with TissueTek O.C.T. mounting medium and freezed in liquid nitrogen as preparation for cryosection with a thickness of 5 µm.

For stainings of myocardium, slides were thawed and fixated with 3.7 % formaldehyde for 15 minutes. After washing with PBS, endogenous peroxidases were blocked with 0.075% H_2O_2 in PBS for 15 minutes and a solution of 10 % mouse serum, 0.1 % Triton X-100 in PBS for one more hour. According to manufacturer's recommendation, anti-F4/80 antibody was diluted to 1:100 and left for staining over night at 4°C. After washing with PBS, 2nd antibody, which was peroxidase labelled was added.

After 1 hour, AEC substrate was applied as suggested by the manufacturer. AEC solution was produced freshly for each application. Staining was completed with hematoxyline solution and finally mounted with DAKO mounting medium. Slides were placed under the Keyence BZ-9000 microscope and 10 pictures of both, the infarct area and the peri-infarct area, of each slide were taken with a magnification factor of 200. Macrophages were counted using ImageJ-software and counts were added for each slide.

For staining of spleen, slides were thawed and fixated with acetaldehyde for 30 minutes. After washing with PBS, unspecific receptors were blocked with 2 % bovine serum albumin (BSA) in PBS for 1 hour. Following the manufacturers instruction, anti-CD11b antibody was diluted 1:50 and left for staining over night at 4°C. After washing with PBS, nuclei were stained with Dapi diluted 1:1000 and slides were mounted with DAKO fluorescence mounting medium. Slides were placed under the Keyence BZ-9000 microscope and 10 pictures of the subcapsular red pulp of each slide were taken with a magnification factor of 200. Monocytes were counted using ImageJ-software and counts were added for each slide.

6.2.4 ELISA

Enzyme-linked immunosorbent assay (ELISA) was used for measurement of MPO in mouse blood and perfusate of peritoneal lavage as described in figure 2. The samples were processed in accordance with the manufacturer protocol. For plate reading, the Multiscan FC produced by Thermo Scientific was used.

6.2.5 Flow cytometry

Flow cytometry was performed for perfusate of peritoneal lavages of K.O. mice and mouse blood one day post-infarct of both K.O. and WT mice. Samples were collected as described above.

First step of processing of samples was lysis of erythrocytes with erythrolysis buffer containing 0.83 % NH_4Cl plus 0.1 % $KHCO_3$ plus 1mM in double-destilled water (ddH_2O). After washing with PBS, cells were fixated with 3.7 % formaldehyde and blocked with 10 % goat serum plus 1mM EDTA in PBS. Primary antibodies were added in concentrations according to the manufacturer's recommendation. For peritoneal lavages, anti-CD45 and anti-F4/80 Alexa Fluor 488 were used as primary antibodies and for blood samples anti-CD45, anti-CD115 PE and anti-Ly6C APC, respectively. After washing, secondary antibodies were added.

As anti-CD45 was the only antibody that was not conjugated to a fluorescence dye and the used antibody had been derived from rabbits, for peritoneal lavage anti-rabbit IgG PE and for blood samples anti-rabbit IgG Alexa Fluor 488 were required. Prior to further procedure, samples were washed with PBS and were then subjected with the BD FACS Canto II for flow cytometric measurement. Analysis was performed with Flowing Software 2. Macrophages were defined as $CD45^+$ $F4/80^+$ and proinflammatory monocytes were defined as $CD45^+$ $CD115^+$ $Ly6C^+$.

6.2.6 Cell counting

Cell counting was performed with the BioRad TC20 Automated Cell counter in accordance with the manufacturer's protocol. To check viability, 10 µl of cell suspension was mixed with µl of trypan blue and transferred on the counting slide. To exclude erythrocytes and debris, counting gates were set to 7 µm and 40 µm.

6.2.7 Real-time PCR

The first step for measurement of the expression of different cytokines was to isolate the RNA from obtained samples. RNA isolation was performed using the RNeasy mini kit provided by Qiagen. As it was expected to have a low yield of RNA, the maximum sample amount of 10^7 cells was used and proceeded in accordance with the manufacturer protocol of Qiagen. Received RNA was quantified via nano drop method with the Thermo Scientific NanoDrop 2000. Subsequently, RNA was transcribed into corresponding cDNA using the Qiagen Quantitect Reverse Transcription Set and a Biometra T3 Thermocycler. To ensure reliable results, RNA input was determined to 1 µg per well. Following qRT-PCR was performed under use of SsoFast EvaGreen Supermix with low ROX provided by BioRad and the BioRad C1000 Touch Thermal Cycler. Required specific primer are listed in 6.1.6. Expression data was normalized to expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and compared using the ΔΔcT method.

6.2.8 Statistics

The statistical analysis in this study was performed with Graphpad Prism 6 software and Microsoft Excel, respectively. To minimize imprecisions following inaccurate pipetting or technical errors, each measurement was recorded in doublets at least, some even in triplets, and arithmetical mean was used to implement further calculation.

Groups were described with the arithmetical mean and to clarify the variance the standard error of the mean (SEM) is delineated as error bars in the figures. To determine the statistical significancy in experiments with two groups (figures 2, 6 and 7) unpaired Student's t-test was applied. In experiments with three groups (figures 3, 4, 5 and 8) one-factor ANOVA and post-hoc analysis with LSD was applied. For alle experiments levels of different significance were specified as $^{*} = p < 0.05$, $^{**} = p < 0.01$ and $^{***} = p < 0.001$. N-Numbers were as indicated as in the figure legend.

7 Results

7.1 Effect of MPO on Macrophage activation

7.1.1 Expression data

In order to characterize the effect of MPO on activation and polarization of macrophages in vitro, we performed experiments with cells derived either from knockout mice (*Mpo*$^{-/-}$) and WT mice to investigate the effect of either treatment with MPO and knockout. We isolated and cultured peritoneal (PM) and bone marrow-derived macrophages (BMDM). Peritoneal macrophages have been isolated by a model of thioglycolate induced peritonitis and mRNA expression analyses for M1- or M2-transcripts were performed. Compared to knockout mice (*Mpo*$^{-/-}$), WT PM showed a significant higher expression of TNFα, but no significant increase in M1-markers iNOS and IL6 or in the M2-markers Arginase or IL10.

BMDM were isolated from *Mpo*$^{-/-}$-mice, incubated with MCSF for 7days and were treated with MPO (10pg/ml). Compared to untreated control, they showed a significant higher mRNA expression level of M1-markers iNOS, TNFα and IL6. Taken together, these experiments reveal that MPO leads to proinflammatory activation of Macrophages.

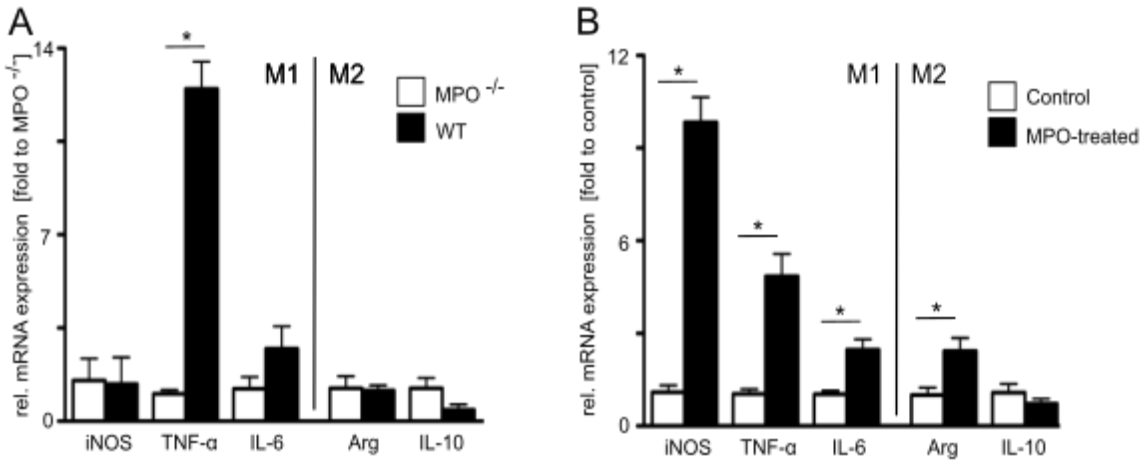

Figure 2: Inflammatory mRNA expression of macrophages: (A) Increased TNF-α mRNA expression in peritoneal macrophages (PMs) derived from WT animals compared to *Mpo*$^{-/-}$ animals. (T-Test, n=4/5). **(B)** Bone marrow derived macrophages (BMDMs) from MPO deficient mice were treated with MPO (10 µg/ml) or vehicel for 24h. Treated cells showed a significantly higher mRNA expression of the M1-markers iNOS, TNF-α and Interleukin-6 and increased M2-marker Arginase compared to untreated cells. (T-Test, n=4/5).

7.1.2 Peritoneal Uptake of MPO

To investigate if i.p. (intraperitoneal) injected MPO can be absorbed to the plasma or only leads to local inflammation, *Mpo*$^{-/-}$-deficient mice were injected with 15µg MPO (human, hMPO) either i.p. or i.v. (intravenous). 2 hours after MPO injection, blood plasma was taken and the peritoneal cavity was lavaged with PBS. Plasma- and peritoneal MPO levels were measured by ELISA and results were compared to a control group that received a NaCl-injection. Upon injection of MPO increased MPO levels could be detected after 2h within the intraperitoneal cavity compared to i.v. injection of MPO or intraperitoneal injection of NaCl (figure 3a).

In plasma (figure 3b) MPO levels showed no significant difference. Both groups showed significantly higher MPO level than the control.

Human MPO was injected and an ELISA-kit specific for human MPO was used to avoid interference with murine MPO.

Taken together, this data reveals that after i.p. of MPO, it reaches increased levels in blood plasma comparable to direct i.v. injection.

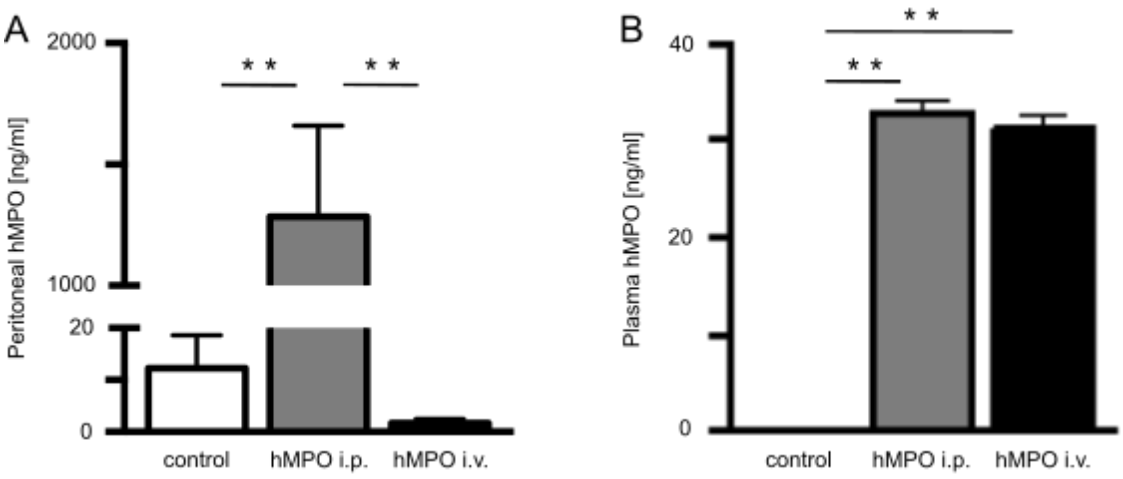

Figure 3: peritoneal uptake of MPO*:* (A) Increased levels of human MPO in peritoneal lavage 2h after i.p. injection of 15 µg hMPO but not after i.v. injection of 15 µg hMPO in MPO-deficient mice. (ANOVA, n = 3/4/3)**. (B)** Increased levels of human MPO in plasma 2h after injection of 15 µg hMPO i.p. or 15 µg hMPO i.v. in *Mpo*$^{-/-}$ deficient mice. (ANOVA, n = 3/4/3)

7.1.3 MPO as a chemoattractant for leukocytes

As MPO has been shown to reach significant levels in plasma after i.p. injection, mice were injected either with MPO, thioglycolate medium or NaCl to further investigate the chemotactic properties of MPO. 3 days after injection, a peritoneal lavage was performed and the number of isolated cells was counted. To include only leukocytes, counting was performed with gates of 7 µm to 40 µm via automatic cell counting. Mice with NaCl injection showed a mean leukocyte number of 3.48*10^5 per ml, mice with MPO injection 4.84*10^5 per ml and mice with thioglycolate medium 8.16*10^5 per ml. The numbers in MPO injected mice were significantly higher than in negative control group with NaCl injection. Given that i.p. MPO injection leads to peritoneal infiltration of leukocytes, MPO might activate leukocytes or has chemoattractant properties,

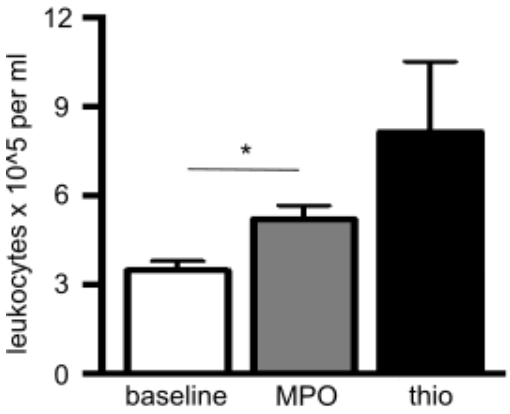

Figure 4: leukocytes in the peritoneum: *Mpo*$^{-/-}$ mice were injected with NaCl, MPO or thioglycolate i.p.. 3 days after injection the peritoneal cavity was lavaged and number of leukocytes were counted by size exclusion of erythrocytes. (ANOVA, p = 0.045 n=15/16/14).

7.1.4 Infiltration of macrophages into the peritoneum

To further identify the number of macrophages after i.p. application of MPO, mice were injected with either MPO, NaCl as negative control or thioglycolate medium as positive control to induce leukocyte infiltration as shown in Figure 4. The peritoneal cavity of the injected mice was lavaged 3 days after i.p. injection and the isolated cells were stained with the leukocyte pan-marker CD45 and macrophage specific marker F4/80. Flow cytometric analysis revealed that in MPO-injected mice, macrophage number in total leukocytes was 1.17-fold higher than in the NaCl-injected animals. The macrophage fraction in thioglycolate-injected mice was 1.29-fold higher compared to the control group but did not significantly differ from the MPO-injected group.

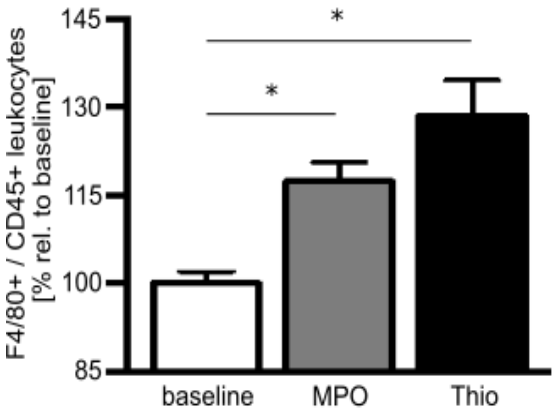

Figure 5: macrophages in the peritoneum: Isolated leukocytes were further analysed via flow cytometry analysis. MPO-treated animals showed a significantly higher percentage of macrophages compared to NaCl-treated animals (baseline) (ANOVA, n=6/7/4).

Taken together, figure 4 and 5 show that injection of MPO not only stimulates leukocyte migration into the peritoneum, but furthermore even sufficiently increases the number of macrophages within the peritoneum.

7.2 Myocardial infarction

7.2.1 Myocardial infiltration of macrophages 2d LAD/3d IR

To investigate the influence of MPO on monocyte/macrophage infiltration to the myocardium after MI, WT and *Mpo*$^{-/-}$ mice were subjected to either permanent (permanent ischemia, PI) or temporary (30min, ischemia/reperfusion, IR) ligation followed by reperfusion of the LAD. After 2 (PI) or 3 (IR) days mice have been sacrificed, hearts were isolated, and heart slices were stained with an F4/80 antibody specific for macrophages.

For PI, *Mpo*$^{-/-}$ mice showed significantly lower counts of F4/80 positive cells per field of view in the infarct (ischemic) zone as well as in the peri-infarct zone compared to WT mice. For IR, *Mpo*$^{-/-}$ mice showed a significantly lower count of F4/80 positive cells per field of view in the infarct zone, but not in the peri-infarct zone, compared to WT mice.

In conclusion, data revealed that *Mpo*$^{-/-}$-deficiency lowers the number of infiltrating macrophages in the infarcted myocardium after ischemic injury.

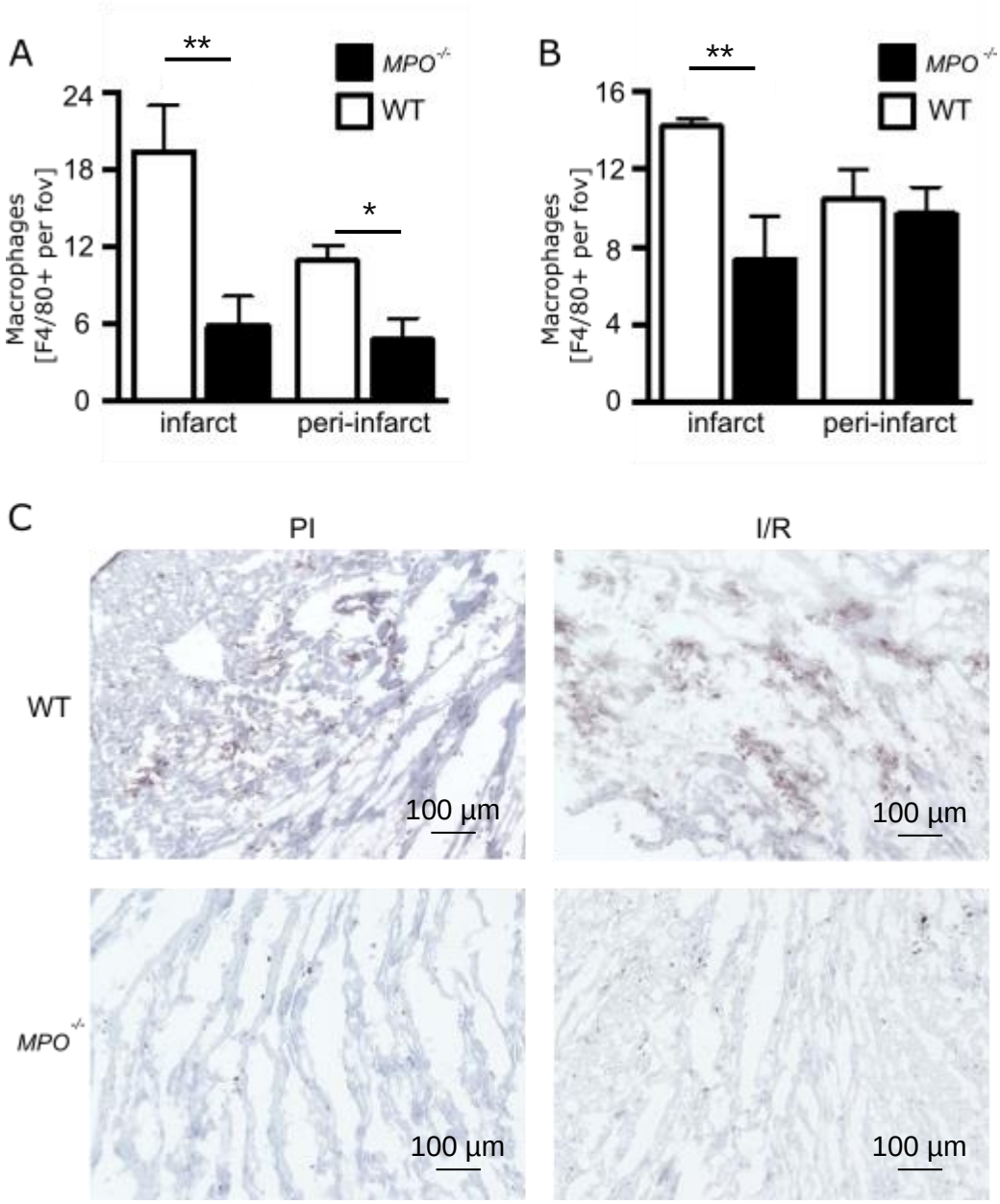

Figure 6: Myocardial infiltration of macrophages: (A) Mean number of macrophages in the myocardium after PI with significantly more macrophages in WT mice compared to *Mpo*$^{-/-}$ mice in infarct and peri-infarct area. (T-test, n=4/3). **(B)** Mean number of macrophages in the myocardium after IR. ($p < 0.01$) (T-test, n=3/4). **(C)** Representative F4/80 stainings of the infarcted area of WT and *Mpo*$^{-/-}$ mice after PI and IR.

7.2.2 Monocytes in blood 1d LAD

As macrophage counts post infarct differ between *Mpo*$^{-/-}$-deficient and WT mice, it seemed interesting whether also monocyte counts in murine blood differ. The expansion of macrophages in myocardium after infarction is thought to be derived from blood monocytes that differentiate into macrophages after infiltration [103,104,119]. Furthermore monocytosis, especially Ly6C^{hi} monocytosis, has been identified as a marker for low outcome after MI in mice as well as in human [123–125].

1 day after PI blood of *Mpo*$^{-/-}$-mice and WT mice was collected and cells were stained for CD45, a leukocyte pan-marker, CD115, a specific monocyte marker, and Ly6C as a marker for inflammatory (Ly6C^{hi}) or regulatory (Ly6C^{lo}) activation. Flow cytometric analysis revealed that there was no significant difference between the counts of Ly6C^{hi} monocytes of *Mpo*$^{-/-}$-mice and WT mice.

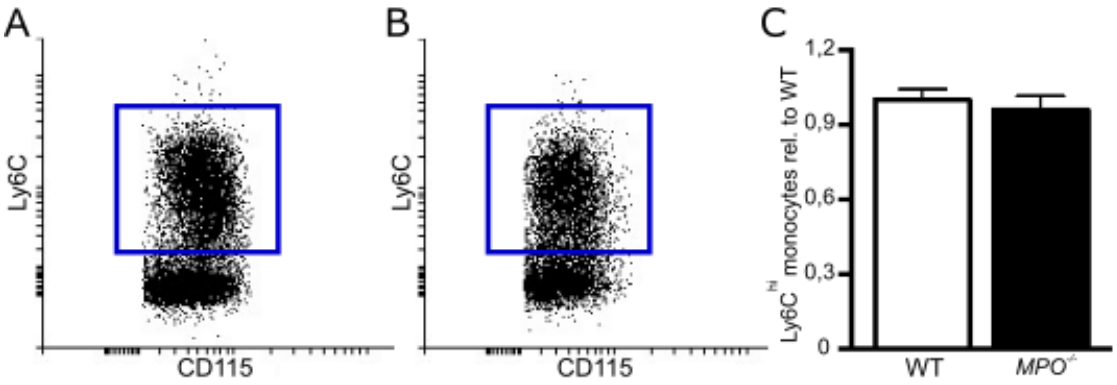

Figure 7: Monocytes in blood: (A) and **(B)** representative dot plots of blood derived monocytes 1d after PI of WT **(A)** and *Mpo*$^{-/-}$ **(B)** mice. **(C)** Flow cytometry showed no significant difference between the groups for the relation of Ly6C^{hi} and Ly6C^{lo} monocytes in the blood 1d after PI. (T-test, n=4/4).

7.2.3 The effect of MPO on splenic monocyte recruitment after MI

Ly6C^{hi} monocyte recruitment after MI is known to be derived from a splenic reservoir during the first days after ischemia. Loss of splenic monocytes has been shown to be a good indicator for the scale of myocardial infiltration of monocytes [85,116].

Mpo$^{-/-}$-mice and WT mice were subjected to permanent ligation of LAD and spleens were harvested 1 day after ligation. Mice subjected to sham treatment were used as control group. Tissue was stained with CD11b antibody specific for monocytes and positive cells in the subcapsular red pulp were counted.

The data revealed that WT mice had significantly lower counts of monocytes in the red pulp 1 day after MI than *Mpo*$^{-/-}$-deficient mice (42.95 positive cells per field of view vs. 69.8 positive cells per field of view). Both groups had also lower counts than untreated mice (139.43 positive cells per field of view), which indicates increased recruitment of cells.

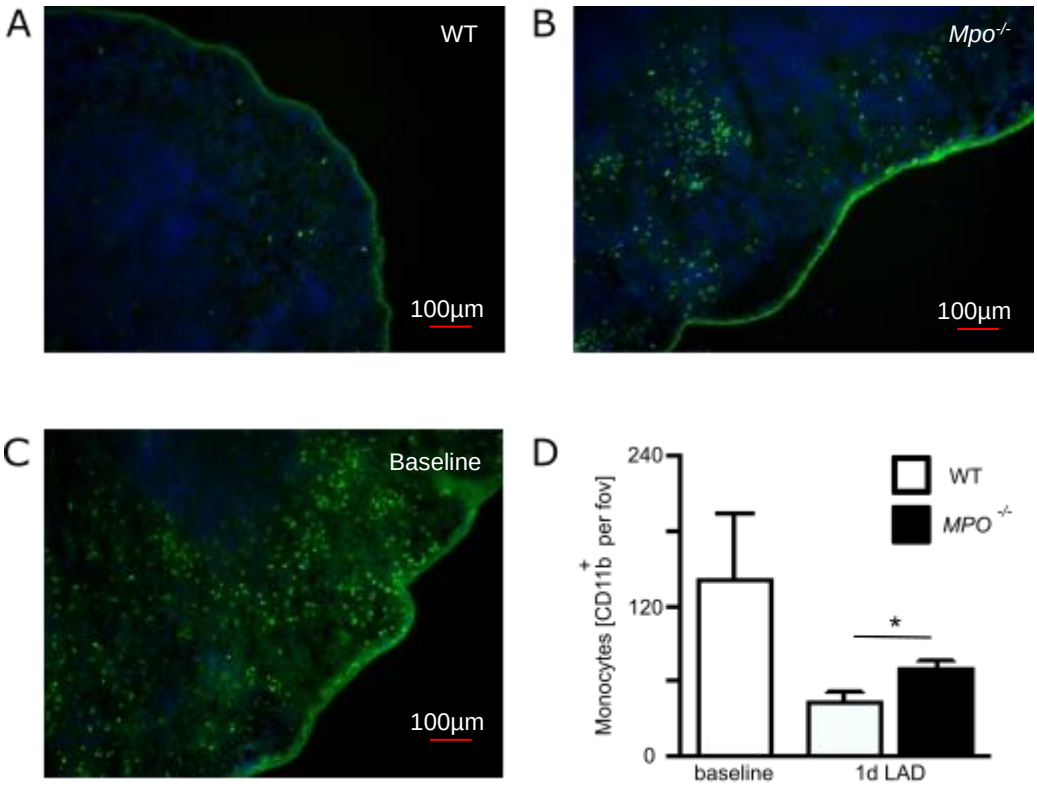

Figure 8 splenic monocytes: (A)-(C) Representative CD11b stainings of the spleen of WT **(A)**, *Mpo*$^{-/-}$ **(B)** or baseline animals **(C)** animals 1 day after PI.
(D) Mean number of monocytes in the spleen of baseline animals and 1d after PI in WT and *Mpo*$^{-/-}$ animals with a significant difference ($p = 0.038$) in the groups with 1d PI. (ANOVA, n=4/4/4).

In summary, MPO activates and supports monocyte recruitment from the spleen and leads to increased infiltration of monocytes and consequently elevated macrophage numbers in the infarcted myocardium.

8 Discussion

MPO is known to play an important role in cardiovascular disease in general and specifically in post-infarct myocardial remodelling. High levels of MPO are shown to facilitate PMN infiltration, promote adverse remodelling and impair cardiac outcome after MI [5–7,80]. Over the past 20 years MPS and especially monocytes and macrophages have been identified as key players in post infarct remodelling, as evidence grew that these cells orchestrate not only inflammation but also cardiac healing and scar formation [119,120,129,130]. Thus, MPO and monocytes/macrophages, are fundamental players in cardiac remodelling. Nonetheless their interplay remains poorly understood.

8.1 MPO attracts macrophages and polarizes them towards an M1-like phenotype

MPO seems to activate and polarize macrophages into a more inflammatory M1-like phenotype indicated by changes of its mRNA expression profile (Figure 2). It is not finally elucidated if MPO interacts directly with a specific receptor or surface protein or whether the downstream products of MPO cause the described alterations. Recent studies indicate the possibility that both options could be right. As mentioned above, macrophages can internalize MPO via the mannose receptor (CD206) and deliver it to the lysosomal compartment, which might be a potential way of interplay between MPO and macrophages [131]. There is furthermore evidence that MPO derived catalytic products like HOCl and hypothiocyanous acid (HOSCN), a highly reactive oxidant formed by MPO under conditions of increased thiocyanate ion levels, have essential impact on macrophages. HOCl has been shown to stimulate apoptotic cell death in macrophages via activation of plasma membrane L-type and T-type Ca^{2+}-channels and endoplasmic reticulum RyR-channels [132]. Even more interesting, HOSCN drives macrophages to significantly enhance the expression of pro-inflammatory cytokines like TNFα, IL6 and IL1β. This effect is crucially dependent on NFκB nuclear translocation. Interestingly, ischemic myocardium does not show increased thiocyanate levels and HOSCN is not part of post infarct remodelling [133]. However, we detected enhanced proinflammatory cytokine expression in macrophages that were exposed to MPO, which identifies NFκB-mediated cytokine expression as a potential signalling pathway.

Moreover, as cells derived from *Mpo*$^{-/-}$-deficient mice had a severe decrease of TNFα expression after induction of peritonitis, this outlines the essential function of MPO in inflammatory macrophage activation.

Chemotactic experiments showed that MPO is capable to attract leukocytes to the peritoneum, and that there were high levels of macrophages amongst these leukocytes. The experiments also reveal that i.p. injected MPO is sufficiently absorbed and plasma MPO reaches equal levels compared to control groups with MPO i.v. injection. Thus, MPO seems not only to be able to trigger local inflammation, but also might have systemic effects. MPO resorption might occur via transcytosis [134]. MPO injection leads to increased peritoneal leukocyte and macrophage infiltration with levels equal to injections with thioglycolate medium. Thioglycolate medium is known to cause strong inflammation and functioned as a positive control in the experiment. MPO, in particular its catalytic oxidation products, are known to show highly destructive properties on tissues leading to apoptotic cell death and initiation of inflammation. [135]. This might be the main reason for peritoneal infiltration. Additionally, MPO might facilitate PMN recruitment from the blood stream as it bounds to the endothelium and attracts PMN independent from its enzymatic properties, which consequently leads to increased PMN infiltration, increased local inflammation and increased infiltration of monocytes and macrophages, respectively [8]. A third contributing mechanism could be a systemic effect of MPO as it reaches sufficient levels in blood quickly after admission to directly activate leukocytes and maybe even damage tissue with subsequently activation of immune cells.

All these experiments demonstrate a pivotal role of MPO in the activation of monocytes and macrophages, respectively. Interestingly, as already mentioned in the introduction, *Mpo*$^{-/-}$-deficient human beings do not have severe immune deficiency but only an increased susceptibility for infection with *Candida albicans*. Therefore the importance of MPO for host defence is topic of a controversial discussion, but its importance for cardiovascular associated diseases seems to be established [63,69–72,136].

8.2 MPO supports inflammatory monocyte migration, infiltration and accumulation in the infarcted myocardium

To further investigate the involvement of MPO in monocyte and macrophage activation upon myocardial infarction, the findings obtained from the experiments described above have been transmitted to a model of myocardial infarction. Macrophage infiltration in the ischemic myocardium 2 days after permanent ligation of the LAD and 3 days after ligation for 30 min followed by reperfusion, respectively, has been tested. Results showed significantly reduced macrophage numbers in the infarcted area of both models and for PI also within the peri-infarct area in *Mpo*$^{-/-}$-deficient mice. Permanent ligation seems to cause a stronger infiltration as WT mice with PI reached tendentially higher numbers of macrophages than WT mice subjected to I/R. Macrophage counts between the test

groups did not differ significantly but tendentially in the peri-infarct area. Interestingly, this difference was nearly absent in *Mpo*$^{-/-}$-deficient mice.

The consequence of increased macrophage counts in the myocardium post infarct are controversial. There are several studies in animals and in humans that show monocytosis after MI, which is most likely combined with elevated macrophage infiltration, resulting in impaired cardiac outcome [117,124–127]. Interestingly, there is also evidence that increased macrophage infiltration can prevent adverse cardiac remodelling. It has been demonstrated that activated human macrophages injected in rat myocardium directly after MI are able to enhance several outcome parameters as scar thickness, fractional shortening and left ventricular diastolic dilatation. [137] Also the cardiac overexpression of CCL-2 (MCP-1) leads to increased monocyte infiltration and subsequently increased macrophage counts in the infarcted myocardium but results in prevented ventricular dysfunction and remodelling [138]. These controversial findings suggest that not the count might be the important parameter, but also the polarization and activation state of infiltrating macrophages respectively. Of note, increased infiltration and stimulated polarization towards an M1-like phenotype seems to worsen cardiac remodelling [123,124].

As already mentioned in the introduction, non-resident cardiac macrophages arise from pro-inflammatory Ly6C^{hi}-monocytes. These monocytes circulate in the blood and infiltrate the infarcted tissue immediately after ischemia and probably proliferate in the tissue. As the number of infiltrating monocytes exceeds the number of circulating monocytes in steady state, there must be a storage of monocytes that can be activated quickly and migrate into the blood stream. This storage has been identified as the splenic red pulp. Bone marrow derived monocytes also contribute to infiltrating monocytes, but to a lesser extent and to a later time point [85,115,116] .

Data from our experiments revealed that *Mpo*$^{-/-}$-deficient mice showed less macrophage counts in the infarcted myocardium. Interestingly, flow-cytometric measurement of Ly6C^{hi}-monocytes in relation to total monocytes in the blood did not significantly differ between WT mice and *Mpo*$^{-/-}$-deficient animals (figure 7). Although myocardial ischemia and especially LAD-ligation have been described as a strong trigger for systemic inflammatory reaction, the stimulus might not have been strong enough to uncover possible differences in the number of total monocytes [85,115,128]. Another possibility is that the effect of MPO on Ly6C^{hi}-monocytosis is too low and could not be sufficiently detected by our experiments as quantity of test groups might not have been large enough. The ratio between Ly6C^{hi}-monocytes in baseline and after myocardial infarction might be a valuable parameter. Another possibility might be that migration of monocytes from the

spleen and infiltration in the infarcted myocardium might keep the balance and therefore no difference in counts could be detected.

Although a difference in monocyte counts in the blood between *Mpo*$^{-/-}$-deficient mice and WT mice was not detectable 1 day after infarct, it still seemed interesting whether monocyte counts in the spleen differed. Surprisingly, *Mpo*$^{-/-}$-deficient mice presented significantly more residing monocytes in the splenic red pulp compared to wild type mice (figure 8). As Swirski et al. demonstrated, migration of monocytes from the spleen is a reliable parameter for the extent of monocyte activation in case of myocardial infarction [85]. This is even more remarkable as elevated macrophage numbers in the myocardium could be detected.

Although MPO seems to play a crucial role in activation and migration in monocytes, there has still been a mobilisation of splenic monocytes in *Mpo*$^{-/-}$-deficient mice. That might be an indication that MPO directly supports or interacts with other crucial key players of monocyte migration. Especially angiotensin II and its interplay with the angiotensin receptor type 1 has been identified as pivotal for migration as application of ACE-inhibitors completely supress monocyte migration [85,115,116]. As a result, further investigations on the influence of MPO on the interplay between angiotensin II and the angiotensin receptor type I might be a possibility to elucidate the pathway of how MPO stimulates monocyte migration.

As a conclusion, MPO seems to have an influence on migration of monocytes from the spleen into the blood and infiltration of the infarcted myocardium. There is valuable evidence that MPO supports monocytosis of proinflammatory Ly6C^{hi}-monocytes and consequently leads to enhanced CCR2-CCL2 mediated tissue infiltration. Although monocytosis was not detectable, it seems probable that increased macrophage counts in the infarcted myocardium arise from splenic monocytes. Another possibility could be enhanced local proliferation of either resident tissue macrophages, which seems less likely as resident tissue macrophages are suspected to die after ischemia, or enhanced local proliferation of already infiltrated and differentiated Ly6C^{hi}-monocytes and M1-like macrophages, respectively [120].

8.3 MPO impairs cardiac remodelling via activation of monocytes and macrophages and polarization towards an inflammatory phenotype

Taken together, we have shown that MPO and the MPS, especially monocytes and macrophages, are fundamental players in cardiac remodelling, have a direct interplay and that MPO supports the activation and differentiation of MPS. A clinical impact of the

results can only be speculated and classified according to the literature. As already mentioned above, the occurrence of monocytosis in an early stage after myocardial infarction is strictly correlated with several parameters of adverse outcome like formation of aneurysm and ventricular dilatation but also with development of pump failure [124]. An interpretation of the reported decrease of macrophages in MPO-free conditions after myocardial infarction seems more difficult as the significance and consequences are rated very differentially in the literature, even contrary [117,124–127,137,138]. As already concluded above, polarization of macrophages might be more important than macrophage numbers or proliferation rate. It might be conceivable that an overshooting polarization towards an M1-like phenotype might lead to enhanced tissue damage with delayed and impaired scar formation followed by adverse outcome. Several reviews have identified macrophage phenotype switch as a pivotal event in post infarct remodelling [129,139]. An extended M1-like macrophage activation and polarization is suspected to cause prolonged inflammation and thus promote adverse remodelling [140,141].

The results of this work demonstrate that MPO not only leads to increased macrophage counts in the infarcted myocardium but also directly activates macrophages to a classical, M1-like activation state in vitro as well as in vivo. Based on this, MPO can be considered to impair cardiac remodelling and outcome via supporting migration of proinflammatory Ly6C^{hi}-monocytes from the spleen, infiltration of these monocytes into the infarcted myocardium and activation towards an more M1-like phenotype. High levels of MPO might contribute to prolonged M1-like response and therefore complicate resolution of inflammation and impairing scar formation. As MPO is known to be part of cardiovascular-associated diseases like atherosclerosis, pulmonal artery hypertension or chronic kidney disease, it seems substantial to further investigate these processes [142–145]. Several MPO inhibiting compounds which have already passed clinical trials emphazise the role of MPO as a potential therapeutic target not only in post-infarct remodelling but also for atherosclerosis and other cardiovascular diseses [53,146]. Notably, similar results have been observed in experiments using the MPO inhibitor PF-1355, as reported by Ali et al. [81].

To further elucidate the link between MPO and monocyte activation and mobilisation, we would recommend utilizing alternative timepoints in future studies, to possibly investigate the influence of MPO on the mobilisation of monocytes from the bone marrow. Furthermore, the interplay between MPO and Ly6C^{low}-monocytes is poorly understood and might be an interesting subject to clarify the potential therapeutical applications of a pharmaceutical MPO inhibitor. Especially the finding, that MPO directly contributes to the

activation and mobilisation of monocytes might be worth to be translated into other pathologies.

In summary, the results of our study identify MPO as a potential pharmaceutical target to prevent cardiac inflammation and adverse cardiac remodelling following MI.

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10 Supplements

10.1 List of figures

10.2 List of tables