Aus dem Institut für Schlaganfall- und Demenzforschung (ISD)

Institut der Ludwig-Maximilians-Universität München

Vorstand: Prof. Dr. Martin Dichgans

MIF proteins and their role in mediating neutrophil activity and survival

DISSERTATION

zum Erwerb des Doktorgrades der Naturwissenschaften

an der Medizinischen Fakultät der

Ludwig-Maximilians-Universität zu München

vorgelegt von

Lisa Schindler

aus Magdeburg

2020

Mit Genehmigung der Medizinischen Fakultät

der Universität München

Betreuer:	Prof. Dr. rer. nat. Jürgen Bernhagen
Zweitgutachter/-in:	Prof. Dr. rer. nat. Sabine Steffens
Dekan:	Prof. Dr. med. dent. Reinhard Hickel
Tag der mündlichen Prüfung:	21.12.2020

Table of Contents

A Declaration/Affidavit

Eidesstattliche Versicherung

Schindler, Lisa

Name, Vorname

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Titel:

“MIF proteins and their role in mediating neutrophil activity and survival”

selbständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

Ich erkläre des Weiteren, dass die hier vorgelegte Dissertation nicht in gleicher oder in ähnlicher Form bei einer anderen Stelle zur Erlangung eines akademischen Grades eingereicht wurde.

München, 26.01.2021
Ort, Datum

Lisa Schindler
Unterschrift Doktorandin bzw. Doktorand

B Abbreviations

°C	Degree Celsius
µM	Micromolar
µl	Microliter
µ	Micro
Ab	Antibody
APCI	Atmospheric pressure chemical ionization
API	Application programming interface
BSA	Bovine serum albumin
ca.	Circa
$CaCl_2$	Calcium-chloride
CD	Cluster of Differentiation
CD74	Major histocompatibility complex, class II invariant chain (li)
CLF	Chemokine like function
CO_2	Carbon dioxide
CREB	cAMP response element-binding protein
C-terminal	Carboxy-terminal
CSF	Colony-stimulating factor
CXCR	CXC chemokine-receptor
CXXC	Cys-Xaa-Xaa-Cys
Da	Dalton
DAPI	4',6-Diamidin-2-phenylindol
ddH_2O	Double-distilled water
D-DT	D-dopachrome tautomerase
DHICA	5,6-dihydroxyindole- 2-carboxylic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Nucleoside triphosphate
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
e.g.	For example

ELR	Glutamine, leucine und arginine
ER	Endoplasmic reticulum
ERK	Extracellular-signal regulated kinase
ESI	Electrospray ionization
FCS	Forward scatter
FPLC	Fast protein liquid chromatography
g	Gram
g	Gravitational acceleration
GMP	Granulocyte–monocyte progenitors
GPCR	G-protein-coupled receptors
GRK	G-Protein- coupled receptor -kinase
GTP	Guanosine triphosphate
h	Hour
HCl	Hydrochloric acid
HED	2-hydroxylethyl disulfide
HMGB1	High mobility group box 1
HPLC	High pressure liquid chromatography
HPP	p-hydroxyphenylpyruvate
HRP	Horseradish peroxidase
H_2SO_4	Sulphuric acid
IL	Interleukin
4-IPP	4-iodo-6-phenylpyrimidine
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	Kilo base
KCl	Potassium chloride
kDa	Kilo Dalton
KH_2PO_4	Potassium dihydrogen phosphate
LC	Liquid chromatography
LPS	Lipopolysaccharide
M	Molar
mAU	Milli-absorbance-units
MFI	Mean fluorescent intensity
mg	Milligram
$MgCl_2$	Magnesium chloride

$MgSO_4$	Magnesium sulphate
MIF	Macrophage migration inhibitory factor
min	Minute
ml	Milliliter
mM	Millimolar
MOMP	Mitochondrial outer membrane permeabilization
MPO	Myeloperoxidase
MS	Mass spectrometer
m/z	Mass-to-charge ratio
n	Quantity
NaCl	Sodium chloride
NAPDH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
ng	Nanogram
nM	Nanomolar
nm	Nanometer
N-terminal	Amino-terminal
O_2	Oxygen
OD	Optical density
o.n.	Over night
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffer saline
PML	Polymorphonuclear leukocytes
PMN	Polymorphonuclear neutrophils
pH	*Potentia hydrogenii*
POD	Peroxidase
pM	Picomolar
Pro	Proline
Pro-1	*N*-terminal proline
PS	Phosphatidylserine
RNA	Ribonucleic acid
RP	Reverse phase
Rpm	Rounds per minute

RT	Room temperature
sec	Second
SDS	Sodium dodecyl sulphate
SD	Standard deviation
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBE	Tris-bromo-EDTA-Puffer
TBS	Tris-buffered hydrochloric acid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TNF	Tumour necrosis factor
TNFR1	TNF receptor 1
Tris	Tris-(hydroxymethyl)-aminomethane
U	Unit
USA	United States of America
UV	Ultraviolet
WB	Western blot
v/v	Volume/volume
w/v	Mass/volume
w/w	Mass/mass

C Publications

The follwing papers were published/ submitted during this dissertation:

Schindler, L., Dickerhof, N., Hampton, M. B., and Bernhagen, J. (2018) Post-translational regulation of macrophage migration inhibitory factor: Basis for functional fine-tuning. *Redox Biol* **15**, 135-142

Mattugini, N., Merl-Pham, J., Petrozziello, E., **Schindler, L.**, Bernhagen, J., Hauck, S. M., and Götz, M. (2018) Influence of white matter injury on gray matter reactive gliosis upon stab wound in the adult murine cerebral cortex. *Glia* **66**, 1644-1662

Roth, S., Singh, V., Tiedt, S., **Schindler, L.**, Huber, G., Geerlof, A., Antoine, D. J., Anfray, A., Orset, C., Gauberti, M., Fournier, A., Holdt, L. M., Harris, H. E., Engelhardt, B., Bianchi, M. E., Vivien, D., Haffner, C., Bernhagen, J., Dichgans, M., and Liesz, A. (2018) Brain-released alarmins and stress response synergize in accelerating atherosclerosis progression after stroke. *Sci Transl Med* **10**(432):eaao1313

Submitted publications:

Schindler, L., Zwissler, L., Krammer, C., Hendgen-Cotta, U., Rassaf, T., Hampton, M.B., Dickerhof, N., and Bernhagen, J. (2020) Macrophage migration inhibitory factor inhibits neutrophil apoptosis by inducing cytokine release from mononuclear cells. *J Leukoc Biol*, in revision.

1 Introduction

1.1 Macrophage migration inhibitory factor (MIF)

First described in 1966, Macrophage migration inhibitory factor (MIF) was initially described as a soluble T leukocyte derived factor that was able to inhibit macrophage migration (1,2). Later on, the expression and purification of recombinant MIF in *E.coli (3)* enabled a deeper understanding of the structure, molecular characteristics and biological functions of the protein.

MIF is a highly conserved chemokine-like protein with a molecular weight of 12.345 kDa, that contains 114 amino acids residues (4). Three oligomerization states of MIF have been discussed in the literature. X-ray analysis revealed a trimeric form in the crystal, whereas another study applying cross-linking analysis indicated a balance between monomer, dimer and trimer at physiological concentrations with a majority of monomers and dimers (4-9). Nevertheless, the structure of MIF is unique compared to other proteins, as it shows chemokine-like functions and binds to the chemokine receptors, but is missing a *N*-terminal cysteine motif (4,5). Consequentially does not belong to one of the typical cytokine and chemokine families and is therefore termed an atypical chemokine.

Within the MIF family, two catalytic activities were characterized: an oxidoreductase and a tautomerase activity. Through its TPOR (thiol-protein oxidoreductase) activity MIF is able to catalyze the reduction of insulin disulfides and 2-hydroxylethyl disulfide (HED) with the use of the co-substrates glutathione (GSH) and dihydrolipoamide (10,11). It is assumed that MIF's well conserved CALC (Cys57-Ala-Leu-Cys60) motif is involved in the oxidoreductase activity. Similarities were shown in the residues between the CXXC cysteines of MIF compared to other TPOR family members such as thioredoxin and glutaredoxin (10). Therefore, it was shown that this catalytic activity plays an important role in the protective effect of MIF in redox provoked cell damage (10-12). The second enzymatic activity of MIF, a tautomerase activity, involves the *N*-terminal proline, that was shown to have a pK_a of 5.6, which is much lower than the normal pK_a of a proline amine (pK_a of around 9) (13,14). Under physiological pH this proline act as a catalytic nucleophile and can convert substrates such as D-dopachrome and 4-hydroxphenyl

pyruvate (13,15,16). Until now, there is no physiological substrate known for MIF's tautomerase activity. Interestingly, this *N*-terminal region is a typical target for small molecule inhibitors, that can inhibit biological and enzymatic activities of MIF (17-22).

1.1.1 MIF – biological activity and role in disease

Under normal conditions, basal MIF levels have been shown to be around 2-6 ng/ml and contrary to other cytokines, it is semi-constitutively expressed, continuously released into circulation following a circadian rhythm (23) and stored in extracellular pools. However, MIF levels differ between human individuals. There are four genotypes being describe differing in there CATT repeats (5, 6, 7, or 8-CATT repeats). It was shown that the 5-CATT allele results in lower concentrations of basal MIF ("low MIF expressors") compared to the other genotypes, which was accompanied by a decreased severeness of rheumatoid arthritis disease in patients (24), that could indicate a concentration dependent disease progression.

MIFs biological and immunological activities have been reviewed intensively for decades. Due to its ability to recruit immune cells (19,25) and trigger the production of other cytokines (26-29), MIF is known as pro-inflammatory mediator. This is supported by MIF's ability to prolong macrophage survival (30) and counter-regulate the anti-inflammatory activities of steroids (31-33).

Following inflammatory stimuli as well as atherogenic and ischemic stress, it was shown that MIF is highly expressed by numerous cell types such as monocytes, macrophages, T cells, and neutrophils, but also endothelial cells, leading to an increased MIF concentration in circulation (34-38). Due to its pro-inflammatory activities, MIF is involved in regulating several acute and chronic inflammatory diseases, including cystic fibrosis, rheumatoid arthritis, cancer, atherosclerosis and other cardiovascular diseases (24,25,39-48). Although most of MIF's activities have been reported to be disease-exacerbating, there are also studies that show a protective effect on cells and tissue, as shown in myocardial ischemia/reperfusion (I/R) injury (49,50).

MIFs inflammatory actions and disease progression are triggered through binding to its receptors CD74, CXCR2, CXCR4 and potentially also CXCR7 (25,51-55).

1.1.2 MIF receptors

The first identified MIF receptor CD74, is a cell membrane receptor, known as the HLA class II histocompatibility antigen gamma chain lacking an intracellular signal transduction domain. Binding of MIF leads to a complex formation with CD44 which results in the activation of several signaling pathways including the ERK1/2-MAPK pathway, Src and NF-κB pathway (55,56). In a myocardial ischemia, it was shown that MIF can induce the activation of CD74 and thereby initiate AMPK signaling, that was accompanied by an uptake of glucose and cardiac protection (57).

MIF also binds to the CXC chemokine receptors CXCR2 and CXCR4, which triggers the recruitment of monocytes, T cells and neutrophils (25). Studies have shown that a CXCR2-ligand such as CXCL8 involves an *N*-terminal Glu-Leu-Arg (ELR) motif in order to facilitate receptor binding (58). A similar motive was also found in MIF and was characterized as a *pseudo*-(E)LR motif (52,59). Alike to CXCR2, CXCR4 is a G protein-coupled chemokine receptor with high ligand specificity. CXCL12, stromal cell-derived factor-1α (SDF-1α), and MIF are known chemokine ligands of CXCR4 (25,60). The MIF/CXCR4 interaction involves an N-like-loop and the *N*-terminal proline residue of MIF, as well as the Arg-Leu-Arg (RLR) motif (61,62). Recently published data also suggests the receptor CXCR7 as a potential MIF receptor, with a functional role in B-lymphocyte migration (51).

Different studies showed that two or three of the MIF receptors can form complexes, which can be required for triggering a particular signaling cascade. Especially the CXCR/CD74 is involved in the initiation of MIF-mediated signaling. Examples are the functional CXCR2/CD74 complex, which was found to have a profound role in MIF-driven atherogenic leukocyte recruitment (25) and the CXCR4/CD74 complex, that was shown to be part of MIF-mediated B cell migration through a ZAP-70-involving pathway (63,64).

1.2 MIF superfamily

MIF-2 or D-dopachrome Tautomerase (D-DT) was first specified in 1993 (65). Crystallographic analysis of MIF (4,5) and later MIF-2 (66) enabled the classification into a MIF superfamily and identified a shared homotrimeric structure (Fig. 1-1). MIF-2

was shown to have significant three-dimensional homology with MIF (66) and the mRNA of both proteins is almost 50% identical (14). Interestingly, MIF and MIF-2 also share an evolutionarily conserved tautomerase activity, which involves the *N*-terminal proline that both proteins share (15). Nevertheless, there are also crucial differences between the proteins. For example, MIF-2 deficits the for MIF typical CXXC and the *pseudo*-(E)LR motif (14), which has been shown to be involved in interactions with the MIF receptors such as CXCR2.

Compared to MIF, the biological function and expression pattern of MIF-2 are still poorly investigated. Merk *et al.* showed that MIF-2 can bind to the MIF receptor CD74 and thereby activates ERK1/2 MAP kinase and other inflammatory signaling pathways (14). As MIF-2 lacks the *pseudo*-ELR motif, it is likely that it might not bind to CXCR2. However, this still remains speculative and the interaction with other MIF receptors have not been explored yet. MIF-2 is expressed in various tissues, as well as in immune cells, and circulating levels of MIF-2 have been shown to be upregulated in sepsis and cancer (14). Like MIF, MIF-2 showed cardioprotective potential by activating the CD74 mediated AMPK signaling pathway (67).

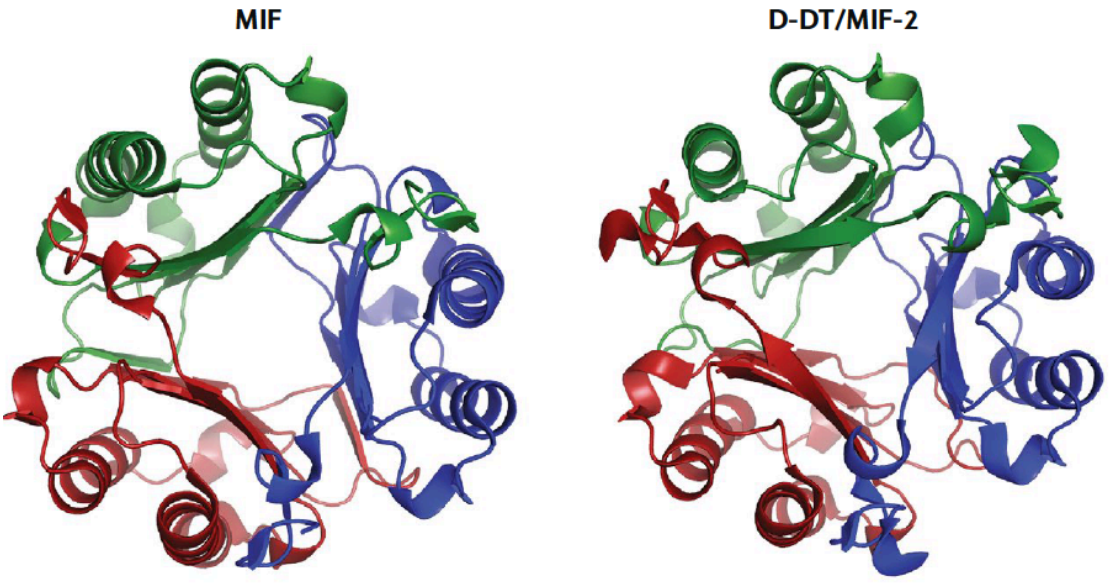

Figure 1-1 Homotrimeric structure of human MIF and MIF-2. MIF superfamily members MIF and MIF-2 share a homotrimeric structure and 3D similarities represented by X-ray crystallographic analysis. Figure taken from Merk *et al.* (68).

Interestingly, other MIF-superfamily members were also found in non-mammalian species such as bacteria, plants, vertebrates and invertebrates (69,70). As an example, in *Arabidopsis thaliana*, a well-studied model plant, three different MIF-like proteins were characterized (*At*MDL1-3) (71). Compared to human MIF, all three *At*MDLs have similar molecular weights and number of residues, including an *N*-terminal proline (71). More recently published data also provided evidence of similarities in biological functions, such as interaction with the known MIF receptors CD74 and CXCR4, as well as the triggering of chemotactic migration of monocytes and T-cells (72). AtMDLs have also been shown to activate CXCR4-dependent activation of PI3K/Akt-signaling pathway in HEK293 cells (72).

1.3 Post-translational modifications of MIF

Numerous studies implicate that MIF undergoes several post-translational modifications (PTM), which can impact the biological actions of MIF (73). Whether this is the case for MIF-2 is still not fully understood.

Figure 1-2 is summarizing the known PTMs and indicates that mostly Cysteines and Serines within the MIF sequence are prone to get modified. Cysteinylation at Cys-60 is supposed to be involved in regulating B and T cell functions (74,75), whereas S-Nitrosation at Cys-81 has been shown to increase oxidoreductase activity and was shown to be involved in cardioprotection (76,77). Additionally, when Ser-91 is additionally phosphorylated, it is supposed to have an impact on the biological activity of cysteinylated MIF (74).

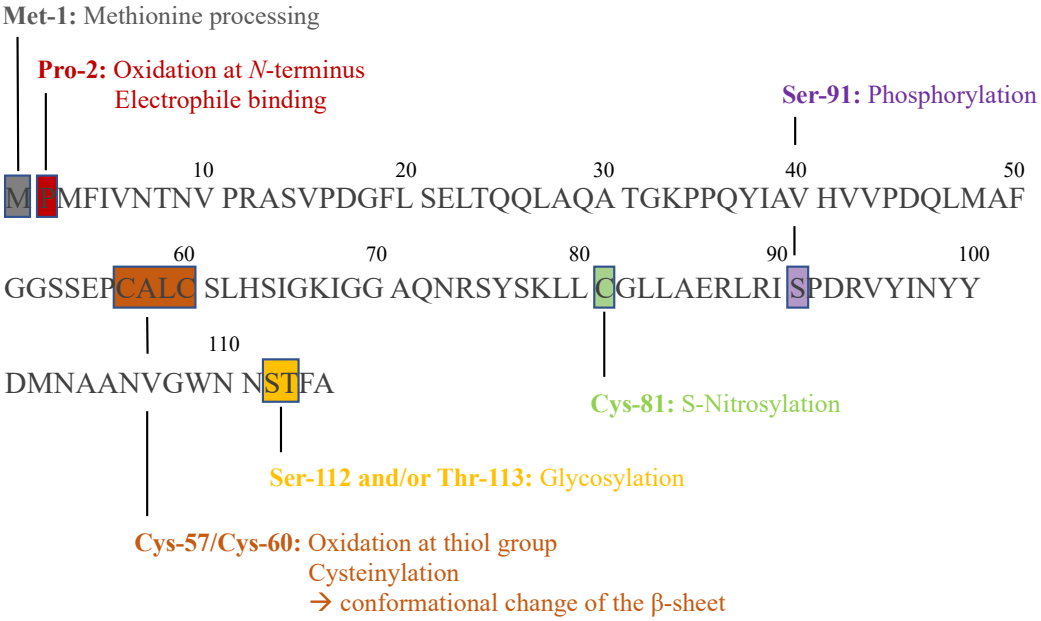

Figure 1-2 Post-translational modifications of MIF. Amino acid sequence of MIF, including different post-translational modifications that are well-defined and highlighted in different colours. Adapted from Schindler *et al.* (73).

1.3.1 Modifications of the *N*-terminal proline

In almost every cell type, MIF's *N*-terminal methionine is post-translationally removed by methionine aminopeptidase (MetAP) which results in an *N*-terminal proline (Pro-2) (3,4). As mentioned before, this proline residue is critical for MIF's tautomerase activity (15) and can be involved in receptor binding (61,78). The *N*-terminal proline can be target for several modifications, including carbamylation, oxidation and modification by dietary electrophiles (73). Likewise, the *N*-terminal site is a preferential domain for pharmaceutical inhibition of MIF's biological and enzymatic activities (20,79).

Covalent binding of isothiocyanates at the *N*-terminal proline inhibited MIF's tautomerase activity and to a certain extend also results in structural changes which affect the binding of MIF to CD74 (80-84). Another study demonstrated that epicatechins, that are oxidized by neutrophil derived myeloperoxidase (MPO), can also suppress the tautomerase activity of MIF (85).

A novel modification of the *N*-terminal proline was described by Dickerhof *et al*. (2015). They were able to show that MPO driven hypochlorous acid oxidizes this proline to a

proline-imine which was correlated with an impairment of MIF's tautomerase activity (86). Although this oxidation suppresses the enzymatic activity, the bioactivity of MIF seems to remain intact. Whether the oxidative modification of the proline results in changes of bioactivity compared to unmodified MIF is still unclear. Nevertheless, the oxidation protected the protein from functional inactivation by small molecule inhibitors such as 4-iodo-6-phenylpyrimidine (4-IPP) (86).

1.4 Granulocytes and their role in the human body

Polymorphonuclear leukocytes (PML), also known as granulocytes, are produced in the bone marrow and form the most abundant white blood cell population in the human body. Their name is derived from their segmented shaped nucleus and is characterized by the presence of granules in the cytoplasm (87). There are three different types of granulocytes: eosinophils, basophils and neutrophils, which develop from stem cells in the bone marrow into myeloblasts and further differentiate into a fully functional granulocytes (Figure 1-3) within 10-14 days (88). While some neutrophils are steadily released into circulation, the bone marrow holds back a large number of immature granulocytes, which can be rapidly mobilized in response to inflammation or infection. Within the granulocytes, the neutrophil population is the most abundant cell type representing about 50-70% of all blood leukocytes (89). The different polymorphonuclear leukocytes differ in structure and function. Both, eosinophils and basophiles are involved regulation of allergic reactions and are also involved in the defense against parasites and viruses (90-92). Nevertheless, both cell types make up only a very small part of the granulocyte population (1-5%). Neutrophils are the largest group of PMLs and display a essential role in the first site defense against invading pathogens such as bacteria. Likewise, it was shown that they are also involved in sterile inflammation and disease.

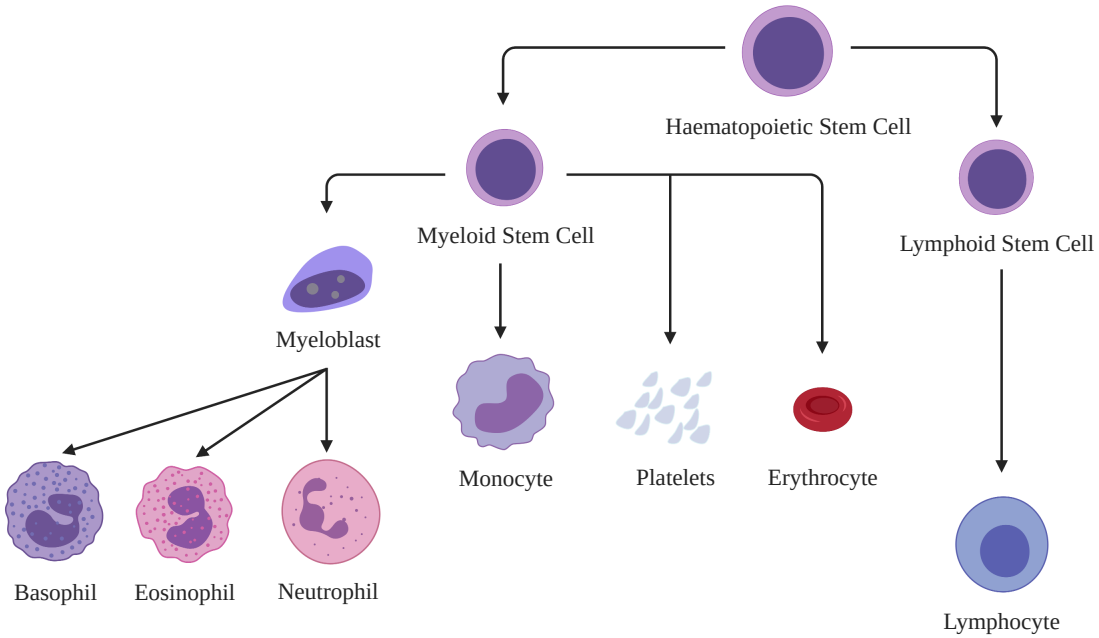

Figure 1-3 Haematopoiesis – development of blood cells. All cellular blood components are derived from a haematopoietic stem cell. While lymphocytes further originate from a lymphoid stem cell, monocytes, platelets and erythrocytes, as well as basophils, eosinophils and neutrophils develop from myeloid stem cells. Adapted from Jagannathan-Bogdan *et al.* (93). Images were designed using BioRender software.

1.4.1 Neutrophils

A healthy human body is producing 10^{11} polymorphonuclear neutrophils (PMN) that are released into the blood stream per day (94). During infection and several inflammatory diseases, the number of circulating neutrophils can dramatically increase (up to 10-fold), followed by a rapid infiltration of neutrophils towards the center of inflammation and infection within the first hour (95).

Circulating mature neutrophils are characterized by a typically segmented nucleus and have a size of approximately 10 µm. Within their cytoplasm different granules and secretory vesicles can be found, which contain several pro-inflammatory proteins. Neutrophils are produced in the bone marrow from hematopoietic stem cells, which further develop into granulocyte–monocyte progenitors (GMPs). The granulocyte colony-stimulating factor (G-CSF) controls the further differentiation from GMPs into myeloblasts (96), which is followed by a maturation process that includes the differentiation into a metamyelocyte, a banded neutrophil, and finally into a

mature neutrophil (97).

Neutrophils that are circulating in the blood stream have been shown to undergo aging processes, characterized by downregulation of CXCR2 and CD62L expression, as well as upregulation of CXCR4 expression (98). It is believed that neutrophils are removed through the infiltration into the liver, spleen or back into the bone marrow if there is no infection or other inflammatory challenge in order to guaranty the homeostasis between neutrophil clearance and production (99-101).

Neutrophils are characterized as short-lived phagocytes and their half-life time has been controversially discussed in literature in the past decades varying from 7-12 h (94) up to 5 days (102). Depending on their prior function and role in the immune system, neutrophil cell death can proceed via apoptosis, necrosis, NETosis, followed by phagocytosis through macrophages and dendritic cells (103), but also via autophagy (104,105). In addition, during inflammatory processes, the neutrophil lifespan can be extended by different factors. Nevertheless, studying the mechanism of neutrophil survival and death *in vivo* and *in vitro* is very difficult due to their short lifespan and sensitivity to get activated.

1.5 Neutrophils in inflammation

Neutrophils are the earliest cells that infiltrate the center of inflammation and infection, whereas other immune cells such as monocytes and macrophages are recruited in a later immune response (106-109). They are not only playing a key role in the "first-line defense" against invading pathogens such as bacteria and fungi (110), but also in inflammatory diseases such as myocardial infarction (111). The understanding and knowledge of neutrophil function and their role in our immune system has essentially enlarged over the past years. Since new techniques have been developed to detect and screen for neutrophils in different biological setting and in *in vivo* studies, new functions and properties of neutrophils have been found.

Neutrophils are recruited from the bone marrow during infection following a chemotactic gradient towards the invading pathogens. For several years it was, without doubt, believed that the only functions of neutrophils are to fight against invading microbial pathogens through three different killing mechanisms: degranulation, phagocytosis and NETosis

(Fig. 1-4). But several recent studies showed that the way how neutrophils combat against microbes is much more complex and involves multiple other processes (110).

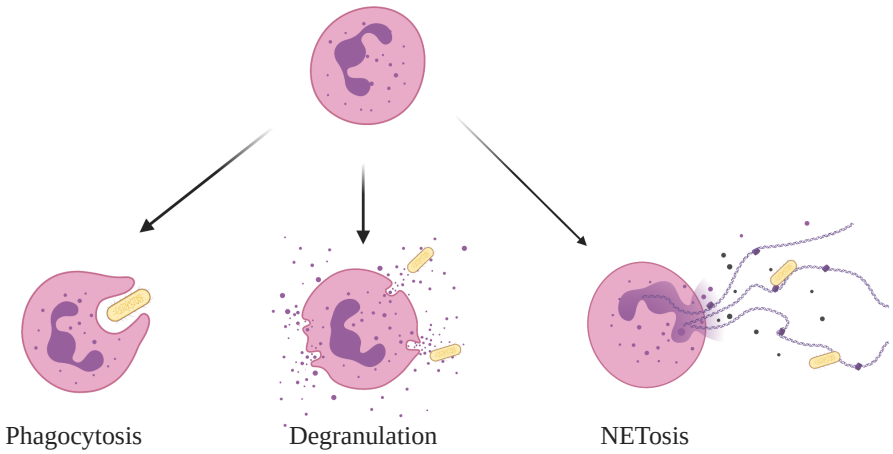

Figure 1-4 Pathogen killing mechanisms of neutrophils. Neutrophils can eliminate pathogens through different mechanisms. Neutrophils can phagocyte microbes, followed by the formation of phagosomes and killing through reactive oxygen species. The release of anti-microbial granules (azurophilic granules, specific granules, tertiary granules) is called degranulation. Neutrophil extracellular traps consist of DNA, histones, proteins and enzymes that trap and kill microbes. Adapted from Kolaczkowska *et al*. (112) Images were designed using BioRender software.

1.5.1 Neutrophils and their crosstalk with other immune cells

Neutrophils are able to modulate the immune response by a multifaceted cross talk with several other cells of the immune system. This can be mediated by the secretion of chemotactic proteins which results in the rapid recruitment of dendritic cells (113,114) and macrophages (115,116) towards the site of infection, and can also result in a direct activation of immune cell function (117,118). Neutrophils have been shown to also produce cytokines that play a role in B cell survival and activation (119).

Just like neutrophils can modulate other immune cells, those immune cells inversely can also act on neutrophils. Cytokines such as GM-CSF, G-CSF, IFN-γ, TNF-alpha and CXCL-8 can be produced by e.g. natural killer cells or macrophages which results in neutrophil priming or prolonged survival (120-123). The understanding of this cytokine-mediated crosstalk between different cells of the immune system is a prerequisite to give a deeper understanding of the process of neutrophil-derived inflammation. Not only in

pathogenic inflammation but also in sterile inflammation the interaction between different immune cells can be essential.

1.5.2 Neutrophils in cardiovascular inflammation

In contrast to macrophages and monocytes, the role of neutrophils in cardiovascular inflammation is not fully understood and is controversially discussed. Myocardial infarction is characterized by an accelerated and increased neutrophil infiltration, based on studies that have been illustrating the pro-inflammatory and tissue damaging role of neutrophils in myocardial ischemia (124,125). Neutrophils can contribute to tissue damage by a number of mechanisms such as the release of reactive oxygen species, activation of endothelial cells and release of mediators such as CXCL8 or myeloperoxidase (MPO) to activate and further recruit additional neutrophils and other immune cells (126-130). However, recent data shows that depletion of neutrophils in myocardial infarction (MI) animal models led to increased heart failure and fibrosis (131). Supporting a potentially protective function, it was described that in myocardial infarction neutrophils are the major source of the protein annexin A1, which is involved in the generation of a reparative macrophage phenotype (132).

1.6 Neutrophil apoptosis and its regulation

When neutrophils undergo apoptosis, characteristic morphological and cellular changes are happening, such as condensation of organelles, shrinking of the cell body, round shape formation of the nucleus, chromatin cleavage and phosphatidylserine (PS) is appearing on the outer cell membrane (133,134). Interestingly and in contrast to necrosis, the cell membrane stays intact and protects the surrounding area from damaging neutrophilic intracellular contents. In addition, apoptotic neutrophils, also downregulate their cellular functions, which can result in declined chemotactic and phagocytic ability, as well as reduced cell adhesion molecule expression (135,136). There are two main pathways of apoptosis in neutrophils: the extrinsic and the intrinsic pathway (137). The extrinsic pathway is dependent on a so-called “death receptor” such as the Fas receptor and TNFR1, that can be activated by structurally related ligands such as CD85L or TNF-α. The death-inducing signaling complex (DISC) initiates the formation of a FAS-associated

death domain (FADD), that leads to caspase 8 activation, which than initiate downstream effector caspases, resulting in apoptosis (138). The intrinsic pathway involves mitochondria and can be initiated by various inducers such as UV radiation, growth factors or ischemia. This pathway is regulated by the Bcl-2 protein family, presented by pro-apoptotic proteins (Bax, Bad, Bak), as well as anti-apoptotic proteins (Mcl-1, A1) (139). These proteins regulate the mitochondrial outer membrane permeabilization (MOMP), through which pro-apoptotic proteins can be released into the cell (140).

During inflammatory processes neutrophils get activated and increase their longevity, to ensure their presence at sites of inflammation (95,122). However, neutrophil apoptosis can be a critical determining state in inflammation, as it turns out to be either promoting ongoing inflammation or its attenuation (141-143). Delayed neutrophil apoptosis can result in a non-resolving inflammation, which can be associated with the development of several human diseases such as cystic fibrosis, sepsis, coronary artery disease and rheumatoid arthritis (144-148). To terminate neutrophil-mediated inflammation, mechanisms are necessary that reduce the expression of inflammatory mediators, that are responsible for neutrophil recruitment, activation and survival. Apoptotic neutrophils can be recognized and phagocytized by macrophages through typical “find me” and “eat me” signals, a process called efferocytosis, and thereby promoting the resolution of inflammation (149).

1.6.1 Regulation of apoptosis by external mediators

Neutrophil homeostasis is a sensitive balance and needs to be controlled precisely. Every neutrophil that is leaving the bone marrow needs to die after a certain time to ensure this balance under physiological conditions. When neutrophils die without an extracellular stimulus, this process is called spontaneous apoptosis. It has been shown that neutrophils are the perfect cells to study programmed cell death, because it is easy to mimic apoptosis *in vitro* by culturing the cells, after isolation from blood, in normal tissue cell media such as RPMI at 37 °C (134). In the past, several studies showed that neutrophil apoptosis can be modulated by several factors such as hypoxia (150), pathogens (151) and several proteins (123,152-154).

In an inflammatory environment, especially macrophages are secreting large amounts of pro-inflammatory cytokines, which have been shown to prolongate neutrophil lifespan

such as IL-1β, G-CSF (121,152) and GM-CSF (153). Another cytokine that has been well reviewed in the past years was TNF-α and its effect on neutrophil apoptosis and survival. However, there is evidence that this cytokine can have pro and anti-apoptotic effects on neutrophils depending on various conditions, such as protein concentration, length of stimulation time and the initial state and genetic background of the stimulated neutrophil (154,155). Likewise, the interleukin 8 (CXCL8), a well-known chemoattractant for neutrophils, was found to play a role in neutrophil apoptosis (156,157). However, these performed studies deliver conflicting results, that need to be further investigated.

1.7 Neutrophil oxidative activity and NET formation

As neutrophils are the first cells of the immune system that respond to infection and injury, they developed special pro-inflammatory and potential anti-inflammatory functions to support the resolution of inflammation.

1.7.1 Role of neutrophil derived oxidants and neutrophil priming

A key role of neutrophils is the defense against invading microorganisms through the release of reactive oxygen species (ROS) (158). This process is also known as oxidative burst and a major component involved in this is the NADPH oxidase (159,160) (Fig. 1-5). Upon activation, neutrophils generate hydrogen peroxide (H_2O_2) and superoxide ($O_2^{-\bullet}$) in a NADPH catalyzed reaction (161). The typical neutrophilic enzyme myeloperoxidase (MPO) can react with hydrogen peroxide to form hypochlorous acid (HOCl) (162-164). HOCl can either have direct antimicrobial effect on pathogens but it is also prone to react with host proteins (165), which can result in protein modification and change of bioactivity.

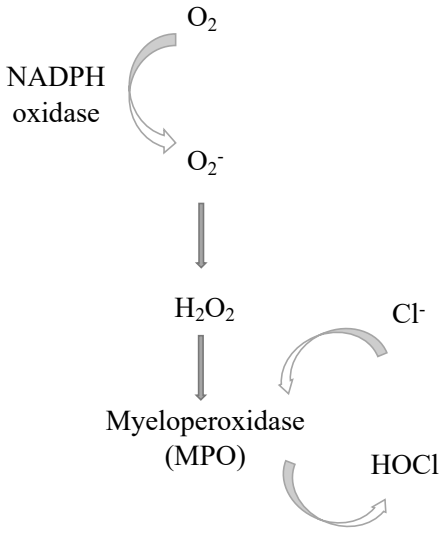

Figure 1-5 Reaction scheme: generation of HOCl and other ROS. NADPH catalyses the generation of superoxide ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). Hypochlorous acid is formed by myeloperoxidase (MPO), H_2O_2 and chloride (Cl^-).

Neutrophil effector function can be prominently boosted by chemokines, cytokines and other stimuli, in a process called neutrophil priming, which can lead to increased ROS production and also phenotypical changes (166). Pro-inflammatory cytokines such as TNF-α and GM-CSF have been shown to enhance the release of ROS after exposure to a stimulus such as PMA or N-formyl peptides (167-170). Also, CXCL8 has been shown to prime neutrophils by activation of NADPH oxidase and enhanced release of superoxide anions (171). Nevertheless, the term "priming" does not only define enhanced respiratory burst activity, but also other phenotypic changes. Primed neutrophils also show enhanced chemotaxis, adhesion and transmigration into tissues, as well as increased phagocytic capacity and NET formation (166).

1.7.2 Neutrophil extracellular traps (NETs)

Neutrophil extracellular traps (NETs) were described as characteristic structures, that are released from activated neutrophils into the ambient environment, composed of chromatin and granule proteins that were first shown to mainly bind and kill microorganisms (170,172,173). The release of NETs is generally known as NETosis and is unlike apoptosis or necrosis, defined by the expansion of neutrophil nuclear material,

chromatin decondensation, granular membrane disintegration, plasma membrane rupture and the final release of neutrophil extracellular traps (174). NETosis process mainly requires the activation of NADPH oxidase and MPO, which results in production of reactive oxygen and nitrogen species (ROS/RNS) (170,175). However, other studies also showed NADPH/MPO-independent pathways that induce NET formation (176).

Several inducers (e.g. bacteria (172), IL-8 (177), hydrogen peroxide (170) and PMA (172)) are known for their potential to induce NETosis. Another prominent and controversially discussed example is the bacterial membrane molecule lipo-polysaccharide (LPS). Some studies observed NET formation when neutrophils were stimulated with low concentrations of LPS (178,179), whereas in other studies, even higher concentrations of LPS failed to induce NET formation (180,181). Clark *et al.* demonstrated that neutrophil-related NET formation and trapping of bacteria by LPS is highly dependent on the presence of platelets and can be significantly decreased when they were depleted (181).

A vast number of studies has contributed NETs to several diseases such as sepsis, cystic fibrosis, but also atherosclerosis and other cardiovascular diseases (174,182). This also highlights the importance of NETs in disease and its potential as a biomarker in clinical studies (182). In contrast to the overall pro-inflammatory action of NETs, it was also shown that aggregated NETs promote the degradation of cytokines and chemokines and thereby support the resolution of neutrophil inflammation (183).

In an infectious and pathogenic inflammatory scenario, bacteria have the ability to degrade NETs by the secretion of DNases (184-186) to avoid being killed. In sterile inflammation NETs might play a more complex role. On the one hand, NETs promote and initiate inflammation, but can also support its resolution (174,183). However, impaired degradation of NETs plays a critical role in autoimmune diseases (187) and can promote ongoing inflammation.

1.8 MIF isoforms and neutrophils

The role of MIF in the immune homeostasis, as well as in inflammation and in disease has been studied since decades (26,188). However, there is only little known about the

role between neutrophils and MIF. Figure 1-6 shows a few direct and indirect effects of MIF on neutrophils and vice versa.

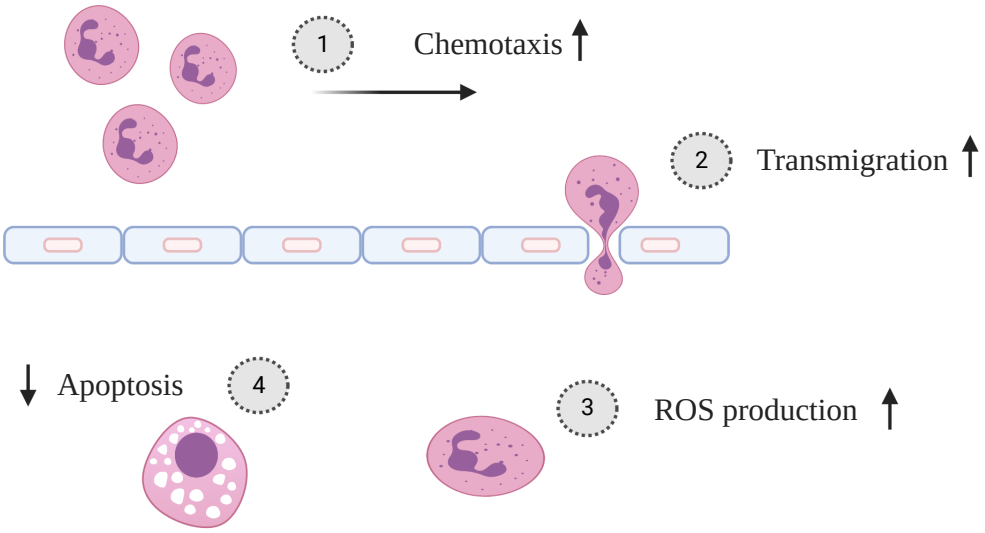

Figure 1-6 MIF affect neutrophil function. Different effects of MIF on neutrophil functions have been demonstrated. (1+2) MIF can induce neutrophil chemotaxis and transmigration (19,25,189,190). (3) In infections, MIF can promote MAPK pathway activation and stimulates ROS production (191). (4) Extracellular MIF can delay neutrophil apoptosis (86,192). Images were designed using BioRender software.

MIF is expressed by many different immune cells, including neutrophils and was shown to be released upon apoptosis induction through TNF-α stimulation (193). Apoptotic neutrophils can undergo secondary necrosis, which was linked to a passive release of MIF (194). MIF was shown to trigger neutrophil migration, depending on activation of the chemokine receptor CXCR2 (25) and MIF depletion exhibited reduced neutrophil trafficking and transmigration in inflammatory conditions (19,189,190). It was also previously reported that MIF can delay neutrophil apoptosis (192), whereat this effect might be triggered through the presence of Peripheral blood mononuclear cell (PBMCs) (86). In *in vitro* models of simulated infections, MIF was shown to promote MAPK pathway activation and stimulates ROS production (191). Until now, there is literally nothing known about the role of MIF-2 on neutrophil function.

1.9 Aim of this study

The atypical cytokine macrophage migration inhibitory factor (MIF) has an essential function in acute and chronic inflammatory diseases such as rheumatoid arthritis, cystic fibrosis, asthma, sepsis, atherosclerosis and myocardial infarction, as well as cancer. MIF is known as a pro-inflammatory mediator and has been shown to be involved in the recruitment of immune cells, as well as the prolongation of macrophage survival and counter-regulation the anti-inflammatory activities of steroids. The MIF homolog D-dopachrome tautomerase (D-DT, or MIF-2) was shown to have similar pro-inflammatory characteristics compared to MIF. However, much less is known about MIF-2 and its role in the immune system.

Neutrophils are the most abundant white blood cell population in the human body and a critical component of our 'first-line-of defense' innate immune response. Correspondingly, they exhibit a key role in the fight against invading pathogens. Moreover, in the last years it has been shown that neutrophils are also involved in sterile inflammatory disease progression. The regulation of neutrophil function such as chemotaxis, myeloperoxidase (MPO) and other oxidative activities as well as apoptosis needs to be controlled precisely, as it can be a critical turnover point in inflammation by either promoting ongoing inflammation or its attenuation. We found that MIF and MIF-2 can be oxidized at the *N*-terminal proline by MPO-driven hypochlorous acid (HOCl) leading to the MIF species oxMIF and oxMIF-2, but their role in MIF bioactivity and inflammation is still unclear.

The aim of my PhD thesis was to characterize the role of MIF isoforms (both unmodified MIF and MIF-2 as well as oxMIF and oxMIF-2) on major neutrophil functions, such as apoptosis, superoxide and HOCl production, as well as phagocytosis and NETosis. I sought to address, how MIF is involved in the delay of neutrophil apoptosis and which parameters promote this anti-apoptotic effect including the characterization of involved survival mediators and receptors. Furthermore, I wanted to clarify the effect of MIF-2 and that of the oxidized isoforms (oxMIF, oxMIF-2) on neutrophil survival compared to MIF. Additionally, I questioned the role of MIF on neutrophil oxidative activities such as the production of superoxide and HOCl and the potential of MIF as a priming agent for the oxidative activity of neutrophils. Furthermore, I aimed at analyzing the direct effect of MIF on promoting induced NETosis and its role on neutrophil phagocytosis.

2 Materials

2.1 Chemicals, reagents and media

Following general chemicals, reagents and media were used in the various experiments and obtained from the manufacturers indicated:

Chemicals and reagents	**Manufacturer**
Acetonitrile	Sigma, Merck, Darmstadt, Germany
Albumin bovine serum (BSA) ≥ 98 %	Carl Roth, Karlsruhe, Germany
Ampicillin	Invitrogen, Karlsruhe, Germany
Ammonium persulfate (APS)	Sigma, Merck, Darmstadt, Germany
AnnexinV	Invitrogen, Karlsruhe, Germany
AnnexinV binding buffer	Invitrogen, Karlsruhe, Germany
Biotin Labelling Kit	Roche, Mannheim, Germany
BetaGlo Kit	Promega, Walldorf, Germany
Catalase from bovine liver	Sigma, Merck, Darmstadt, Germany
Cytochrome C from equine heart	Sigma, Merck, Darmstadt, Germany
DAPI	Sigma, Merck, Darmstadt, Germany
D-dopachrome tautomerase, human (D-DT; MIF-2)	Bernhagen Laboratory, Munich, Germany; Hampton Laboratory, Christchurch, New Zealand
Dextran	Sigma, Merck, Darmstadt, Germany
Dimethylsulfoxid (DMSO)	Sigma, Merck, Darmstadt, Germany
Diphenyleneiodonium chloride (DPI)	Sigma, Merck, Darmstadt, Germany
Disodium hydrogen phosphate (Na_2HPO_4)	Sigma, Merck, Darmstadt, Germany

Chemicals and reagents	**Manufacturer**
Dithiothreitol (DTT)	Sigma, Merck, Darmstadt, Germany
Ethanol	Carl Roth, Karlsruhe
Fetal calf serum (FCS)	Sigma, Merck, Darmstadt, Germany
N-formyl-Met-Leu-Pheu (fMLP)	Sigma, Merck, Darmstadt, Germany
Ficoll	GE Healthcare, Uppsala, Sweden
Fluorescein-5-isothiocyanate (FITC)	Sigma, Merck, Darmstadt, Germany
Glycine	Fluka Chemie, Buchs, Switzerland
Hanks Buffered Saline Solution (HBSS)	Sigma, Merck, Darmstadt, Germany
Heparin	Ratiopharm, Ulm, Germany
Hydrochloric acid (HCl)	Fluka Chemie, Buchs, Switzerland
Isopropanol	KMF Laborchemie, Lohmar, Germany
Lipopolysaccharide (LPS)	Sigma, Merck, Darmstadt, Germany
Macrophage migration inhibitory factor, human (MIF)	Bernhagen Laboratory, Munich, Germany; Hampton Laboratory, Christchurch, New Zealand
Methanol	Carl Roth, Karlsruhe, Germany
Phorbol 12-myristate 13-acetate (PMA)	Sigma, Merck, Darmstadt, Germany
Polymyxin B sulfate salt	Sigma, Merck, Darmstadt, Germany
Potassium chloride (KCl)	Sigma, Merck, Darmstadt, Germany
Potassium dihydrogen phosphate	Sigma, Merck, Darmstadt, Germany
Propidium iodide (PI)	Invitrogen, Karlsruhe, Germany
R19-S	Hampton Laboratory, Christchurch, New Zealand

Chemicals and reagents	**Manufacturer**
RPMI 1640 media	Gibco, Thermo Fisher Scientific, Watham, MA, USA
Sodium chloride (NaCl)	Sigma, Merck, Darmstadt, Germany
Sodium hydroxide (NaOH)	Sigma, Merck, Darmstadt, Germany
Sodium dodecylsulfate (SDS)	Sigma, Merck, Darmstadt, Germany
Superoxide Dismutase (SOD) from bovine erythrocytes	Sigma, Merck, Darmstadt, Germany
SYTOX green	Invitrogen, Karlsruhe, Germany
TMB substrate	Thermo Fisher Scientific, Watham, MA, USA
Tris-HCl	Carl Roth, Karlsruhe, Germany
Triton X-100	Fluka Chemie, Buchs, Switzerland
Tween 20	Fluka Chemie, Buchs, Switzerland
Yeast Nitrogen Base selective medium	Formedium, Norfolk, United Kingdom
Yeast Extract-Peptone-Dextrose (YPD) medium	Formedium, Norfolk, United Kingdom
Zymosan from *Saccharomyces cerevisiae*	Sigma, Merck, Darmstadt, Germany

2.2 ELISA Kits

Following ELISA kits were used in indicated experiments and obtained from the manufacturers indicated:

ELISA kit	**Manufacturer**
Human CXCL12/SDF-1 DuoSet ELISA	R&D Systems, Minneapolis, USA
Human G-CSF DuoSet ELISA	R&D Systems, Minneapolis, USA
Human GM-CSF DuoSet ELISA	R&D Systems, Minneapolis, USA
Human IL-1ß/IL-F2 DuoSet ELISA	R&D Systems, Minneapolis, USA
Human IL-6 DuoSet ELISA	R&D Systems, Minneapolis, USA
Human IL-8/CXCL8 DuoSet ELISA	R&D Systems, Minneapolis, USA

2.3 Buffers and solutions

2.3.1 Common buffers and solutions

Buffer	**Compositions**
PBS (pH 7.2)	137 mM NaCl 2.7 mM KCl 1.5 mM KH_2PO_4 8.1 mM KCl in ddH_2O
PBS-T	0.05 % Tween 20 in PBS
TBS (pH 7.3)	20 mM Tris-HCl 150 mM NaCl in ddH_2O

Buffer	Compositions
TBS-T	0.05 % Tween 20 in TBS

2.3.2 Buffers and solutions for protein expression and purification

Buffer	Compositions
LB-media	1 % (w/v) NaCl 1 % (w/v) Bacto-Trypton 2 % (v/w) yeast extract
LB-agar	LB-media + 1,5 % (w/v) Bacto-agar
Low salt buffer, pH 7.5 [for MIF]	50 mM Tris-HCL, pH 7.5 150 mM NaCl
High salt buffer, pH 7.5 [for MIF]	50 mM Tris-HCL, pH 7.5 2 M NaCl
Low salt buffer, pH 7.4 [for MIF-2]	50 mM Tris-HCL, pH 7.4 50 mM NaCl
High salt buffer, pH 7.4 [for MIF-2]	50 mM Tris-HCL, pH 7.4 1 M NaCl
Dialysis buffer 1	20 mM NaH_2PO_4 5 mM DTT
Dialysis buffer 1	20 mM NaH_2PO_4

2.3.3 Buffers and solutions for HPP-assay

Buffer/Solution	Compositions
50 mM ammonium acetate buffer, pH 6.0	0.3854 g ammonium acetate + 100 ml ddH_2O incubate at RT over night; store at 4 °C

Buffer/Solution	Compositions
8 mM HPP solution	14.3 mg HPP + 10ml 50mM ammonium acetate buffer, pH 6.0
0.5 M boric acid, pH 6.2	47.2 mg boric acid + 10 ml ddH_2O

2.3.4 Buffers and solutions for leukocyte isolation

Buffer/Solution	Compositions
5% (w/v) dextran	25 g dextran + 4.5 g NaCl + 500 ml ddH_2O
2.7% (w/v) NaCl saline	27 g NaCl + 1000 ml ddH_20
5% (v/v) FBS/PBS	2.5 ml FBS + 47.5 ml PBS

2.3.5 Buffers and solutions for ELISA DuoSet® assay

Buffer/Solution	Compositions
Wash buffer	0.05 % Tween® 20 in PBS (pH 7.2-7.4)
Reagent diluent	1.0 % BSA in PBS (pH 7.2-7.4)
Substrate solution	1:1 mixture of Color Reagent A (H_2O_2) and Color Reagent B (Tetramethylbenzidine)
Stop solution	2 M H_2SO_4 in ddH_20

2.4 Antibodies and inhibitors

2.4.1 Antibodies

Antibody	Dilution/ Concentration	Use	Manufacturer
CD14	1:4 per $1x10^7$ cells	Isolation of monocytes/lymphocytes	Miltenyi Biotec, Bergisch Gladbach, Germany
CD61	1:4 per $1x10^7$ cells	Isolation of monocytes/lymphocytes	Miltenyi Biotec, Bergisch Gladbach, Germany
CD235a	1:4 per $1x10^7$ cells	Isolation of monocytes/lymphocytes	Miltenyi Biotec, Bergisch Gladbach, Germany
Ly6G	1:200	Immunofluorescence microscopy	Abcam, Cambridge, United Kingdom
MIF 3D9	1:200	Immunofluorescence microscopy	Bernhagen Laboratory, Munich, Germany
Neutrophil elastase	1:200	Immunofluorescence microscopy	R&D Systems, Minneapolis, USA
Rabbit anti-mouse; Alexa Fluor 555	1:500	Immunofluorescence microscopy	Pierce, USA
Mouse anti-rat; Alexa Fluor 488	1:500	Immunofluorescence microscopy	Pierce, USA
Goat anti-mouse; Alexa Fluor 647	1:500	Immunofluorescence microscopy	Pierce, USA

2.4.2 Inhibitors

Inhibitor	Description	Use	Manufacturer
4-iodo-6-phenylpyrimidine	Small molecule Inhibitor (4-IPP)	Block enzymatic- and bioactivity of MIF	Santa Cruz Biotechnology (Dallas, TX)
SB225002	CXCR2 antagonist; 'anti-CXCR2'	Block CXCR2 on neutrophils and PBMCs	Sigma Aldrich (St. Louis, MO)
NBP1-76867PEP	blocking peptide; 'anti-CXCR4'	Block CXCR4 on neutrophils and PBMCs	NovusBiologicals (Littleton, CO)
LN2	anti-CD74 antibody; 'anti-CD74'	Block CD74 on PBMCs	Abcam (Cambridge, UK)

2.5 Consumables

Consumables	Manufacturer
Microtube	Eppendorf, Hamburg, Germany
Cryotubes	Nalgene, Rochester, USA
Parafilm "M" – Laboratory Film	American National Can, USA
Pipette tips 1000 µl	Sarstedt, Nümbrecht, Germany
Pipette tips 200 µl	Sarstedt, Nümbrecht, Germany
Pipette tips 10 µl	Sarstedt, Nümbrecht, Germany
Plastic vials	Sarstedt, Nümbrecht, Germany
Syringes (2 ml, 10 ml, 50 ml)	Dispomed, Gelnhausen, Germany
VACUETTE Blood Collection Tubes	Greiner BioOne, Frickenhausen, Germany
96-Well Optical Btm Plt PolymerBase Black w/Lid Cell Culture Sterile PS	Thermo Scientific, Asheville, USA

Consumables	Manufacturer
Whatman filter paper	Greiner BioOne, Frickenhausen, Germany
Centrifuge tube (15 ml, 50 ml)	Thermo Scientific, Asheville, USA
Microtiter plates (24 well, 96 well)	Greiner Bio-One, Kremsmünster, Austria

2.6 Devices

Device	Manufacturer
-20 °C Freezer Premium NoFrost	Liebherr, Bulle, Switzerland
-80 °C Freezer HeraFreeze HFU T	Thermo Scientific, Asheville, USA
-152 °C Freezer ULT	Thermo Scientific, Asheville, USA
4 °C Fridge Premium BioFresh	Liebherr, Bulle, Switzerland
ÄKTApure	GE Heathcare, Chicago, USA
Analytic balance PCB	Kern&Sohn, Balingen, Germany
Analytic balance Analytical	Mettler Toledo, Columbus, USA
Centrifuge accuSpin Micro 17	Thermo Scientific, Asheville, USA
Centrifuge Avanti JCN-26	Beckman Coulter, Brea, USA
Centrifuge Megafuge 16R	Thermo Scientific, Asheville, USA
Clean bench Biowizard Silverline	Kojair, Mänttä-Vilppula, Finland
Clean bench HERAsafe	Heraeus, Hanau, Germany
FC500 MPL flow cytometer	Beckman Coulter, Brea, USA
FACSVerse™	BD Bioscience, Franklin Lakes, USA
Fraction collector F9-R	GE Heathcare, Chicago, USA
French Press, EmulsiFlex-C5	Avestin, Ottawa, Canada

Device	Manufacturer
Heating magnetic stirrer	VELP Scientifica, Usmate, Italy
Spectrophotometer U3900/3900H	Hitachi, Tokyo, Japan
Homogenisator EmulsiFlex C-5	ATA Scientific, Taren Point, Australia
Incubator shacking, for bacteria	GFL, Burgwedel, Germany
Incubator HERAcell VIOS 160i	Thermo Scientific, Asheville, USA
Ice machine	Manitowoc, Manitowoc, USA
LEICA DMi8	Leica, Wetzlar, Germany
Olympus IX81 (fluorescent microscope)	Olympus, Tokyo, Japan
Ph meter FiveEasy	Mettler Toledo, Columbus, USA
Pipettes	Gilson, Middleton, USA
Pipettor accu-jet® pro	Brand, Wertheim, Germany
PolarStar fluorescence plate reader	BMG Labtech, Ortenberg, Germany
Rocking shaker VIBRAX VXR basic	Janke&Kunkel, Staufen, Germany
Schott flask	Schott, Mainz, Germany
Vortex VV3	VWR, Radnor, USA
Water bath type 1004	GFL, Burgwedel, Germany

2.7 Software

Software	Use	Manufacturer
GraphPad 6	Data and statistical analyses Generate graphs for publication and dissertation	GraphPad Software, La Jolla, Ca, USA

Software	**Use**	**Manufacturer**
ImageJ	Counting cells (Migration Assay)	Schneider C. *et al*. (2012), *Nature methods* (9)7 (195)
Fiji	quantify the fluorescence signal	Schindelin, J. *et al*. (2012), *Nature methods* 9(7) (196)
FlowJo	Data analysis of FACS samples	FlowJo, Becton, Dickinson & Company, Franklin Lakes, USA

3 Methods

3.1 Protein chemistry techniques

3.1.1 Purification of human MIF

Recombinant MIF protein was overexpressed in *E. coli* BL-21(DE3) and a FPLC was used for the purification process using an established protocol (3).

In the beginning, the bacterial pellet was carefully resuspended in Buffer A (50 mM Tris-HCL, pH 7.5, 150 mM NaCl) and cells were lysed using a French Press. Cell lysate was centrifuged at 30,000 x g for 30 min at 4 °C using a Beckman ultra-centrifuge. The lysate was filtered (0.2 µm filter), before applying to an equilibrated MonoQ column using the ÄKTA purifier FPLC system at a flow rate of 1 ml/min. MIF is not binding the column and is eluted directly. Individual fractions were collected, pooled and protein concentrations were measured using Bradford assay (chapter 3.1.3). Subsequently, a reverse phase chromatography technique was used to further purify the MonoQ MIF factions. A C8 SepPak RP column was equilibrated with 10 ml of methanol and washed with 10 ml of water at a flow rate of 5 ml/min. A maximum of 2 mg of protein solution was loaded onto the column and was pressed through the column a flow rate of 0.6-0.8 ml/min. Afterwards, the column was washed with water and 20% acetonitrile, to remove low- and non-binding proteins. The bound MIF was eluted from the column using 60% acetonitrile. The eluted protein was lyophilized and stored at -20 °C until further use.

After lyophilization, the protein was renatured. Therefore, 1 mg lyophilized MIF protein was dissolved in 3300 µl GuaHCl/NaPP and 33.3 µl DTT (1 M) and incubated at RT for approximately 2 h. The protein solution was transferred into a dialysis tube and dialyzed for 5 h against dialysis buffer 1. Afterwards, the protein was dialyzed another three times against dialysis buffer 2. The protein concentration was measured using Bradford Assay. Containing LPS concentrations were measured by using the limulus amoebocyte assay (LAL, Lonza, Cologne, Germany) following the manufacturer's instructions and has been determined to be below 0.02-0.1 ng/µg protein.

3.1.2 Purification of human MIF-2

Similar to MIF proteins, MIF-2 was overexpressed in transfected *E. coli* BL-21(DE3) and purified using a FPLC technique. The following established protocol was used (14).

The bacterial pellet was resuspended in Buffer A (50 mM Tris-HCL, pH 7.4, 50 mM NaCl) and cells were lysed as described above (chapter 3.1.1). The filtered lysate was subjected to an equilibrated Q Sepharose anion exchange column and the protein was purified by using the ÄKTApurifier FPLC system at a flow rate of 1 ml/min Buffer A. MIF-2 was eluted from the column by using the following buffer gradient protocol, reaching 100% Buffer B (50 mM Tris-HCl, pH 7.4, 1 M NaC1) in 100 min. MIF-2 containing fractions were collected and pooled for further purifications. The protein concentration was measured using Bradford Assay (chapter 3.1.3).

A hydrophobic chromatography system was used to increase the purity of the protein. In contrast to MIF, a C18 column is used. The preparation of the column and protein purification was performed as described before (chapter 3.1.1). Containing LPS concentrations were obtained to be <0.02-0.1 ng/µg protein.

3.1.3 Measurement of protein concentration – Bradford assay

Protein concentrations were measured by the Braford assay (197) using a spectrophotometer. First, the reaction solution concentrate (BioRad (Munich) 5x Bradford solution) was diluted 1:5 with ddH_2O. Subsequently, 980 µl Bradford solution and 20 µl sample were mixed in a cuvette and incubated for 5 min at RT in the dark. The absorbance was measured at 595 nm against the corresponding reference solution and the protein concentration was calculated using a calibration standard curve.

3.1.4 HPP keto-enol activity assay

Both MIF and MIF-2 share an evolutionary conserved intrinsic hydroxyphenyl pyruvate (HPP) keto-enol activity which is supposed to be facilitated by the *N*-terminal proline, that both proteins hold (14). In order to measure the enzymatic activity of MIF and its isoforms MIF-2, oxMIF and oxMIF-2 this HPP Keto-enol Activity Assay was performed. The assay measures spectrophotometrically a complex between borate and the enol form

of HPP at an absorbance of 306 nm (198). Assay was performed for each preparation of MIF and MIF-2, to verify enzymatic activity.

In preparation, the HPP buffer was prepared one day before the assay was performed by dissolving 8 mM of HPP in 50 mM ammonium acetate buffer (pH 6.0) and was equilibrated at RT overnight. Just before the assay was performed, HPP buffer was diluted to a final concentration of 0.8 mM in 0.44 M boric (pH 6.2). Subsequently, the protein was added at a final concentration of 250 nM. The complex formation of borate and HPP was estimated by an increasing absorbance at 306 nm for 240 sec in 5 sec intervals. Sodium phosphate buffer was used as a reference control.

3.2 Cell isolation and cell activity assays

3.2.1 Neutrophil and PBMC isolation

Human neutrophils and PBMCs were isolated from fresh drawn blood of healthy donors through an established detran sedimentation and Ficoll separation protocol (86). Heparin was used as an anticoagulant at a concentration of 10 units per 1 ml of blood. For the following cell isolation process, all solutions were warmed to room temperature before use. Heparinized blood was diluted 1:3 in a 50 ml Falcon tube with PBS and mixed gently by inversion. Afterwards, 5% dextran was added to the blood/PBS solution to a final concentration of 1% dextran and was kept at RT approximately 15 min after gentle inversion. Dextran sedimentation is used for the separation of the majority of the red blood cell population, which settle down first and leave a leukocyte-enriched upper layer. To gain an optimal neutrophil yield, a longer sedimentation time should be avoided as neutrophils might sediment as well. Subsequently, the upper layer, containing PBMCs and neutrophils, was removed by using a Pasteur pipette and was gently transferred to a 15 ml Falcon tube. To separate neutrophils and PBMCs, a Ficoll separation was performed. Therefore, 3 ml of Ficoll was carefully underlaid the leukocyte solution. This was followed by a centrifugation step in a swing out rotor at 1000 x g for 20 min at RT with no brake. After centrifugation two major cell layers were visible – the upper PBMC containing layer and the lower neutrophil and red blood cell containing layer. First, the PBMC layer was removed by using a sterile Pasteur pipette without touching the tube

walls. Following, each neutrophil containing pellet was resuspended in 500 µl PBS and transferred to a new 50 ml Falcon. To purify the neutrophils from contaminating red blood cells a water lysis was performed by adding 20 ml of water and gently mixing by inversion. After 2 minutes, 10 ml of 2.7% NaCl solution was added to reach physiological saline conditions and to reduce osmotic pressure. Shortly after, neutrophils were pelleted by centrifugation in a swing out rotor for 5 min at 450 x g at RT. The supernatant was aspirated carefully, and the cell pellet was resuspended in RPMI media supplemented with 5% FCS. Cells were counted by using a hemocytometer and neutrophil purity was determined by FACS analysis using an established protocol. The neutrophil culture showed a purity between 97-99%. To avoid activation, cells should be kept on RT and used as soon as possible for following experiments.

3.2.2 Monocyte and lymphocyte isolation

Human monocytes and lymphocytes were isolated using an antibody coupled MicroBeads system from Miltenyi Biotec containing a LS Column and a MidiMACS separator. The purification of monocytes is based on a positive selection with a CD14 antibody, whereas lymphocytes are purified by negative depleting using CD14, CD61 and CD235a antibodies. Using the manufactures protocol, PBMCs were collected as described above (chapter 3.2.1) and counted by using a hemocytometer. Cells were first incubated with CD14 antibody in a ratio of 5:1 for 15 min at 4 °C. Afterwards, cells were pelleted by centrifugation and washed twice with 5% FCS/PBS and resuspended in 5% FCS/PBS at an appropriate volume. For the isolation of $CD14^+$ cells (monocytes), the column was positioned in a magnetic MidiMACS separator and cells were applied to the column. Unlabeled cells (lymphocytes and $CD14^-$ monocytes) were washed from the column by applying 3x3 ml of 5% FCS/PBS and were used for further purification. Subsequently the column was detached from the MidiMACS separator and $CD14^+$ cells were eluted by adding 5 ml of 5% FCS/PBS. Eluted cells were pelleted by centrifugation and resuspended in 1 ml RPMI media supplemented with 5% FCS. The remaining cell solution containing $CD14^-$ cells was incubated with a CD61 and CD235a antibody solution in a ratio of 5:1 for 15 min at 4 °C. Next, cells were washed twice with 5% FCS/PBS and resuspended in 5% FCS/PBS. Lymphocytes were isolated by negative selection. As described above, a LC column was positioned in a magnetic MidiMACS

separator and the cell suspension was applied. While $CD61^+/CD235a^+$ cells attach to the column, $CD61^-/CD235a^-$ cells (lymphocytes) with pass through. The lymphocyte cell suspension was centrifuged, and the cell pellet was resuspended in 1 ml RPMI media supplemented with 5% FCS. Cells were counted by using a hemocytometer and cell purity was determined by FACS analysis using an established protocol (86). The lymphocyte and monocyte cultures showed a purity between 95-99%.

3.2.3 Processing of human serum

For autologous serum processing, blood from individual donors was collected in tubes coated with microscopic silica particles, that activate the coagulation process (Greiner Bio-One). The serum tube was incubated at 37 °C for approximately 1 h. To collect the serum, tube was centrifuged at 1,200 x g and the clear supernatant was transferred into a sterile 1.5 ml microtiter tube. To remove any contaminating red blood cells, tube was again centrifuged at 12,000 x g for 30 s and supernatant was transferred to a new sterile microtiter tube. Serum was kept on ice until further use or stored at -20 °C.

3.2.4 Annexin V-FITC labelling

Annexin V-FITC/PI labelling was performed using the following established protocol (86). First, 500 µl of cells were centrifuged for 5 min at 450 x g at RT. The cell pellet was resuspended in 50 µl Annexin-V binding buffer (1x) containing 1 µl of Annexin V-FITC and incubated in the dark for 10 min at RT. After the incubation, 5 µl Propidium Iodide (PI) (1 mg/ml) and 200 µl of Annexin-V binding buffer was added and transferred to a 96-well plate for FACS analysis. Counted neutrophils (10,000 cells/measurement) were gated for positive and negative staining of FITC labeled Annexin-V and PI. Gating strategies considered Annexin-V and PI negative cells to be “viable”, Annexin-V positive and PI negative to be “apoptotic” (Fig. 3-1) and Annexin V positive and PI positive cells to be “necrotic”.

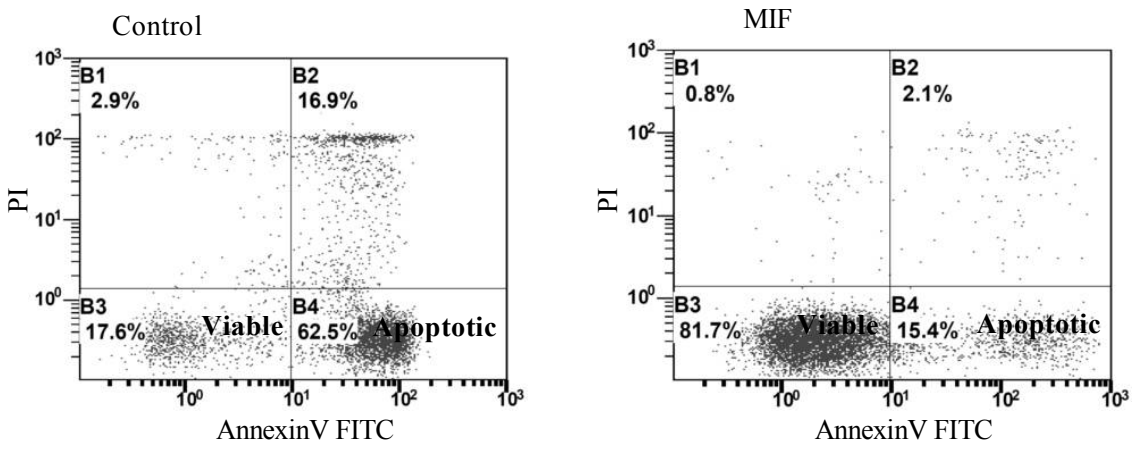

Figure 3-1 Gating strategies neutrophil apoptosis assay. Representative FACS data showing viable and apoptotic neutrophils after 21 h incubation with and without MIF (10 µg/ml). Gating strategies considered Annexin-V and PI negative cells to be "viable", Annexin-V positive and PI negative to be "apoptotic" (Schindler *et al.* (2020), *J Leukoc Biol,* in revision).

3.2.5 Neutrophil apoptosis assays

To verify whether MIF or MIF-2 and their oxidized isoforms are able to affect neutrophil apoptosis, neutrophil apoptosis was measured using Annexin V/Propidium iodide (PI) staining, followed by FACS analysis. The methods/treatments explained in this chapter are submitted for publication as part of the following manuscript: Schindler, L. *et al.* (2020), *J Leuko Biol*.

Neutrophils and PBMCs were isolated as described before (chapter 3.2.1). Next, human neutrophils were either cultured alone or co-cultured with PBMCs with increasing concentrations of MIF and MIF-2 (0, 0.5, 2.5, 5, 7.5 and 10 µg/ml) in RPMI 1640 media supplemented with 10% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B. The effect of LPS and the ability of polymyxin B to block LPS-mediated effects were tested in a separate experiment. A controls LPS (1 ng/ml) and LPS plus polymyxin B (20 µg/ml) were added to a neutrophil/PBMC 1:1 co-culture. Every apoptosis assay was performed in a 24-well plate and cells were incubated at 37 °C/ 5% CO_2 in a humidified incubator for 21 h. In a separate experiment, MIF and MIF-2 were also pre-treated with 5-fold molar excess of hypochlorous acid (oxMIF, oxMIF-2) for 15 min at RT before adding to the cells using the same concentrations as described before for non-treated MIF and MIF-2. Cell viability was analyzed using AnnexinV/PI staining as described before (chapter 3.2.4).

In another experiment, PBMCs alone were incubated with MIF and MIF-2 (10.0 µg/ml), as well as LPS (1 ng/ml) with or without polymyxin B (20 µg/ml) in RPMI 1640 media supplemented with 10% heat-inactivated FCS (v/v) for 21 h. Afterwards, conditioned supernatants were collected by centrifugation and stored at -80 °C for further experiments. Later, PBMC-conditioned supernatants (600 µl/well) were added to freshly isolated neutrophils ($5x10^5$/ml) and were incubated for 21 h as described above followed by Annexin V-FITC/PI labelling and FACS analysis.

To investigate the ideal ratio of PBMCs to neutrophils and the population, that is mainly responsible for the anti-apoptotic effect of MIF, neutrophils were also incubated with different ratios of total PBMC cultures or isolated monocytes and lymphocytes with 10 µg/ml MIF in RPMI 1640 media supplemented with 10% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B. After 21 h neutrophils were analyzed for their viability using Annexin-V/PI staining.

The small molecule inhibitor 4-iodo-6-phenylpyrimidine (4-IPP) has been shown to block MIF's enzymatic and bioactivity through binding the *N*-terminal proline (79,199,200). Previously published data showed that oxidation of MIF led to failed binding of 4-IPP (86). In order to verify whether 4-IPP can also block MIF's biological activities in the delay of neutrophil apoptosis, MIF and oxMIF were pre-treated with 10-fold molar excess of 4-IPP for 15 min at RT before adding them to a neutrophil-PBMC co-culture system. As a control, non-treated MIF was used. Neutrophils and PBMCs were incubated with the different treatments for 21 h before viability was measured using FACS analysis.

For receptor-blocking experiments, neutrophils and PBMCs were pre-treated with different agents to block the MIF surface receptors CXCR2 (SB225002), CXCR4 (NBP1-76867PEP) and CD74 (LN2). Cells ($1x10^7$/ml) were pre-treated with SB225002 (25 µg/ml), NBP1-76867PEP (25 µg/ml) or LN2 (10 µg/ml) for 30 min at RT. Afterwards, neutrophils ($5x10^5$/ml) and PBMCs ($5x10^5$/ml) were incubated in the presence or absence of MIF (10 µg/ml) in RPMI 1640 media supplemented with 10% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B. Cell viability was analyzed by Annexin V-FITC/PI labelling and FACS analysis.

To verify the effect of Interleukin-8 (CXCL8) in the system, PBMCs were pre-treated with MIF and MIF-2 (10 µg/ml) for 21 h at 37 °C. Afterwards, cells were pelleted by centrifugation and supernatants were collected. Anti-hCXCL8/IL-8 (0.4 µg/ml) was

added to the conditioned supernatants and incubated for 15 min at RT. Monoclonal IgG1 antibody was used as negative control and recombinant human IL-8 (20 ng/ml) served as a positive control sample. Neutrophils ($5x10^6$/ml) were added to the differently treated conditioned supernatants and incubated for 21 h at 37 °C/5% CO_2 in a humidified incubator and neutrophil apoptosis was measured as described below.

3.2.6 Continuous neutrophil cytochrome c assay

Neutrophils have been shown to get stimulated by various stimuli which results in the production of superoxide (175). The cytochrome c assay is the easiest way to determine the respiratory burst activity of intact neutrophils. This established assay measures the superoxide-mediated reduction of cytochrome c which can be detected in a change of absorbance at 550 nm. To activate neutrophils, Phorbol 12-myristate 13-acetate (PMA) and *N*-Formylmethionyl-leucyl-phenylalanine (fMLP) were used, which both are known as a stimulators of the NADPH oxidase and the respiratory burst in neutrophils (175). The method explained in this chapter will be submitted for publication as part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

Neutrophils were isolated as described above (chapter 3.2.1) and pre-incubated with increasing concentrations of MIF and oxMIF (0, 0.1, 0.25, 0.5, 1 and 10 µg/ml) in HBSS buffer supplemented with 1% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B for 10 min up to 4 hours on a rotator at 37 °C. Afterwards, cells were centrifuged at 450 x g and the pellet was resuspended in pre-warmed HBSS buffer. PMA (100 ng/ml) or fMLP (100 nM) were used to induce the production of superoxide. The assay was performed in the presence of Catalase (20 µg/ml) to scavenge hydrogen peroxide production, which can also oxidize the reduced cytochrome c leading to a false positive result. The reduction of cytochrome c (40 µM) by superoxide production was measured at 550 nm every 18 s for 5 min using the Hitachi spectrophotometer. Superoxide dismutase (SOD) is able to inhibit the reduction of cytochrome c and was therefore used as a control treatment. The assay was performed in a plastic cuvette with a slit width of 0.5 nm at 37 °C. The rate of superoxide production was calculated (nmoles superoxide/min/10^6 cells) using the following formula: $\varepsilon=21.1\ x10^3\ M^{-1}cm^{-1}$.

In separate experiments, MIF and oxMIF were incubated with a ten-fold molar excess of the small molecule inhibitor 4-IPP for 15 min at RT before adding neutrophils as described before.

To determine relevant neutrophil surface receptors, mediating MIF affected superoxide production, receptor blocking experiments were performed by pre-treatment of neutrophils with SB225002 (‘anti-CXCR2, 25 µg/ml) or the CXCR4 blocking peptide NBP1-76867PEP (‘anti-CXCR4’, 25 µg/ml) for 30 min at RT before MIF or oxMIF were added and assay procedure was performed as described.

3.2.7 Preparation of zymosan particles for R19-S assays

Zymosan A (from *S. cerevisiae*) is a glycan derived from the yeast cell wall and was used as a phagocytosable particle in the following assays. Zymosan A (5 mg/ml ≙ $3x10^8$ particles/ml) was resuspended in PBS and sonicated for 5 min. Afterwards, zymosan was placed into boiling water for 10 min and washed twice before it was resuspended in PBS (20 mg/ml ≙ $1.2x10^9$ particles/ml). The zymosan stock was stored in aliquots at -20 °C until further use.

For FITC labelling, zymosan particles were pelleted by centrifugation and resuspended in 0.1 M sodium carbonate buffer (pH 9), before sonification in a waterbath for 2 min. FITC (1 mg/ml in DMSO) was added at a final concentration of 30 µg/ml and incubated at 37 °C in the dark for 30 min. After labelling, zymosan was washed twice with HBSS and store on ice in the dark until further use (used within 1 h).

Prior phagocytosis by neutrophils, zymosan particles needed to be opsonized. Therefore, labelled and non-labelled zymosan was incubated with 50% autologous human serum for 30 min at 37 °C and was washed twice and resuspended in Hanks buffer, before stored on ice until further use (used within 3 h).

3.2.8 R19-S assay

R19-S is a non-fluorescent rhodamine-based dye which can get converted into a highly fluorescent rhodamine structure (R19) through oxidation by HOCl. This switch is unique for the reaction with HOCl and cannot be seen within other oxidants (201-203). It has

been shown to react with HOCl produced by activated neutrophils (202) and can be used to monitor HOCl production in neutrophils using live cell imaging or FACS analysis. To activate neutrophils in this assay, opsonized zymosan was used. The method explained in this chapter will be submitted for publication as part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

For FACS analysis, neutrophils were isolated as described above (chapter 3.2.1) and $1x10^6$ cells were pre-incubated in the presence or absence of 10 µg/ml MIF or oxMIF for 30 min or 60 min in HBSS buffer supplemented with 1% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B at 37 °C. In the meantime, opsonized zymosan (see chapter 3.2.7) and R19-S samples were prepared. R19-S was dissolved in acetonitrile at 50 °C and concentration was measured at 308 nm using a spectrophotometer. Neutrophils were centrifuged and the pellet was resuspended in HBSS buffer to a final density of $1x10^6$/ml neutrophils. Before adding the opsonized zymosan, R19-S (10 µM final concentration) was added to each sample and incubated for 5 min at 37 °C in the waterbath. Subsequently, the opsonized zymosan was added to each sample at a concentration of $5x10^6$ particles/ml and incubated at 37 °C for 5, 10, 15 and 30 min. Diphenyleneiodonium chloride (DPI), a scavenger of NADPH oxidase (10 µM final) was added as a negative control to suppress the response on zymosan. Red fluorescence intensity of R19 (Ex_{488}/Em_{575}) in the neutrophil population was measured at the indicated timepoints using a flow cytometer. FITC-labeled zymosan ($5x10^6$/ml) was used to measure the rate of zymosan phagocytosis by measuring green fluorescence intensity (Ex_{459}/Em_{519}) at indicated timepoints via FACS.

3.2.9 NETosis assay – SYTOX® green

SYTOX® green is a fluorescent dye that only binds to extracellular DNA, as it cannot permeate the plasma membrane. Formation of NETs is one of the neutrophil key functions to combat different invading pathogens (172). One characteristic is release of DNA in response to antimicrobial components (174). The method explained in this chapter will be submitted for publication as part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

NET formation by neutrophils was measured using the SYTOX green plate assay as described before (204). Briefly, neutrophils were isolated as described above (chapter 3.2.1) and $1x10^6$ cells/ml were pre-incubated in the presence or absence of 10 µg/ml MIF or oxMIF for 60 min in HBSS buffer supplemented with 1% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B at 37 °C. Cells were pelleted and resuspended in HBSS before inducing NET formation by 20 nM PMA in a 96-well plate format. SYTOX green (30 nM) was added at various time points (30, 60, 120, 180 and 240 min) and fluorescence emission was measured using a PolarStar fluorescence plate reader (Ex_{485}, Em_{520}). Background signal from a sample containing MIF alone was subtracted. To test whether MIF itself could induce NET formation, neutrophils were -incubated with MIF (10 µg/ml) without adding PMA and fluorescent signal was measured after adding SYTOX green (30 nM) at 30, 60, 120, 180 and 240 min.

In a separate experiment, we wanted to investigate the role of MIF as a potential nuclease (205). For this, neutrophils ($1x10^6$/ml) were incubated with PMA (100 ng/ml) in a 96-well plate for 4 h at 37 °C to preform NETs. Supernatants were removed and each well was carefully washed with HBSS before adding 10 µg/ml MIF for 24 h at 37 °C. SYTOX green (30 nM) was used to measure remaining DNA. HBSS or HBSS containing DNAse (0.01, 0.1, 10 Units) served as negative and positive controls, respectively.

3.3 Immunocytochemistry and immunohistochemistry

3.3.1 R19-S assay and FITC-zymosan phagocytosis

To verify the effect of MIF and oxMIF on the production of HOCl in neutrophils, the R19-S assay (chapter 3.2.8) was performed using immunocytochemistry. The method explained in this chapter will be submitted for publication as part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

Neutrophils were isolated as described above (chapter 3.2.1) and were pre-incubated ($1x10^6$/ml) with 10 µg/ml MIF or oxMIF for 60 min in HBSS buffer supplemented with 1% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B at 37 °C. R19-S and opsonized zymosan were prepared as described above (chapter 3.2.7). After the incubation time,

cells were pelleted and resuspend in 500 µl HBSS buffer and kept on ice for about 20 min. R19-S (10 µM final concentration) and methionine (1 mM final concentration), that was used so scavenge extracellular HOCl, were added to the ice-cold cell suspension. Finally, zymosan ($1.2x10^7$ particles/ml) was added and immediately 100 µl were transferred into a 96-well microscopy plate and centrifuged for 1 min at 1000 rpm. The fluorescent microscope chamber was set to 37 °C. The plate was placed into the fluorescent microscope and time lapse images (frames) of DIC, Cy3 (for R19) and FITC (for zymosan) channels were taken every 18 s over 60 min with a 20x magnification. Fiji by Image J software was used to quantify the fluorescence signal of single phagosomes and intensity over time.

3.3.2 NET formation

Immunocytochemistry analysis of NETs released by human neutrophils was applied to visualize the effect of MIF on neutrophil DNA and its potential role in NET degradation (206). The method explained in this chapter will be submitted for publication as part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

NET formation was measured using fluorescent signal of extracellular SYTOX green and neutrophil elastase. Therefore, neutrophils ($3x10^5$/ml) were pre-incubated ($1x10^6$/ml) in the presence or absence of 10 µg/ml MIF or oxMIF for 60 min in HBSS buffer supplemented with 1% heat-inactivated FCS (v/v) and 20 µg/ml polymyxin B at 37 °C, before NETosis was induced by adding 100 ng/ml PMA in a 24-well plate on a coverslip for 4 h at 37 °C. To prepare coverslips for staining, paraformaldehyde was carefully added into each well at a final concentration of 4% PFA and incubated for 20 min at RT. Afterwards, each sample was washed with PBS four times and 0.5% Triton X-100 in PBS was added to permeabilize the cells for 10 min, followed by another three washing steps. Each sample was blocked with 10% BSA/PBS for 1 h at 37 °C, before the primary antibody (hNE) was added at a concentration of 1:500 in 3% BSA/PBS for 1 h at 37 °C. Next, samples were washed and the secondary antibody (goat anti-mouse AF647) was added at a concentration of 1:500 in 3% BSA/PBS for 1 h at 37 °C, followed by another washing step. In addition, SYTOX green was added to each sample at a final concentration of 200 nM and was incubated in the dark for 5 min at RT. At the end, each

coverslip was placed face down on a small drop of Fluormount G and left to dry. Neutrophil elastase and SYTOX green signals were analyzed using a fluorescent microscope and images of Cy5 (NE), DIC and FITC (SYTOX green) channels were taken with a 40x magnification.

3.3.3 Cardiac ischaemia reperfusion injury – cryo sections

Myocardial infarct tissue from mice was kindly provided by Ulrike Hendgen-Cotta and the Department of Cardiology and Angiology from the University Hospital Essen at the Westdeutsches Herz- und Gefäßzentrum Essen in Germany. The *in vivo* myocardial I/R injury (30 min ischemia/ 24 h reperfusion) was performed as described and cryosections were prepared (76).

Immunofluorescent microscopy was performed to identify the potential co-localization of neutrophils and MIF in myocardial I/R injury. Therefore, frozen cryosections were airdried for 10-15 min at RT, before adding acetonitrile for 10 min to fix the tissue samples. The samples were washed twice and blocked wit 5% horse serum/PBS for 60 min at RT. Afterwards, each sample was incubated with primary antibodies (Ly6G/ MIF 3D9) over night at 4 °C, followed by four washing steps. The secondary antibodies (Alexa Fluor 555/488) were added for 60 min at RT and samples were washed with PBS. Nuclear counterstain was performed using DAPI for 10 min at RT. Finally, each sample was washed again, and the stained tissue was covered with mounting anti-fate media and left to dry. Co-localization was analyzed using a fluorescent microscope and images of Cy3 (MIF), DIC and FITC (Ly6G) channels were taken.

3.4 Molecular biological and immunological assays

3.4.1 Enzyme-linked immunosorbent assay (ELISA)

The ELISA assay is an antibody-based technique to quantitatively and qualitatively identify proteins, peptides or hormones in solutions (207,208). This assay was used to screen supernatants of stimulated PBMCs for six different proteins: Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin 8 (CXCL8), stromal cell-derived factor 1 (SDF-1),

Granulocyte-colony stimulating factor (G-CSF) and Granulocyte macrophage colony-stimulating factor (GM-CSF). Assays were purchased from R&D Systems and were performed using the manufactures protocol. Capture and Detection antibody were both provided in every kit for each individual ELISA. The assay procedure was identical for each test kit and is described below. The methods/treatments explained in this chapter are submitted and under revision for publication in the following paper: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

Human PBMCs were isolated as described above (chapter 3.2.1) and were incubated with increasing concentrations of MIF, MIF-2, oxMIF and oxMIF-2 for 21 h at 37 °C/5% CO_2 in a humidified incubator. Afterwards, cells were pelleted by centrifugation and supernatants were collected. Supernatants were stored at -20 °C until the ELISA assay was performed.

To start the ELISA assay, a 96-well plate was coated with 100 µl/well of the capture antibody diluted in PBS over night at RT. Afterwards, the plate was washed 3 times with wash buffer (400 µl/well) and 300 µl/well of reagent diluent was added to block the plate for a minimum of 1 hour. In the meantime, samples were diluted 1:10 in reagent diluent and a standard curve with provided recombinant protein for each individual assay was prepared. Afterwards, 100 µl/well of sample and standard was added to the plate and incubated for at least 2 h at RT. The Plate was washed another three times (400 µl/well) with wash buffer, followed by adding 100 µl/well of detection antibody solution into each well. After an incubation time of 2 hours at RT, the plate was washed again 3 times. Before adding the substrate solution, each well was incubated with 100 µl of a Streptavidin-HRP conjugate for 20 min at RT in the dark. The plate was washed again 3 times and 100 µl/well of substrate solution was added. After 5-10 min, depending on the respective ELISA kit, 50 µl/well stop solution was added, and optical density of each sample was measured using a microplate reader set to a wavelength of 450 nm.

3.5 Microbiological assays

3.5.1 Yeast assay

Receptor binding of MIF and oxMIF to CXCR2 was determined using a yeast based signaling activity assay. The genetically modified yeast strain *Saccharomyces cerevisiae* (CY12946) expressing a functional human receptor (CXCR4) was previously described (61,209). Here we used the same protocol and tools to generate a human CXCR2 yeast transformant. Agonist (MIF or oxMIF) mediated activation of CXCR2 signaling was measured through β-galactosidase activity assay described previously (62,72). Therefore, yeast cells expressing the human receptor CXCR2 were grown overnight at 30 °C, in Yeast Nitrogen Base selective medium (Formedium, UK). Following, the cells were diluted to an OD600 of 0.2 in Yeast Extract-Peptone-Dextrose (YPD) medium and were further grown until cell solution reached an OD600 of 0.5 – 0.6. Next, the cells were incubated with different concentrations of MIF and oxMIF (5, 10 and 20 µM) for 90 min. Finally, for each individual sample the OD600 was measured again and the CXCR2 mediated signaling cascade activation was quantified by β-galactosidase activity using the commercial BetaGlo Kit (Promega, Germany) and a standard microplate reader.

3.6 Statistics

Graphs were plotted and statistical analysis was performed using GraphPad Prism 6. Each dot represents the mean of duplicate measurements for each independent experiment from individual donors. Differences between groups were determined by either one-way ANOVA or unpaired student's t-test. A p-value <0.05 was considered significant.

4 Results

This thesis addresses the role of MIF proteins and their oxidized isoforms on main neutrophil functions, such as apoptosis, superoxide production, HOCl production and NET formation. Therefore, I characterized the potential of MIF as a priming agent on neutrophil activity. It was further tested, whether MIF and neutrophils are co-localized in an *in vivo* myocardial I/R injury model.

Results have been submitted and are under revision in part in the following papers:

Schindler, L., Zwissler, L., Krammer, C., Hendgen-Cotta, U., Rassaf, T., Hampton, M.B., Dickerhof, N., and Bernhagen, J. (2020) Macrophage migration inhibitory factor inhibits neutrophil apoptosis by inducing cytokine release from mononuclear cells. *J Leukoc Biol*, in revision.

Results will be shortly submitted in the following papers:

Schindler, L., Hampton, M.B., Bernhagen J. and Dickerhof, N. (2020) Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity, to be submitted.

In each chapter, published/submitted data will be individually indicated by referring to the corresponding paper.

4.1 MIF isoforms delay neutrophil apoptosis

Modified neutrophil apoptosis can have a significant impact on the progression of inflammation. For example, delayed neutrophil apoptosis can result in non-resolving inflammation, a driver of many diseases such as atherosclerosis, rheumatoid arthritis and cancer (143). Previous studies indicated that MIF can attenuate neutrophil apoptosis (86,192). The results illustrated in this chapter have been submitted and are under revision for publication in the following manuscript: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

This part of my dissertation describes the role of MIF protein isoforms on neutrophil survival and its potential in delaying neutrophil apoptosis. Our group recently published the effect of MIF on delaying neutrophil apoptosis (86), however, there is no evidence so far of MIF-2 having the same effect. Therefore, neutrophils and PBMCs were cultured alone or co-cultured with MIF and MIF-2, followed by neutrophil viability measurement using FACS. Surprisingly, there was no effect of MIF and MIF-2 on neutrophil survival, when neutrophils were cultured alone in the absence of PBMCs (Fig. 4-1A). By adding peripheral blood mononuclear cells, the effect of MIF and MIF-2 mediated delay of neutrophil survival increased significantly and peaked at a concentration of 10 µg/ml for both proteins (Fig. 4-1A, B). When comparing both proteins and their effect on neutrophils survival, no significant differences could be observed (Fig. 4-1A, B).

The recombinantly produced proteins (MIF and MIF-2), that were used in this study, contained low concentrations of LPS (<0.02-0.1 ng/µg protein). However, as lipopolysaccharide (LPS) has been proposed as a neutrophil survival mediator (123), each individual experiment was performed with of the well-known LPS-neutralizing agent polymyxin B, to exclude false positive results of MIF proteins on neutrophil survival. Therefore, I additionally used LPS as a positive control treatment and was able to effectively block the anti-apoptotic effect of LPS on neutrophils by adding polymyxin B (Fig. 4-1A). Nevertheless, when polymyxin B was added to the MIF and MIF-2 treatment groups, there was no significant reduction of the pro-survival effect (Fig. 4-1A).

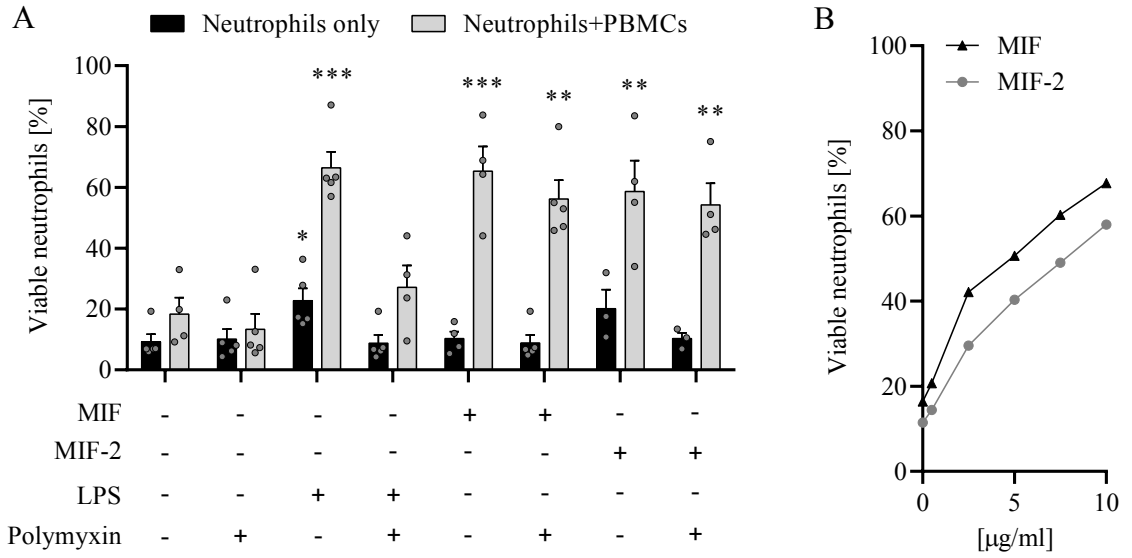

Figure 4-1 MIF and MIF-2 prolong neutrophil survival. Neutrophil viability was measured after 21 h. (**A**) Neutrophils ($5x10^5$/ml, black bars) or Neutrophils+PBMCs (both at $5x10^5$/ml, grey bars) were incubated with or without LPS (1 ng/ml), MIF (10 µg/ml), MIF-2 (10 µg/ml) and polymyxin B (20 µg/ml). (**B**) Neutrophils and PBMCs (both at $5x10^5$/ml) were incubated with increasing concentrations of MIF and MIF-2 (0-10 µg/ml). Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least four independent experiments. Statistical differences were determined within each group to control (containing no MIF) using one-way ANOVA and are indicated by *p<0.05, **p<0.01, ***p<0.001 (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*).

Hypochlorous acid (HOCl) was shown to oxidize the *N*-terminal proline of MIF and MIF-2, which results in a loss of enzymatic activity (86). Whether this has an impact on MIF's ability to promote neutrophil survival was still unclear. MIF and MIF-2 were pre-treated with HOCl (oxMIF, oxMIF-2) before neutrophil survival assay was performed. Similar to MIF and MIF-2, both oxidized isoforms were still able to delay neutrophil apoptosis in the presence of PBMCs in a concentration dependent manner (Fig. 4-2 B), which was not affected by polymyxin B (Fig. 4-2A). However, similar to non-oxidized MIF and MIF-2, there was no effect seen when neutrophils alone were incubated with oxMIF and oxMIF-2 (Fig. 4-2 A).

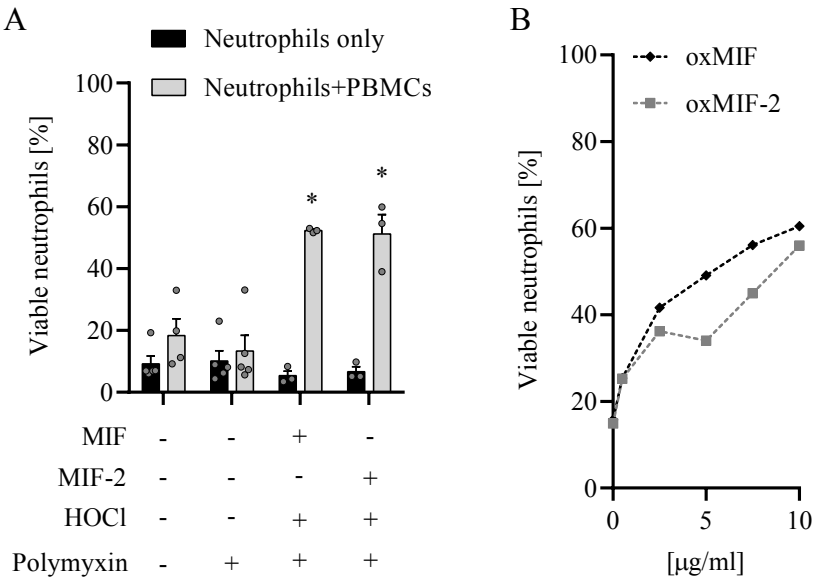

Figure 4-2 oxMIF and oxMIF-2 prolong neutrophil survival. Neutrophil viability was measured after 21 h. (**A**) Neutrophils ($5x10^5$/ml, black bars) or Neutrophils+PBMCs (both at $5x10^5$/ml, grey bars) were incubated with oxMIF and oxMIF-2 (10 µg/ml), with or without polymyxin B (20 µg/ml). (**B**) Neutrophils and PBMCs (both $5x10^5$/ml) were incubated with increasing concentrations of oxMIF and oxMIF-2 (0-10 µg/ml). Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least three independent experiments. Statistical differences were determined within each group to control (containing no MIF) using one-way ANOVA and are indicated by *$p<0.05$ (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*)

4.1.1 PBMCs support MIF-triggered neutrophil survival

To further investigate the role of PBMCs in MIF-mediated neutrophil survival, I used different ratios of PBMCs to neutrophils, to verify the minimum number that is needed to have a significant effect of MIF on promoting neutrophil survival. The results illustrated in this chapter have been submitted and are under revision for publication in the following manuscript: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

In the following experiment, where neutrophils were cultured in the presence of different numbers of PBMCs, I was able to show that a ratio of 0.05:1 PBMCs : neutrophils was sufficient for MIF to have a significant effect on neutrophil survival (Fig. 4-3A). With an increasing number of PBMCs, the pro-survival effect of MIF reached a maximum of around 80% survival compared to control where only 20% viable cells were detected after 21 h (Fig. 4-3A).

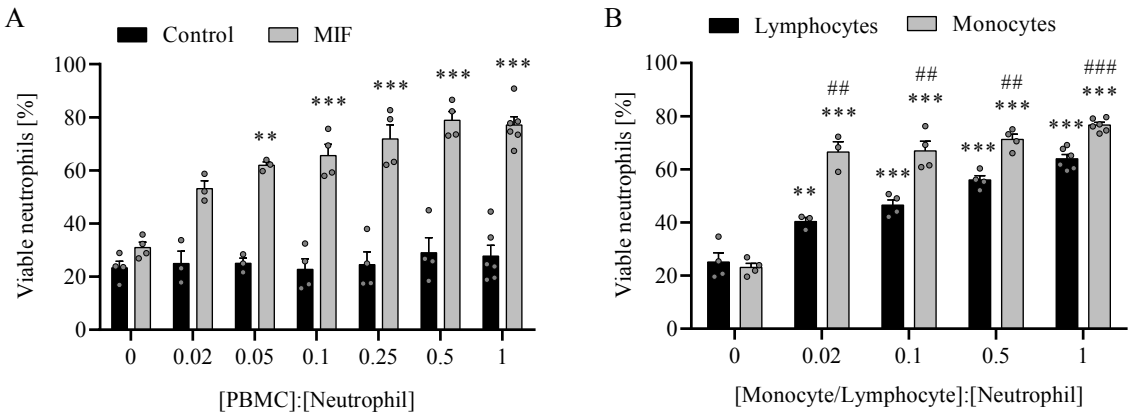

Figure 4-3 PBMCs are required for the MIF-mediated effect on neutrophil survival. Neutrophil viability was measured after 21 h. (**A**) Different ratios of PBMCs (up to $5x10^5$/ml) and neutrophils ($5x10^5$/ml) were incubated in the presence (grey bars) or absence (black bars) of MIF (10 µg/ml) and polymyxin B (20 µg/ml). (**B**) Different ratios of lymphocytes (black bars) and monocytes (grey bars) were incubated with MIF (10 µg/ml) and polymyxin B (20 µg/ml). Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least four independent experiments. Statistical differences were shown to control of each group using one-way ANOVA and are indicated by *p<0.05, **p<0.01, ***p<0.001. Statistical differences between monocytes and lymphocytes when present at the same ratio to neutrophils were determined using unpaired student's t-test and are indicated by ##p<0.01 and ###p<0.001 (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*).

The isolated PBMC culture consist of a mixture of approximately 80% lymphocytes and 20% monocytes which was verified by FACS analysis. To prove which cell population is mainly responsible for mediating the MIF effect, the PBMC sub-populations were fractionated by using an antibody-based isolation method and incubated with MIF at different cell ratios. Both cell types were able to promote the MIF-triggered survival effect, showing a positive correlation between viable neutrophils and number of monocytes or lymphocytes (Fig. 4-3B). However, monocytes showed a significantly higher effect on neutrophil survival at any given ratio of mononuclear cells to neutrophils (Fig. 4-3B) compared to the lymphocyte co-culture. Due to these findings, all following co-culture experiments were performed at a 1:1 cell ratio of PBMCs and neutrophils to simplify the procedure.

Supporting, the hypothesis that MIF and MIF-2 treatment of PBMC cultures is initializing the release of soluble survival factors, an experiment was performed where PBMCs were separately incubated with MIF and MIF-2 (10 µg/ml). The conditioned supernatants were further used to incubate neutrophils alone. MIF and MIF-2 conditioned PBMC supernatants prolong neutrophil survival to the same extent as the co-culture system (Fig. 4-4).

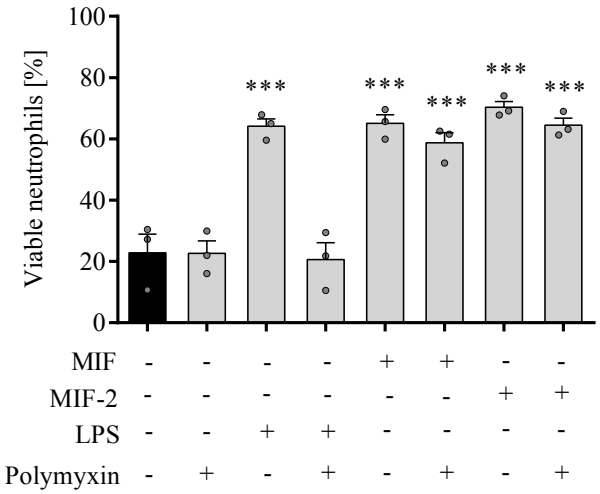

Figure 4-4 Supernatants of MIF and MIF-2 treated PBMCs prolong neutrophil survival. Neutrophil survival was measured after 21 h. PBMC-conditioned supernatants (LPS (1 ng/ml), MIF (10 µg/ml), MIF-2 (10 µg/ml) and polymyxin B (20 µg/ml)) were incubated with neutrophils ($5x10^5$/ml) for 21 h. Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least three independent experiments. Statistical differences between treated samples (grey bars) and control (black bar) were determined using one-way ANOVA and are indicated by ***$p<0.001$ (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

Similar to the experiments shown before, an LPS (1 ng/ml) control treatment was included with or without polymyxin B (20 µg/ml) to exclude false positive results. The addition of polymyxin B showed a significant reduction of LPS mediated neutrophil survival back to control levels. When adding polymyxin B to MIF and MIF-2 groups, there was no significant reduction of neutrophil survival seen (Fig. 4-4).

4.1.2 MIF isoforms induce release of inflammatory mediators

As a consecutive step, experiments were designed to analyze supernatants of PBMCs that were stimulated with MIF and MIF-2 and their oxidized counterparts, since those showed significant effects on delaying neutrophil apoptosis. The results illustrated in this chapter have been submitted and are under revision for publication in the following manuscript: Schindler, L. *et al.* (2020), *J Leukoc Biol*. Various cytokines have been shown to inhibit neutrophil apoptosis (121-123,154,210). Therefore, ELISA analysis was used to determine whether the indirectly PBMC-mediated effect on neutrophil survival was due to the release of soluble survival factors. Here levels of the cytokines IL-1β, IL-6, CXCL8

(IL-8), CXCL12 (SDF-1), G-CSF and GM-CSF in MIF, MIF-2, oxMIF and oxMIF-2 treated PBMCs were measured.

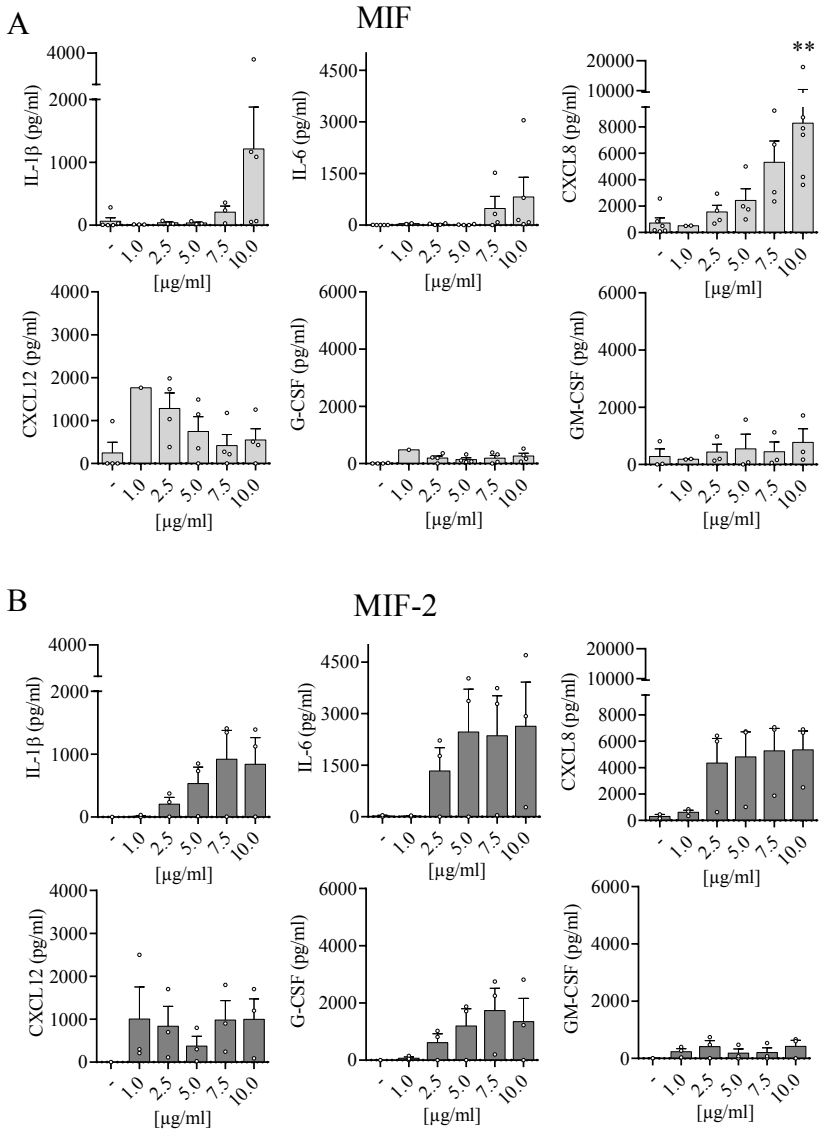

Figure 4-5 Cytokines released by PBMCs after incubation with MIF and MIF-2. PBMCs ($5x10^5$/ml) were incubated with (**A**) MIF (0-10 µg/ml), (**B**) MIF-2 (0-10 µg/ml) and polymyxin B (20 µg/ml). Levels of IL-1β, IL-6, CXCL8 (IL-8), CXCL12 (SDF-1), G-CSF and GM-CSF were measured by ELISA. Each data point shows individual donors and the mean ±SEM of duplicated measurements from three independent experiments. Statistical differences between treated samples and controls in each group were determined using one-way ANOVA and are indicated by **p<0.01. (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*). Experiments were performed with the help of Leon Zwissler.

First, I was able to demonstrate that both MIF and MIF-2 induced the release of inflammatory mediators from PBMCs. Levels of IL-1β, IL-6 and CXCL8 showed positive correlation with increasing protein concentrations (Fig. 4-5A, B). However,

within each group, statistical significance was only reached for CXCL8 in supernatants of MIF-treated PBMCs (Fig. 4-5A). Surprisingly, CXCL12 levels reached a maximum at low concentrations of MIF proteins, but there was no significance measurable. In the MIF treatment group G-CSF or GM-CSF levels were not elevated. Nevertheless, G-CSF levels in the MIF-2 group were visibly higher compared to the MIF group, even though MIF and MIF-2 showed the same pro-survival capacity on neutrophils (Fig. 4-5A, B).

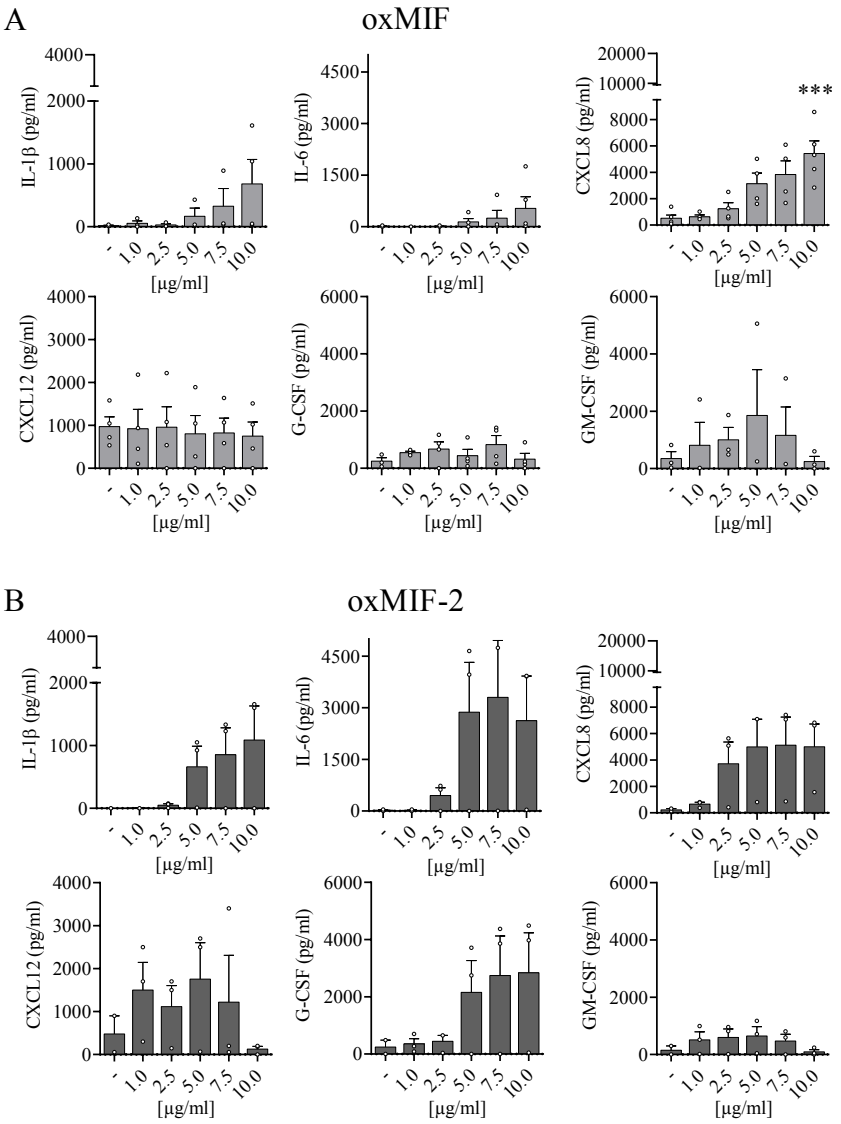

Figure 4-6 Cytokines released by PBMCs after incubation with oxMIF and oxMIF-2. PBMCs ($5x10^5$/ml) were incubated with (**A**) oxMIF (0-10 µg/ml) and (**B**) oxMIF-2 (0-10 µg/ml) and polymyxin B (20 µg/ml). Levels of IL-1β, IL-6, CXCL8 (IL-8), CXCL12 (SDF-1), G-CSF and GM-CSF were measured by ELISA. Each data point shows individual donors and the mean ±SEM of duplicated measurements from three independent experiments. Statistical differences between treated samples and controls in each group were determined using one-way ANOVA and are indicated by ***p<0.001. (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*). Experiments were performed with the help of Leon Zwissler.

Next, I compared the cytokine profiles of supernatants where PBMCs were stimulated with the oxidized isoforms of MIF and MIF-2 and showed noticeable differences compared to the non-oxidized isoforms (Fig. 4-5, 4-6). The most pronounced difference in the MIF/oxMIF group was seen in levels of GM-CSF with the highest level at an oxMIF concentration of 5 µg/ml (Fig. 4-6A). Furthermore, in the MIF-2/oxMIF-2 group, the oxidized MIF-2 led to a concentration-dependent upregulation of G-CSF (Fig. 4-6B), which was slightly less in the MIF-2 conditioned media. However, similar to the non-oxidized MIF group, the only significant upregulation was seen for CXCL8 in the supernatant of oxMIF stimulated PBMCs (Fig. 4-6A). There were no significant differences in the levels of any other given cytokine compared to particular concentrations of MIF isoforms. Through these ELISA screens I cannot fully provide a conclusive homolog- and isoform-based cytokine picture. However, this data indicates distinct effects of the different MIF isoforms on PBMCs, even though they had the same effect on neutrophil survival (chapter 4.1).

4.1.3 CXCL8 is involved in MIF mediated neutrophil survival

Previous findings showed that after MIF exposure, PBMCs released a significant amount of CXCL8 (Fig. 4-5). In this part of my thesis I wanted to study, whether CXCL8 is the sole component, that is responsible for the inhibition of neutrophil apoptosis triggered by MIF treatment. Two different experiments were performed to answer this question. The results illustrated in this chapter have been submitted and are under revision for publication in the following manuscript: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

First, conditioned media from MIF and MIF-2 treated PBMCs were incubated with a neutralizing antibody directed against CXCL8. As shown in Fig. 4-7 A, CXCL8 neutralizing antibody significantly decreased the pro-survival effect of the MIF-conditioned PBMC supernatants by approximately 60% and the MIF-2 conditioned supernatant by about 50% compared to isotype control. The difference between MIF and MIF-2 were not significant, again supporting that these proteins act similarly.

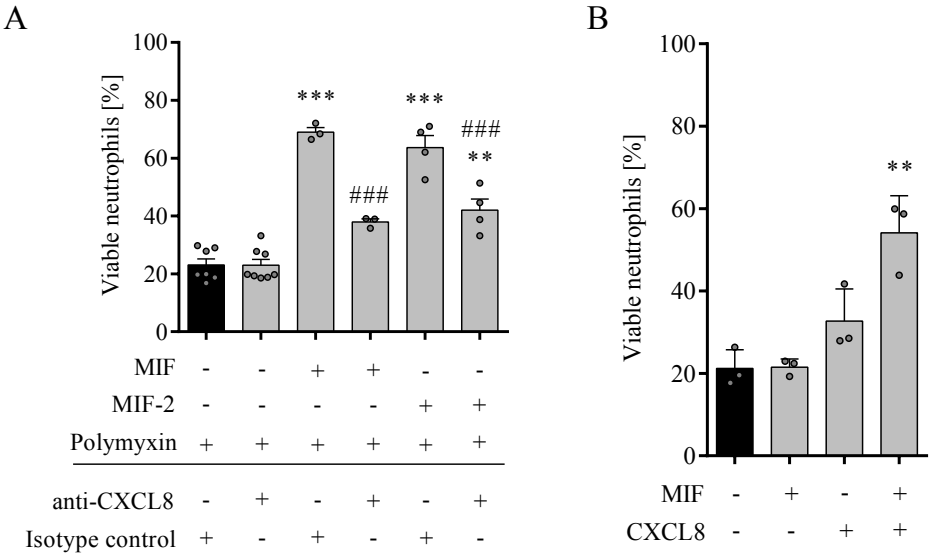

Figure 4-7 CXCL8 and MIF mediate the delay of neutrophil apoptosis. Neutrophil survival was measured after 21 h. (**A**) Conditioned media from MIF and MIF-2 (10 µg/ml) treated PBMCs were incubated with a neutralising antibody against CXCL8 (0.4 µg/ml for MIF-2 and 1.2 µg/ml for MIF) or a monoclonal IgG1 antibody as isotype control, before neutrophils ($5x10^5$/ml) were added. (**B**) Neutrophils ($5x10^5$/ml) were incubated with recombinant CXCL8 (20 ng/ml), MIF (10 µg/ml) or MIF and CXCL8 together. Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least three independent experiments. Statistical differences between treated samples (grey bars) and controls (black bars) in each group were determined using one-way ANOVA and are indicated by **p<0.01 and ***p<0.001. Statistical differences between MIF/MIF-2 with or without neutralizing antibody were determined using student's t-test and are indicated by $^{###}$p<0.001 (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*).

Second, I tested whether purified, recombinant human CXCL8 itself could inhibit neutrophil apoptosis at concentrations measured by ELISA in the MIF-treated PBMC supernatants (20 ng/ml). After incubation of neutrophils with CXCL8, the percentage of viable neutrophils only increased slightly, but was not significant (Fig. 4-7B). Surprisingly, when I incubated neutrophils with CXCL8 and MIF together and I was able the observed a significant survival effect on neutrophils, that was comparable to that elicited by MIF-conditioned PBMC supernatant (Fig. 4-7B). Taken this data together, it is highly possible that MIF and CXCL8 have a synergistic effect on promoting neutrophil survival.

4.1.4 MIF receptor CXCR2 is required for neutrophil survival

MIF's biological and inflammatory actions are triggered through binding to its receptors CD74, CXCR2 and CXCR4 (25,52,54,55,61). The results illustrated in this chapter have been submitted and are under revision for publication in the following manuscript: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

In this part, I wanted to determine which receptor is responsible for the pro-survival effect of MIF. Therefore, I first analyzed which MIF receptors are present on the surface of neutrophils and PBMCs. Flow cytometry analysis showed that especially CXCR2 is highly expressed on neutrophils, whereat CXCR4 is low expressed and CD74 was not detected (Fig. 4-8A). Those findings also confirm previously published data (107,211). In comparison to PBMCs, where CD74 and CXCR4 are expressed, the amounts detected for CXCR2 were very low (Fig. 4-8B).

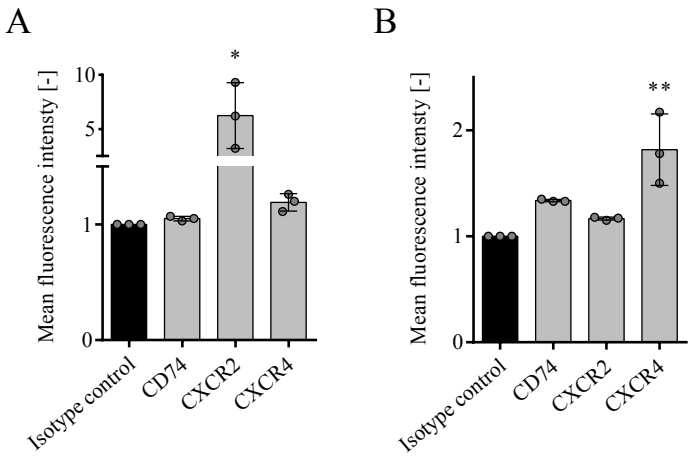

Figure 4-8 MIF receptors CD74, CXCR2 and CXCR4 on neutrophils and PBMCs. Surface expression of the MIF receptors CD74, CXCR2, CXCR4, and CXCR7 on (**A**) neutrophils and (**B**) PBMCs measured by flow cytometry using specific fluorescently labelled antibodies. The MFI of each individual receptor is compared to its respective isotype control. Each data point shows the mean ±SEM of duplicated measurements from three independent experiments. Statistical difference compared to isotype control within each group was determined using one-way ANOVA and is indicated *p<0.05, **p<0.01 (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*).

To further identify which receptor is directly involved in MIF's activity to prolong neutrophil survival, receptor blocking studies were performed. Each individual receptor on neutrophils and/or PBMCs was blocked by using a monoclonal antibody (LN2) directed against CD74 (64), a blocking peptide (NBP1-76867PEP) directed against

CXCR4 and small molecule antagonist (SB225002) directed against CXCR2 (212). Blocking of CD74 and CXCR4 on both, neutrophils and PBMCs, had no significant effect on the MIF-mediated pro-survival effect on neutrophils (Fig. 4-9A). However, blocking CXCR2 had a dramatic effect on neutrophil survival, whereat the detected percentage of viable neutrophils dropped from around 80% (MIF control group) down to 20% (Fig. 4-9A). Intestinally, I also detected a significant decrease of survival in the absence of exogenously added recombinant MIF, when CXCR2 was blocked (Fig. 4-9A).

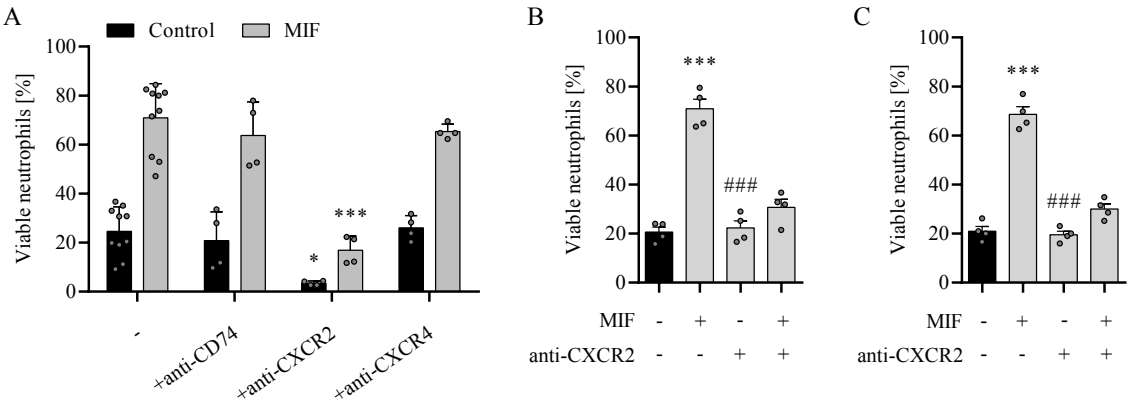

Figure 4-9 CXCR2 is required for the anti-apoptotic effect of MIF. Neutrophil survival was measured after 21 h. (**A**) Neutrophils and PBMCs (both $5x10^5$/ml) were pre-incubated with LN2 ('anti-CD74', 10µg/ml), SB225002, ('anti-CXCR2', 10 µM) or NBP1-76867PEP ('anti-CXCR4', 25 µg/ml) for 30 min before adding into MIF (10 µg/ml; also containing 20 µg/ml polymyxin B) containing media. (**B**) Neutrophils ($5x10^5$/ml) were pre-incubated with SB225002 (10 µM) for 30 min, before conditioned PBMC supernatants were added and viable neutrophils determined after 21 h. (**C**) PBMCs ($5x10^5$/ml) were pre-incubated with SB225002 (10 µM) for 30 min, before adding MIF (10µg/ml) and polymyxin B (20 µg/ml). The conditioned supernatants were added to neutrophils ($5x10^5$/ml) and incubated for 21 h. Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least four independent experiments. Statistical differences between treated samples (grey bars) and untreated controls (black bar) in each group were determined using one-way ANOVA and are indicated by **p<0.01 and ***p<0.001. Statistical differences between MIF treatment on CXCR2-blocked and non-blocked PBMCs or neutrophils were determined using unpaired student's t-test ###p<0.001 (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*).

To further distinguish whether blocking CXCR2 on neutrophils or on PBMCs let to the dramatically decreased survival, the receptor was blocked on both cell types separately. When CXCR2 was blocked on neutrophils, before incubating them with MIF-conditioned PBMC supernatant, the survival decreased significantly compared to non-blocked neutrophils (Fig. 4-9B). Likewise, when I blocked CXCR2 on PBMCs and incubated

them with recombinant MIF (10 µg/ml), their supernatant could no longer inhibit neutrophil apoptosis (Fig. 4-9C).

4.1.5 oxMIF exhibits preserved binding activity to CXCR2

The biological receptors for MIF are well characterized (25,51-55), but whether oxidized MIF binds the same receptors is still unclear. Since MIF and its oxidized counterpart inhibit neutrophil apoptosis similarly in the presence of PBMCs, I hypothesized that also oxMIF bind CXCR2 equally. To test our hypothesis, a receptor-binding assay was performed using an established genetically modified yeast strain that expresses human CXCR2 (72). This assay allows the specific analysis of MIF and oxMIF mediated activation of CXCR2, which results in β-galactosidase (lacZ) expression, that can be enzymatically measured in the change of luminescence using a commercial BetaGlo Kit. Experiments were performed with the help of Priscila Bourilhon.

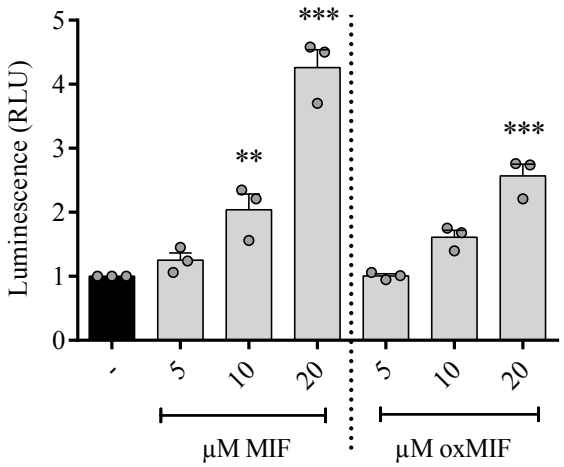

Figure 4-10 Yeast-based reporter system support binding of MIF and oxMIF to CXCR2. MIF and oxMIF binding to CXCR2 activates signalling and expression of the lacZ gene, accompanied by β-gal activity. Different concentrations of MIF and oxMIF were tested (5,10, 20 µM). Binding and reporter activity are indicated as relative luminescence, normalized to the untreated control (black bar). Data shows the mean ±SEM of duplicated measurements from three independent experiments (n=3). Statistical differences between treated samples (grey bars) and untreated control (black bar) were determined using one-way ANOVA and are indicated by **p<0.01 and ***p<0.001. Experiments were performed with the help of Priscila Bourilhon and Leon Zwissler.

This data indicates, that MIF triggered CXCR2 mediated β-galactosidase activity in a dose dependent manner, showing a significant upregulation of luminescence already at

10 µM of MIF. In comparison, performed receptor binding assays using oxMIF show a reduced but still significant binding capacity of oxMIF to CXCR2 (Fig. 4-10). However, binding of MIF seems to be much stronger compared to oxidized MIF, which could be explained through modification of the *N*-terminal proline to a proline imine in the HOCl treated protein (oxMIF).

4.1.6 *N*-terminal proline oxidation prevents 4-IPP binding

The small molecule inhibitor 4-IPP was shown to bind to the *N*-terminal proline of MIF and thereby blocked its enzymatic and bioactivity (79). In previous studies, we showed that oxidation of the *N*-terminal proline of MIF by HOCl protected MIF from covalent binding of 4-IPP (86). This finding was confirmed by mass spectrometry, but whether 4-IPP will fail to inhibit the pro-survival effect on neutrophils needed to be confirmed. The results illustrated in this chapter have been submitted and are under revision for publication in the following manuscript: Schindler, L. *et al.* (2020), *J Leukoc Biol*.

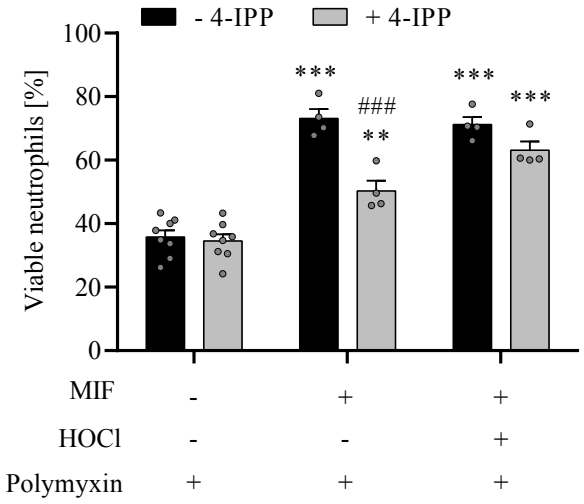

Figure 4-11 *N*-terminal proline oxidation prohibit binding of 4-IPP. Viable neutrophils were measured after 21 h. Neutrophils and PBMCs (both $5x10^5$/ml) were incubated in the presence or absence of MIF (10 µg/ml), oxMIF (10 µg/ml) pre-incubated with or without a 10-fold molar excess of 4-IPP for 15 min. Each data point shows individual donors and the mean ±SEM of duplicated measurements from four independent experiments. Statistical difference to the control without MIF within each group was determined using one-way ANOVA and is indicated by **p<0.01 and *** p<0.001. Statistical differences between each MIF isoform and its 4-IPP-treated counterpart (black vs grey bars) were determined using unpaired student's t-test ###p<0.001 (under revision: Schindler, L. *et al.* (2020), *J Leukoc Biol*).

I was able to show, that neutrophil survival mediated by MIF could be significantly blocked by 4-IPP treatment (Fig. 4-11). However, adding the small molecule inhibitor to oxMIF had no effect on inhibitory effect on its neutrophil survival activity. Oxidized MIF that was pre-treated with 4-IPP was still able to promote neutrophil survival in the same extent compared to oxMIF without prior treatment. This indicated that *N*-terminal proline oxidation prevents 4-IPP binding to oxMIF.

4.2 MIF accelerates superoxide production in stimulated neutrophils

The production of superoxide is one of the major functions of neutrophils in their fight against invading pathogens. Different groups showed that PMA (213) and fMLP can both induce superoxide production in neutrophils rapidly by activating NADPH oxidase (175). However, the effect of MIF on ROS production is still unclear, but cystic fibrosis studies indicate a promotive effect of MIF (191). Here, I wanted to investigate whether MIF has the potential to act as a priming agent on neutrophils oxidative activity. The results illustrated in this chapter will be part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

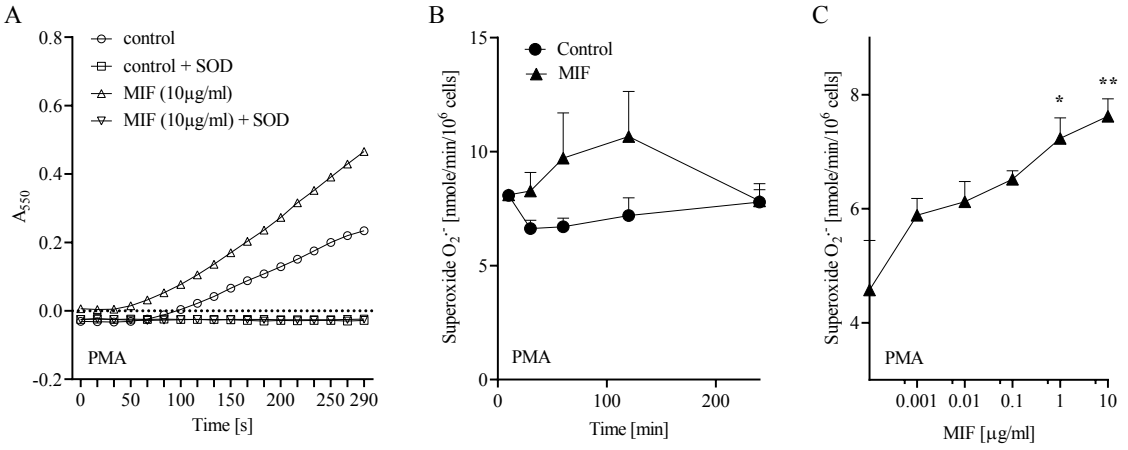

Figure 4-12 MIF accelerate PMA induced neutrophil superoxide production. Neutrophils (1x10^6/ml) were incubated in the absence of presence of MIF (10 µg/ml) and 20 µg/ml polymyxin B for up to 4 h. Superoxide production in response PMA (100 ng/ml) was assessed by measuring the reduction of cytochrome C (40 µM) by an increase in absorbance at 550 nm (**A**) Representative A550 traces recorded after the addition of PMA, respectively. SOD (20 µg/ml) was added to block any superoxide-independent response. (**B**) Neutrophils (1x10^6/ml) were pre-incubated with MIF (10 µg/ml) for up to 4 h and superoxide

production was measured. (**C**) Neutrophils ($1x10^6$/ml) were incubated with increasing concentrations of MIF for 60 min before measuring the rate of superoxide production. Each data point shows the mean ±SEM of duplicated measurements from four independent experiments. Statistical difference to the control was determined using one-way ANOVA and is indicated by *p<0.05 and **p<0.01 (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

To study the impact of MIF and oxidized MIF on neutrophil derived superoxide production a cytochrome c assay was used. MIF pre-treatment was shown increase superoxide production in neutrophils after PMA activation (Fig. 4-13) and indicate a bell-shaped time-response curve, with a maximum at 2 h (Fig. 4-13B). However, pre-treatment of MIF for 4 h had no effect on superoxide production compared to control after PMA activation. In addition, prior MIF stimulation was shown to be dose-dependent, with a significant increase of superoxide production at a concentration of >1 µg/ml MIF (Fig. 4-13C). Interestingly, MIF pre-treatment also decreased the lag time, that is typical for PMA induced superoxide production (Fig. 4-13A).

To verify whether the previously shown effect can also be seen when neutrophils were activated through a more physiological mediator, I used fMLP to induce superoxide production. Similar to the PMA induced neutrophil activation, the rate of superoxide production was increased after MIF pre-treatment in response to fMLP. However, already low concentrations of MIF (100 ng/ml) had a significant effect on superoxide production and higher concentrations showed no further increase (Fig. 4-14C). In contrast to PMA activation, MIF pre-incubation maintained the superoxide production rate in response to fMLP, whereat control cells showed decreased superoxide production over time (Fig. 4-14B). The same effect was seen in neutrophils pre-treated with oxMIF, suggesting remained biological function (Fig. 4-14D), although oxidation led to loss of enzymatic activity.

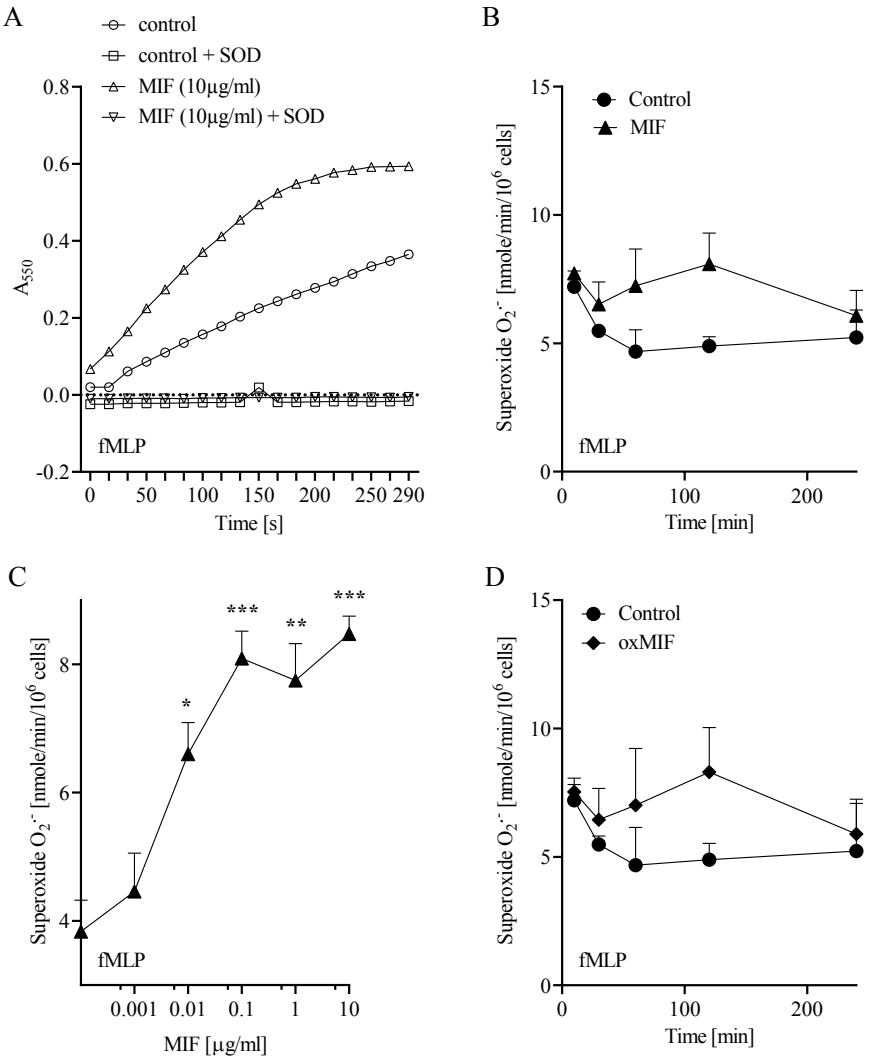

Figure 4-13 MIF accelerate fMLP induced neutrophil superoxide production. Neutrophils ($1x10^6$/ml) were incubated in the absence of presence of MIF (10 µg/ml) or oxMIF (10 µg/ml) and 20 µg/ml polymyxin B for up to 4 h. Superoxide production in response to fMLP (100 nM) measured by reduction of cytochrome C (40 µM). (**A**) Representative A550 traces recorded after the addition of fMLP, respectively. SOD (20 µg/ml) was added to block any superoxide-independent response. (**B**) Neutrophils ($1x10^6$/ml) were incubated with MIF (10 µg/ml) for up to 4 h and superoxide production was measured. (**C**) Neutrophils ($1x10^6$/ml) were incubated with increasing concentrations of MIF for 60 min before measuring the rate of superoxide production. (**D**) Neutrophils ($1x10^6$/ml) were incubated with oxMIF (10 µg/ml) for up to 4 h and superoxide production was measured. Each data point shows the mean ±SEM of duplicated measurements from four independent experiments. Statistical difference to the control was determined using one-way ANOVA and is indicated by *p<0.05, **p<0.01 and ***p<0.001 (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

In a next step, I wanted to verify if this effect is directly caused by MIF. Therefore, MIF was blocked with 10-fold molar excess of the small molecule inhibitor 4-IPP, before pre-treating neutrophils and further activation. MIF accelerated superoxide production was significantly reduced by the inhibitor 4-IPP, whereat this effect was more pronounced

using lower concentrations of MIF (Fig. 4-15A). However, when MIF was oxidized with a 5-fold molar excess of HOCl, 4-IPP cannot block the oxMIF induced effect on superoxide production (Fig. 4-15B). As I reported before, oxidation of MIF by HOCl prohibit the binding of 4-IPP and potentially also other inhibitor that are directed against the *N*-terminus.

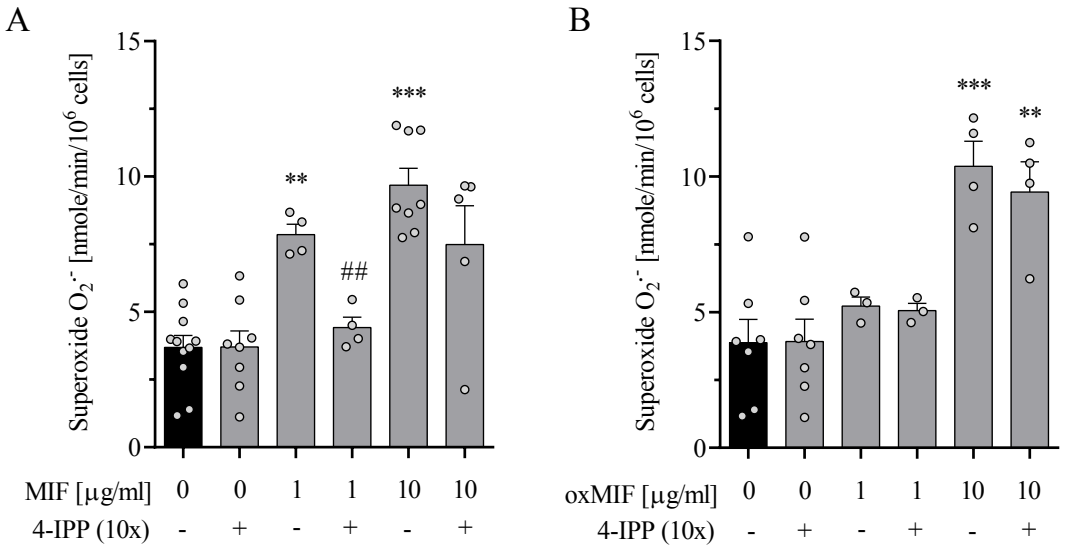

Figure 4-14 4-IPP can block MIF-mediated effects on superoxide production but oxidized MIF preserves its biological function. MIF (**A**) and oxMIF (**B**) (1 µg/m, 10 µg/ml) were pre-treated with ten-fold molar excess of 4-IPP for 15 min before added to neutrophils (1x10^6/ml). After 1 h incubation, superoxide production was induced by fMLP and measured using the cytochrome c assay. Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least three independent experiments. Statistical difference to control within each group was determined using one-way ANOVA and is indicated by **p<0.01 and ***p<0.001. Statistical differences between each MIF concentration and its 4-IPP-treated counterpart were determined using unpaired student's t-test ##p<0.01 (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

Since blocking CXCR2 had a significant effect on neutrophil apoptosis, I wanted to determine whether this effect could be also seen in MIF-mediated acceleration of superoxide production. Therefore, surface CXCR2 was blocked on neutrophils through incubation with the small molecule antagonist SB225002 ('anti-CXCR2'), before incubating with MIF. As indicated in Fig. 4-16, blocking CXCR2 on neutrophils had no significant effect and accelerated superoxide production remains to be unchanged compared to non-blocked controls.

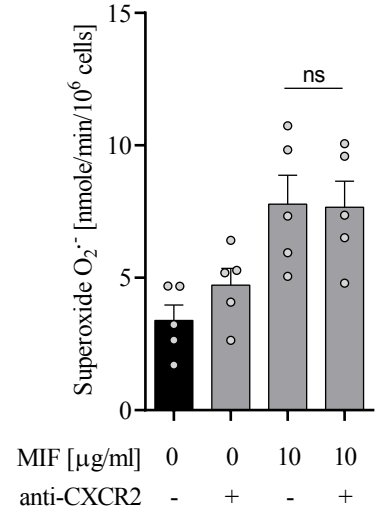

Figure 4-15 CXCR2 blocking had no effect on superoxide production. Neutrophils (1x10^6/ml) were pre-incubated with SB225002 (10 µM) for 15 min at RT and afterwards incubated with MIF (10 µg/ml) and 20 µg/ml polymyxin B for 60 min at 37 °C. Superoxide production induced by fMLP and measured using cytochrome c assay. Each data point shows individual donors and the mean ±SEM of duplicated measurements from five independent experiments. Statistical difference to the control was determined using one-way ANOVA and is indicated to be not significant (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

4.3 MIF affects intracellular hypochlorous acid production in neutrophils

The ability of neutrophils to produce HOCl, a downstream oxidant produced by the neutrophil enzyme myeloperoxidase, in phagosomes is another major defense systems to kill invading pathogens (163,164). Previously, the rhodamine-based non-fluorescent probe R19-S has been shown to react with HOCl, which results in a fluorescent product (R19) and can be used to measure HOCl production in neutrophils via flow cytometry and live cell imaging (202). The results illustrated in this chapter will be part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

In the first experiment, I wanted to analyze whether MIF treatment could affect HOCl production in neutrophils. Therefore, HOCl production was induced through incubation with zymosan particles and increase in fluorescence of R19 was measured using FACS analysis. Neutrophils that have been pre-treated with MIF or oxMIF respond faster to the presence of zymosan particles and developed a more rapid R19 fluorescence compared

to control cells (Fig. 4-17B). The most significant difference was seen in very early timepoints (5 min), also noticeable in a representative FACS histograms (Fig. 4-17A, B). However, a maximum of 80% fluorescent cells was reached after 15 min within all groups, including the control treatment (Fig. 4-17B), indicating that MIF pre-treatment had no effect on the amount of HOCl producing neutrophils.

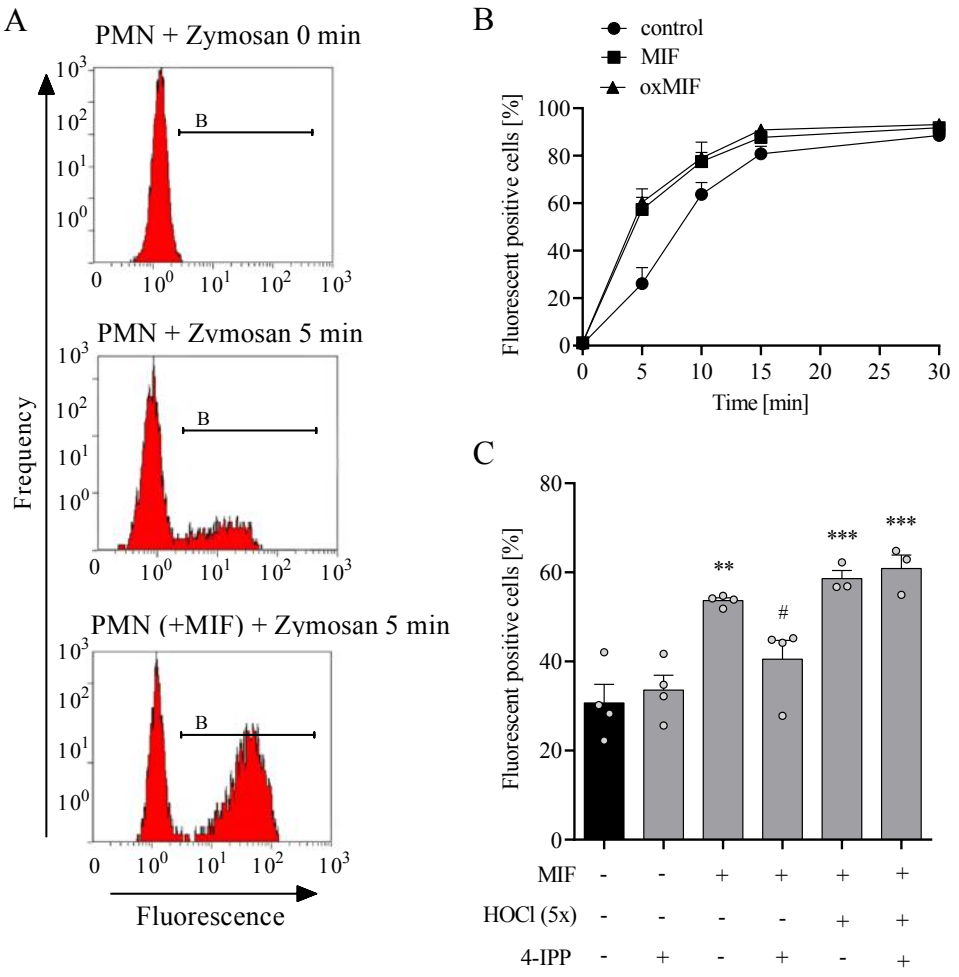

Figure 4-16 MIF and oxMIF accelerate HOCl production in neutrophils stimulated with zymosan. Neutrophils (1x10^6/ml) were incubated in the presence or absence of MIF or oxMIF (10 µg/ml) and 20 µg/ml polymyxin B for 1 h. R19-S (10 µM) was added before adding opsonized zymosan (5x10^6/ml). (**A**) Representative flow cytometry histograms after 0 and 5 min. (**B**) Fluorescence was measured after 0, 5, 10, 15 and 30. Fluorescence-positive cells as a percentage of total gated neutrophils (**C**) MIF and oxMIF (10 µg/ml) were treated with a ten-fold molar excess of 4-IPP for 15 min at RT before adding to neutrophils fluorescence positive cells were determined after 5 min. Each data point shows individual donors and the mean ±SEM of duplicated measurements from at least three independent experiments. Statistical difference to control was determined using one-way ANOVA and is indicated by **p<0.01 and *** p<0.001. Statistical differences between MIF isoform and its 4-IPP-treated counterpart were determined using unpaired student's t-test #p<0.05 (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

In addition, I wanted to address if this effect is also directly mediated through MIF and can thereby be blocked by 4-IPP, as shown in previous experiments. MIF and oxMIF were pre-treated with 10-fold molar excess of 4-IPP, before neutrophils were added. Plotted data indicates that the effect of MIF in accelerating the HOCl production in neutrophils can be blocked by 4-IPP significantly (Fig. 4-17C). Nevertheless, there was no inhibitory effect of 4-IPP seen in the group where neutrophils were incubated with oxMIF (Fig. 4-17C).

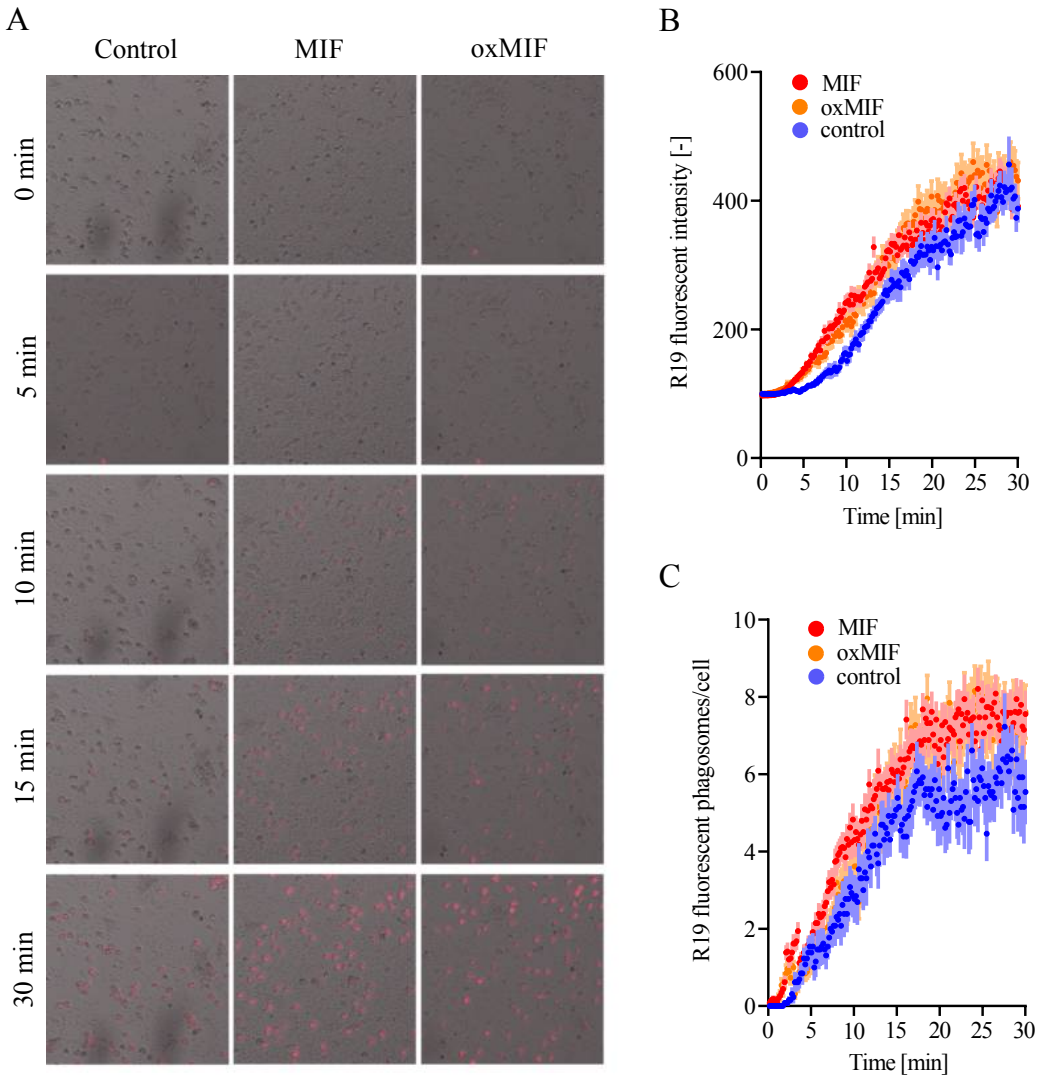

Figure 4-17 HOCl production of individual neutrophils after MIF and oxMIF incubation by live-cell fluorescence microscopy. Neutrophils ($1x10^6$/ml) were pre-treated in the presence or absence of MIF or oxMIF (10 µg/ml) for 1 h, before zymosan ($1x10^7$/ml) was added in the presence or R19-S (10 µM). (**A**) Representative time laps images of a movie were taken after 0, 5, 10, 15 and 30 min and show a merge of DIC and Cy3 (red fluorescence). (**B**) Analysis of fluorescent phagosomes per cell and (**C**) red fluorescent intensity of R19-S live cell movie. Data shows representative images and analysis of duplicated measurements (n=2) (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor

(MIF) enhances neutrophil phagocytic and oxidative activity. Analysis was performed with the help of Leon Smyth.

To support the previous finding, a similar experiment was performed using live cell microscopy. Representative images were taken after 0, 5, 10, 15 and 30 min, to quantify R19 fluorescent cells and intensity. In accordance to the FACS data, R19 fluorescence appeared faster in MIF and oxMIF pre-treated neutrophils compared to control (Fig. 4-18A). However, due to the experimental setup, a visual difference between the MIF groups and the control group was not seen before 10 min (Fig. 4-18A) after zymosan was added to induce HOCl production. Analysis of the fluorescent phagosomes per cell revealed a more rapid development of R19 fluorescence (red) within the first minutes (Fig. 4-18B). Consistent to the fluorescent intensity, the number of R19 fluorescent phagosomes was increased in neutrophils that have been exposed to MIF or oxMIF compared to control (Fig. 4-17C), indicating increased HOCl production per single neutrophil.

Furthermore, to address whether accelerated HOCl production is accompanied by increased phagocytotic activity, following studies were performed using FITC labeled zymosan to measure the rate of phagocytosis. First, flow cytometry analysis was performed, and intracellular fluorescent signal was measured. MIF and oxMIF pre-treated neutrophils showed a greater proportion of FITC positive cells after 5 min compared to control cells indicating a faster uptake of zymosan (Fig. 4-19A). However, and in contrast to the HOCl production, the difference in the rate of phagocytosis at any given timepoint of MIF/oxMIF-treated neutrophils to control was not significant (Fig. 4-19A). Secondly, to confirm the FACS data, fluorescent live cell microscopy was used. Two different factors were monitored (Fig. 4-19B): phagocytosis through FITC labeled zymosan (green) and HOCl production by fluorescent R19 (purple). Similar to the FACS data, I was able to show that MIF and oxMIF pre-treatment did not affect the ability of neutrophils to phagocyte zymosan particles, indicated by the number of R19 positive cells (Fig. 4-19C). However, the number of phagosomes per cell increased in neutrophils that were pre-treated with MIF and oxMIF compared to control cells (Fig. 4-19D), which was accompanied with an increased HOCl production.

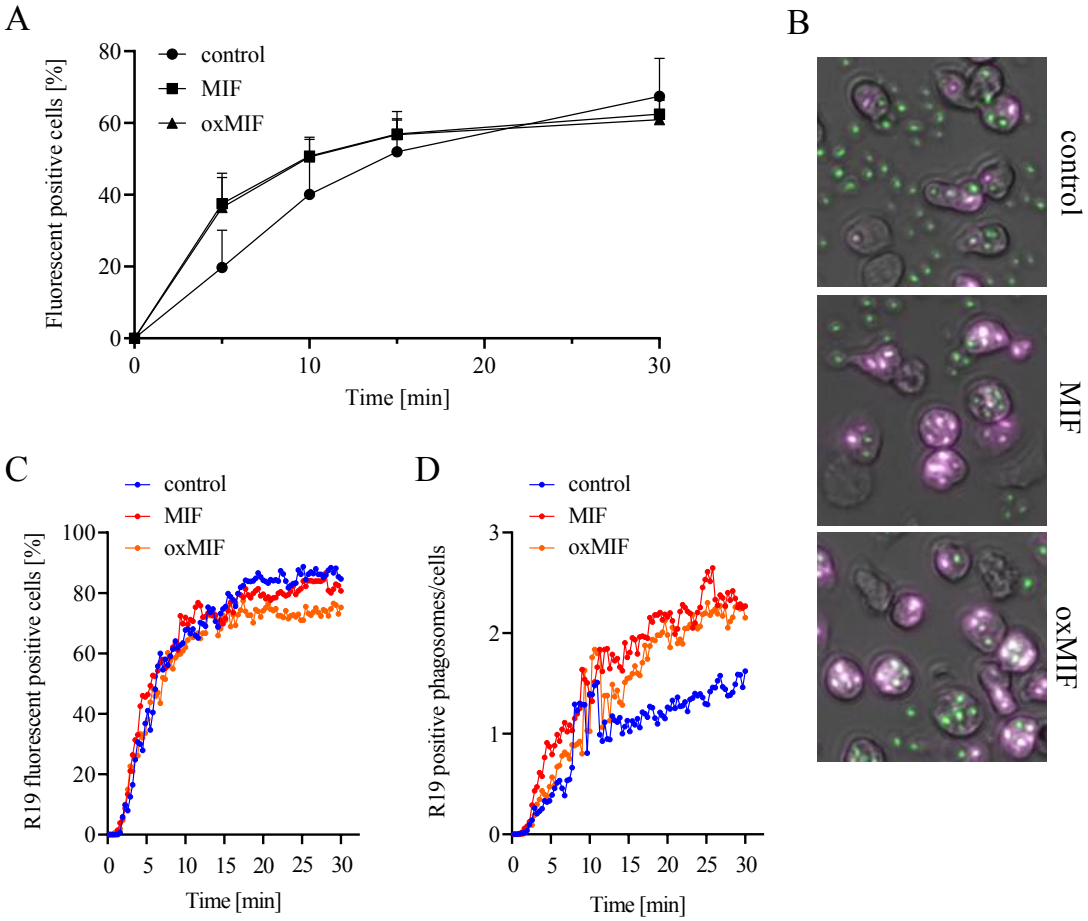

Figure 4-18 Effect of MIF and oxMIF on neutrophil phagocytosis. Neutrophils (1x10^6/ml) were pre-treated in the presence or absence of MIF or oxMIF (10 µg/ml) for 1 h, before FITC labelled zymosan was added. (**A**) Fluorescence was measured after 0, 5, 10, 15 and 30 min using FACS analysis and proportion of positive cells is shown as a percentage of total gated neutrophils (n=3). (**B**) Representative time laps images of a movie were taken after 15 min and show a merge of DIC, Cy3 (purple) and FITC (green); overlay (white). (**C**) Live cell microscopy was used to track phagocytosis and HOCl production. Time-lapse frames from a movie were taken and analysed after 0, 5, 10, 15 and 30 min of R19 and FITC-labelled zymosan particles. Data shows representative images and analysis of duplicated measurements (n=2). Statistical difference to control was determined using one-way ANOVA and is indicated to be not significant for (A) (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity. Analysis was performed with the help of Leon Smyth.

4.4 MIF mediates increased NET formation induced by PMA

Another strategy of neutrophils to kill invading pathogens is the formation of extracellular traps (NETs) (172). Different factors and cytokines have been shown to induce NETs directly such as PMA, CXCL8 and LPS (172). Here, I wanted to address the role of MIF and its oxidized isoform on NET formation. The results illustrated in this chapter will be

part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

To verify whether MIF or oxMIF can directly induce NET formation, neutrophils were incubated with MIF and oxMIF for up to 4 h and extracellular DNA was measured using SYTOX green. MIF isoforms were not able to actively induce NET formation. However, fluorometric analysis of extracellular DNA showed a slight decreased signal in neutrophils incubated with MIF and oxMIF (Fig. 4-20A). This could be also seen due to decreased death in the MIF-treated neutrophils. Immunocytochemistry was used to further confirm fluorometric analysis. Additionally to the SYTOX green staining, samples were analyzed for neutrophil elastase, which has been shown to be present within NETs (206). Representative microscopy images indicate that MIF and oxMIF itself cannot induce NET formation. The nuclei in the MIF and oxMIF treated samples showed the typical multilobed shape, whereat the nuclei in the control group was observed to be more rounded, which can be explained by elevated neutrophil death in the control samples (Fig. 4-20B).

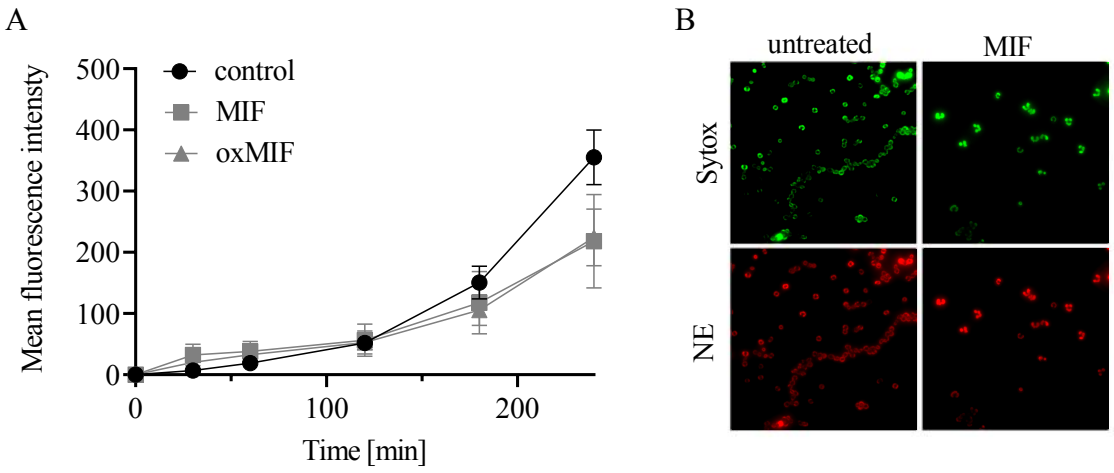

Figure 4-19 Direct effect of MIF and oxMIF on NET formation. (**A**) Neutrophils ($1x10^6$ cells/ml) were incubated in the presence or absence of MIF and oxMIF (10µg/ml) for up to 4h. Extracellular DNA was measured using SYTOX green dye and plotted as mean fluorescent intensity. (**B**) Representative images of neutrophils incubated with MIF and control were taken after 4 h incubation and show channels of Cy5 (NE; red) and FITC (DNA; green). Each data point shows the mean ±SEM of duplicated measurements from three independent experiments. Data shows representative images of duplicated measurements (n=3). Statistical differences between MIF isoform and control were determined as non-significant by using student's t-test (will be submitted Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

Previously published data showed that PMA was able to induce NET formation efficiently (172). To verify whether MIF or oxMIF can alter PMA induced NETosis, neutrophils were pre-incubated with MIF and oxMIF. NET formation was measured fluorometrically using a SYTOX green dye at 0, 30, 60, 120, 180 and 240 min after PMA was added to the neutrophil culture. Pre-treatment with MIF and oxMIF resulted in an increased fluorescent signal, which is referable to an increased amount of extracellular DNA (Fig. 4-21A). To confirm this finding immunocytochemistry was performed by fluorescent labeling of extracellular neutrophil elastase and DNA (Fig. 4-21B).

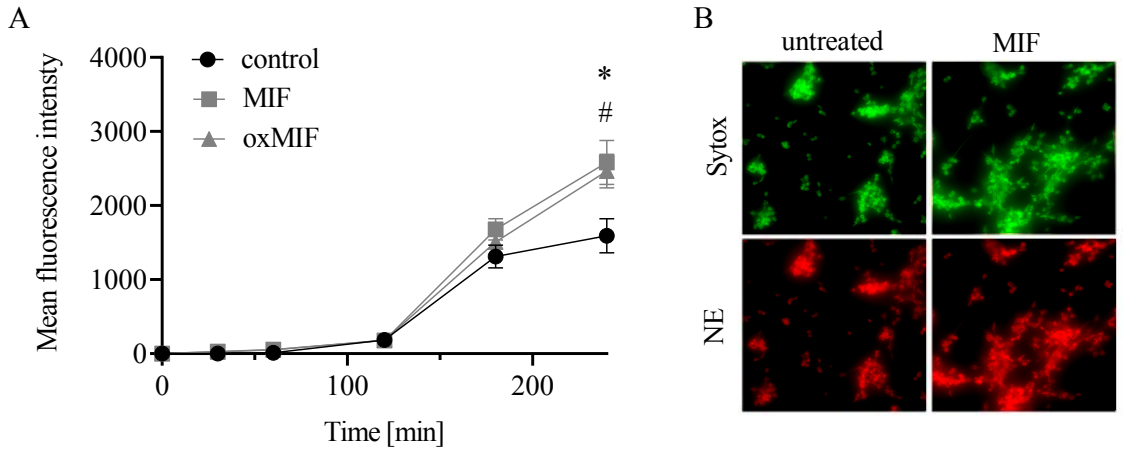

Figure 4-20 Effect of MIF and oxMIF in PMA induced NET formation. Neutrophils (1x10^6 cells/ml) were pre-incubated in the presence or absence of MIF and oxMIF (10µg/ml) for 60 min, before NETosis was induced by PMA (20nM) stimulation. (**A**) Extracellular DNA was measured after indicated timepoints using SYTOX green dye and plotted as mean fluorescent intensity. (**B**) Representative images of neutrophils pre-incubated with MIF and control, before PMA stimulation and show channels of Cy5 (NE; red) and FITC (DNA; green). Each data point shows the mean ±SEM of duplicated measurements from three independent experiments. Statistical differences between MIF and control were determined using unpaired student's t-test *p<0.05; differences between oxMIF and control #<0.05 (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

4.4.1 NET degradation

Neutrophil extracellular traps have been implicated in disease progression and tissue damage (214). DNase was shown to completely degrade neutrophil extracellular DNA (172) and was effectively used as a therapy treatment in patients with cystic fibrosis (215), where NETs contribute to disease progression (216). Previous studies have

identified MIF's potential to act as a nuclease (205), but whether they can degrade NET associated DNA was not investigated yet. The results illustrated in this chapter will be part of the following manuscript: Schindler, L. *et al.* (2020), Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

To investigate whether MIF can degrade DNA released from activated neutrophils, I pre-formed NETs by PMA treatment before MIF was added for 24 h. In the samples that were incubated with MIF, the detected signal of neutrophil DNA was reduced compared to control (Fig.4-22), indicating a DNA degradation through MIF. However, comparing the effects of MIF with the treatment of NETs with DNase, I have seen a much more efficient degradation of DNA by down to 0.01 units of DNase (Fig. 4-22). Still, this biological function of MIF is new and need to be further investigated.

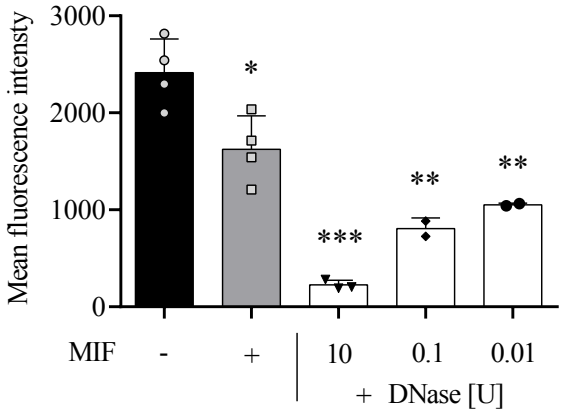

Figure 4-21 MIF degrades preformed NETs. Neutrophils ($1x10^6$ cell/ml) were incubated with PMA for 4 h to preform NETs, followed by incubation with MIF (10 µg/ml) and different concentrations of DNase (0.01, 0.1 and 10 units) for 24 h. Extracellular DNA was measured using SYTOX green dye and plotted as mean fluorescent intensity. Each data point shows the mean ±SEM of duplicated measurements from 2-4 independent experiments. Statistical differences between control and different treatments were determined using unpaired student's t-test *$p<0.05$, **$p<0.01$ and *** $p<0.001$ (will be submitted: Schindler, L. *et al.* (2020*),* Macrophage migration inhibitory factor (MIF) enhances neutrophil phagocytic and oxidative activity.

4.5 Neutrophils and MIF co-localize in myocardial I/R injury – preliminary data

In myocardial reperfusion injury, neutrophils were found to accumulate in the infarcted myocardial area and are assumed as a major source of ROS (217), which can result in acute inflammation and the initiation of myocardial apoptosis (218). Here I wanted to

address, the potential of the co-localization of MIF and neutrophils in an *in vivo* myocardial I/R injury model. In this setting, MIF might play a role in the increased release of neutrophil-derived ROS. However, this is still under investigation and needs to be further validated. With the preliminary data shown in Fig. 4-23, I was able confirm the co-localization of MIF and neutrophils by specific antibodies in myocardial I/R injury. In the infarcted area, high numbers of neutrophils were detected, accompanied with increased levels of MIF. This indicates, that it is very likely that MIF can also affect neutrophil activity *in vivo*. However, further experiments need to be performed.

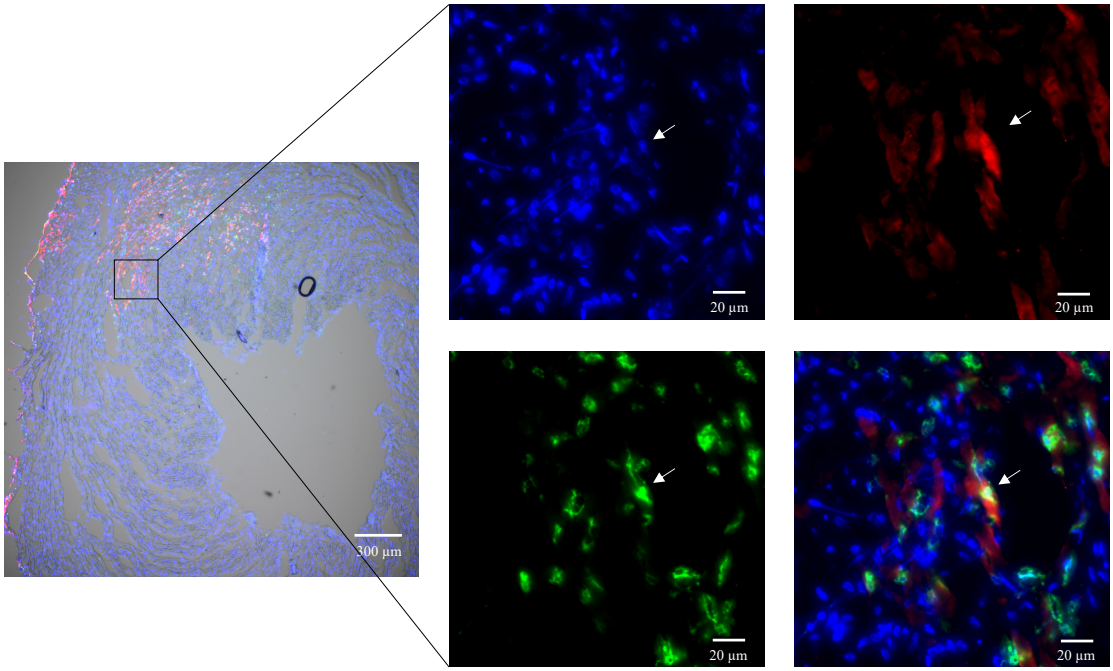

Figure 4-22 Co-localisation of MIF and neutrophils in myocardial I/R injury. Immunofluorescent microscopy staining of neutrophils (green) and MIF (red), as well as DNA (blue) from WT mice after 30 min myocardial ischemia and 24 h reperfusion. Data shows representative images and analysis of duplicated measurements (n=2).

5 Discussion

5.1 MIF proteins prolong neutrophil survival

Previous studies have shown that MIF can prolong neutrophil survival *in vitro* (192). However, there was subsequent evidence that this effect is not a direct effect on neutrophils but is indirectly triggered through an effect of MIF on PBMCs as verified in co-culture experiments (86). Here, I wanted to address and support the importance of PBMCs in MIF-mediated neutrophil survival. Additionally, there was no evidence so far, whether MIF-2 can also affect the delay of neutrophil apoptosis *in vitro*. Due to the high degree of structural and functional similarities, I hypothesized a similar effect of MIF-2 on neutrophil survival. MIF-2 shares 34% sequence identity with MIF, including the *N*-terminal proline and high structural similarities to the MIF protein superfamily (14,65). Indeed, in this thesis, MIF and MIF-2 were shown to promote neutrophil survival in a concentration-dependent manner. My data indicated that a co-culture with at least 5% PBMCs and neutrophils was necessary to mediate the pro-survival effect. MIF as well as MIF-2 had no effect on neutrophil survival in pure neutrophil cultures. This two-cell type model was supported by data showing the release of pro-survival factors from mononuclear cells after MIF and MIF-2 exposure. The interplay and crosstalk between neutrophils and mononuclear cells through cytokines is in line with the importance of cell-cell interactions between different immune cell types in an inflammatory event with various cross-inhibitory or cross-activatory activities (219,220). Although obtained so far only in *in vitro*-settings, my data suggest that through the delay of apoptosis, inflammation could be elevated and/or prolonged, when pro-inflammatory mediators such as MIF and MIF-2 are present.

During inflammation, the levels of circulating neutrophils increase dramatically, to provide a first-line action at sites of inflammation and injury (107,219). In this process, activated neutrophils expand their longevity and are able to control the immune response through their oxidative activities such as the production of superoxide and HOCl, as well as neutrophil extracellular traps (NETs) formation (221,222). The secretion of MIF is highly upregulated in various cell types during inflammation and was shown to exert a number of pro-inflammatory activities (26,30,33,188), including the recruitment of

neutrophils, as shown in *in vitro* and *in vivo* settings (25,31,211). In fact, it is very likely that MIF proteins and neutrophils are co-localized at sites of inflammation and infection. Interestingly, MIF and MIF-2 share a conserved *N*-terminal proline, that was shown to have a pK_a of 5.6, which is much lower than the usual pK_a of around 9 (normal for proline amide) and is involved in the evolutionarily conserved tautomerase activity of the proteins (13,14). Additionally, the region of the tautomerase pocket is structurally involved in the binding of MIF to CD74 and CXCR4, as it contributes to the binding site between MIF and these receptors (61,78). In previous *in vitro* experiments, we were able to show that neutrophil-derived oxidizing agents like HOCl are able to modify the *N*-terminal proline of MIF and MIF-2, resulting in proline imine formation (86). Subsequently, these MIF isoforms were termed oxMIF and oxMIF-2 and are distinct from a cysteine-modified MIF species termed oxMIF by the Kerschbaumer group (223). Oxidation by HOCl results in a loss of tautomerase activity (86), however other functional consequences were not determined yet. Neutrophil apoptosis studies showed that oxMIF as well as oxMIF-2 prolong neutrophil survival to the same extent as their non oxidized counterparts. They also stimulate the expression and secretion of inflammatory mediators such as GM-CSF and IL-1β. Even though oxMIF and oxMIF-2 showed the same pro-survival properties as MIF and MIF-2, the evaluated ELISA data of stimulated PBMCs assume a difference in the induced signaling cascade. However, additional studies need to be performed to clarify the differences of individual MIF isoforms.

5.1.1 CXCL8 and MIF are major drivers of neutrophil survival

Among other cytokines, CXCL8 is a well-known chemoattractant for neutrophils and there are numerous studies showing a link between CXCL8 and disease progression (224,225). Previously, MIF was demonstrated to stimulate the expression of CXCL8 in B-cell chronic lymphocytic leukemia (B-CLL) cells, which thereupon initiates an anti-apoptotic pathway through Bcl-2 proteins (54). Our data demonstrated that MIF stimulation of PBMCs induced the secretion of significant amounts of CXCL8, suggesting that CXCL8 might be an important mediator of MIF-promoted neutrophil survival. Other studies, investigating the role of CXCL8 on neutrophil survival revealed conflicting and differing results (156,157). Here, I show that CXCL8 alone prolonged the

survival of neutrophils, but not to the same extent as supernatants of MIF-treated PBMCs. However, by exposing neutrophils to both MIF and CXCL8, I was able to mimic the inhibitory effect of the supernatants, indicating, that MIF and CXCL8 exert a synergistic effect on neutrophils. Surprisingly, blocking CXCL8 in supernatants from MIF-treated PBMCs did not fully inhibit the pro-survival effect on neutrophils. This might be because the doses of CXCL8 antibody used in this setting were insufficient to bind all the CXCL8 present in the supernatants or because low levels of other cytokines such as IL-6 and GM-CSF measured in the supernatants could compensate for the lack of CXCL8. This supported the theory that CXCL8 is not the sole mediator that drives MIF triggered neutrophil survival. In fact, IL-6 and GM-CSF have previously been shown to inhibit neutrophil apoptosis (123,226). Further studies are needed to get a deeper understanding in the function of other cytokines in mediating the effect of MIF on neutrophil survival.

5.1.2 CXCR2 is an important receptor in MIF-mediated neutrophil survival

CXCL8 and MIF are both ligands of the well-known neutrophil receptor CXCR2 (25). Here, I provide biological evidence suggesting that MIF modified by HOCl can also bind CXCR2, leading to the possibility that the CXCR2/MIF/oxMIF interaction might be important in triggering PBMC-driven neutrophil survival. Receptor blocking studies confirmed the significance of CXCR2 in MIF-mediated neutrophil survival. However, receptor blocking might also affect the CXCL8/CXCR2 axis, keeping the synergistic effect of MIF and CXCL8 in mind. In a disease-related setting, neutrophils were found to be involved in the development of atherosclerosis, as they were found in early and advanced atherosclerotic lesions (227). The arterial recruitment and transmigration of neutrophils is facilitated through CXCR2 and chemotactic mediators (228). Interestingly, MIF as well as CXCL8 were demonstrated to be important mediators of atherogenesis by triggering the recruitment of leukocytes (25,53,111). In atherosclerosis-prone *ApoE*$^{-/-}$ mice, increased levels of MIF were detected in the vascular walls, especially in present macrophages, accompanied by reduced atherosclerotic lesion when using neutralizing antibodies directed against MIF (25), suggesting an important role of CXCR2/MIF mediated leukocyte recruitment. Extended neutrophil lifespan and the maintenance of their pro-inflammatory occupation such as the release of cytokines and the generation of

oxygen species, could contribute to atherogenesis and plaque destabilization. Through the co-appearance of neutrophils and mononuclear cells in the atherosclerotic lesion, it is likely that MIF mediated neutrophil survival is essential in atherosclerosis and may hold therapeutic potential, through specific blocking of MIF-mediated pro-inflammatory actions on delaying neutrophil apoptosis and thereby ensuring resolution of inflammation.

The following graphical summary (Fig. 5-1) shows the pro-survival effect of MIF isoforms (MIF, MIF-2, oxMIF and oxMIF-2) on neutrophils through a PBMC-dependent mechanism and the release of survival mediators such as CXCL8 and potentially also IL-6, G-CSF and GM-CSF. I also provided evidence that the receptor CXCR2 is highly involved in MIF and MIF-2 mediated neutrophil survival.

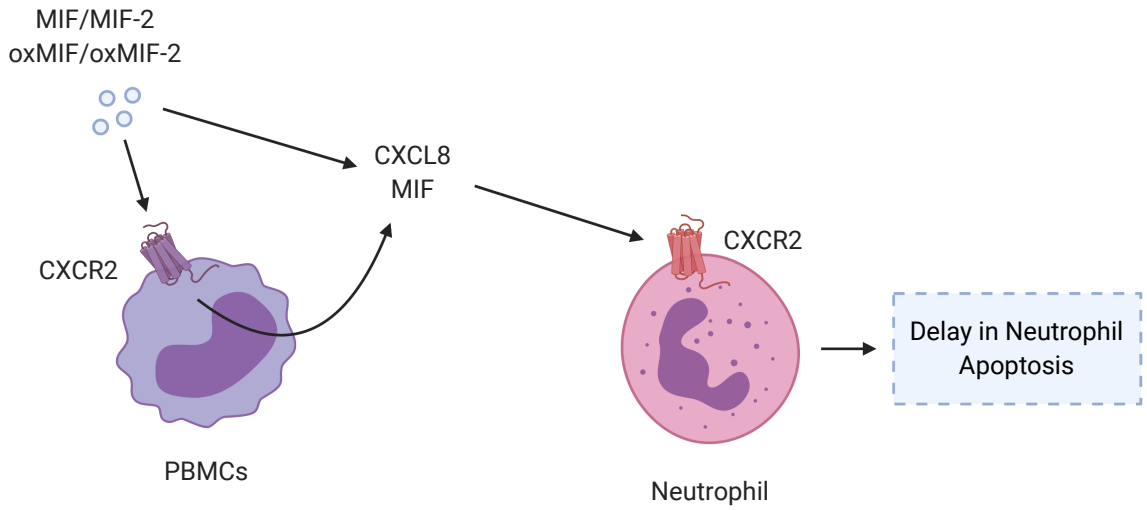

Figure 5-1 Graphical summary of proposed mechanism for MIF and MIF-2 mediated delay of neutrophil apoptosis. MIF and MIF-2, as well as their oxidized counterpart initiate the release of CXCL8 and other cytokines (latter not shown) from PBMCs via the receptor CXCR2. Synergistic action of MIF and CXCL8 delay neutrophil apoptosis mainly through the receptor CXCR2 but potentially also other receptors (latter not shown). Images were designed using BioRender software

5.1.3 4-IPP blocks the pro-survival effect of MIF but not oxMIF

Dysregulation of neutrophil apoptosis has been implicated with the progression of several diseases (141,142). In fact, delayed neutrophil apoptosis can result in non-resolving inflammation which was shown to occur in chronic inflammatory diseases (143). Phagocytosis of neutrophils by macrophages is fundamental to mediate resolution and initiate tissue repair (149). The extended occurrence of activated neutrophils at site of

inflammation and infection creates an aggravated inflammatory environment, accompanied by the generation of pro-inflammatory signals (222). As mentioned before, upregulated levels of MIF have been implicated with the progression and a severe outcome in various diseases (35). Within the last decades, the discovery of small molecule inhibitors opened up new vistas, allowing the development of new therapeutic strategies (229). The small molecule inhibitor 4-IPP has been shown to inhibit MIF's tautomerase activity and is able to block MIF-induced CD74-depenent signaling pathways (79). In this study, I was able to block MIF's pro-survival activity through pre-treatment with 4-IPP significantly. Since the pro-survival effect of MIF is potentially triggered through CXCR2, it could be likely that 4-IPP can also affect this interaction. However, as 4-IPP targets the *N*-terminal proline, it was shown by mass spectrometry that oxidation of MIF by HOCl prohibit the binding of 4-IPP (86) and was thereby not able to inhibit the pro-survival effect of oxMIF on neutrophils. This indicates that in a neutrophil-derived oxidative microenvironment, inhibitors targeting the *N*-terminus of MIF isoforms might fail to block the pro-inflammatory actions of those modified proteins. Nevertheless, it is not fully clear whether the modification of MIF proteins by HOCl can also occur *in vivo*. Approaches designing a specific antibody against proline imine failed so far and need to be further investigated.

5.2 MIF accelerates superoxide formation in neutrophils

A hallmark of the inflammatory action of neutrophils is the production of reactive oxygen species (ROS), such as superoxide radicals, hydrogen peroxide and hypochlorous acid (164), in a process that is called oxidative burst. Dependent on the activation of NADPH oxidase complexes, it is contributing to oxidative stress and can initiate and exacerbate the development of inflammatory processes. Studies showed that neutrophils lacking components of the NADPH oxidase complex decrease their ability to efficiently kill pathogens (230,231). ROS have also been linked to the progression of sterile inflammation in diseases such as atherosclerosis and cardiovascular diseases (232). Antioxidants can be used to prevent the formation of free radicals, providing a high potential for the inhibition and treatment of oxidative damage (233). For example, superoxide dismutase (SOD) can respectively dismutase superoxide radicals and is able to decompose hydrogen peroxide (234). Here I show that pre-stimulation with MIF and

oxMIF can accelerate the fMLP and PMA-induced superoxide anion production in human neutrophils in a concentration and time dependent manner. This effect was dependent on NADPH oxidase, supported by the efficient blocking of superoxide release through diphenyleneiodonium (DPI), a scavenger of NAPDH oxidase. Similar, using additional superoxide dismutase (SOD) fully blocked the production of superoxide in neutrophils. Even though MIF was shown to accelerate the production of superoxide, this effect was also blocked by SOD. As I have also shown that MIF triggers the delay of neutrophil apoptosis, it is likely that MIF can be an effective priming agent for neutrophils. Priming of neutrophils has been proven for several agents and is accompanied with induced phenotypic changes, that leads to increased neutrophil activity and can be potentially linked with diseases such as rheumatoid arthritis, chronic kidney disease and bacterial infections (166). Priming can be accompanied with increased expression of adhesion molecules and altered neutrophil shape (235). However, priming itself does only induces weak ROS production, but results in rapid and increased ROS formation after exposure to other stimuli such as fMLP (236). Interestingly, the priming process was supposed to be irreversible, however studies showed that under certain conditions neutrophils can also be de-primed, resulting in basal neutrophil function (237). This de-priming might be conductive in the resolution of inflammation, promoting the reverse migration into circulation. Pre-stimulation of neutrophils with MIF for longer than 2 h resulted in a retrogressive effect on superoxide production after fMLP and PMA activation back to control levels. This might also facilitate MIFs role in acute and chronic inflammation by differently regulating the immune response depending on the time- and dose of accessible MIF. Short exposure of MIF could have priming properties, whereas longer exposer to MIF can results in de-priming of neutrophils.

The inhibition of accelerated superoxide production could be a potential target for therapeutic approaches. On the one hand, cytokines known as neutrophil priming agents could be blocked, whereat on the other hand, the inhibition of enzymes involved in ROS production, such as NADPH oxidase can be a target for the inhibition of the respiratory burst in neutrophils. Here I showed that the small molecule inhibitor of MIF, 4-IPP, can efficiently block the MIF induced enhanced superoxide production in neutrophils activated with fMLP. Similar to previous experiments, where MIF that was prior oxidized by hypochlorous acid (oxMIF), 4-PP was no longer able to be block the biological

activity, again supporting that in a neutrophil derived oxidative microenvironment the usage of particular inhibitors should be considered.

Another chemokine that was shown to induce superoxide production in neutrophils is CXCL8 (238). Interestingly, MIF and CXCR8 share one distinct receptor that is highly expressed on neutrophils – CXCR2 (25,239). However, CXCL8 induced superoxide production was shown to be not affected by blocking CXCR2 with specific antibodies (212). Similar results were shown in this study, where I blocked CXCR2 on neutrophils with the small molecule antagonist SB225002. Pre-stimulation of neutrophils in the presence of the inhibitor had no effect on MIF promoted acceleration of superoxide production. As CXCL8 can also act on another receptor, CXCR1 blocking strategies showed an inhibitory effect of the CXCL8 induced respiratory burst (238). This hypothesizes that the MIF promoted effect on induced superoxide production can be mediated via another receptor, such as CXCR4 or CXCR7, which is also present on neutrophils. Whether this is the case, still needs to be validated.

5.3 MIF accelerates HOCl production in neutrophil phagosomes

The phagocytosis of pathogens is an important defense mechanism of various immune cells in the human organism. Within these, neutrophils are known to ingest bacteria, forming an intracellular compartment called phagosome (240). Inside the phagosome, pathogens are killed by generating oxidants such as superoxide, hydrogen peroxide and hypochlorous acid (241). Low concentrations of HOCl can already effectively kill a wide range of microorganisms (241,242). Interestingly, not only pathogens can be phagocyted, also in sterile inflammation, apoptotic neutrophil can be removed by macrophages through phagocytosis, which is called efferocytosis (243). Especially at sites of inflammation neutrophils and macrophages show increased phagocytic activity. This effect could be explained by the specific priming and activation of neutrophils through pro-inflammatory cytokines such as TNF-α, CXCL8 or GM-CSF (166). In this study, I demonstrated that pre-treatment with MIF and its oxidized isoform can act directly on neutrophils and accelerate the rate of HOCl production, as well as phagocytosis of zymosan particles. However, the signaling events underlying MIF pre-treatment is still unclear and needs to be further addressed. Nevertheless, these data suggest that MIF can act as a priming agent for neutrophils in a pro-inflammatory environment.

During the respiratory burst, it was shown that approximal 30% of the generated superoxide is converted to HOCl (244,245). MIF triggered neutrophil priming could result in increased tissue damage through accelerated HOCl production. However, this hypothesis needs to be further addressed. Interestingly, MIF modified by HOCl is still able to promote its priming ability on neutrophil activity. This indicates that in an oxidative microenvironment, modification of the *N*-terminal proline has no effect on MIF's ability to boost the neutrophil-derived oxidative burst.

Hypochlorous acid was found to stimulate the expression and secretion of pro-inflammatory cytokines and chemokines in other immune cells such as macrophages (246). Additionally, it was found that HOCl can trigger extracellular trap (ET) formation in macrophages, however it is not known whether this ET release is similar to neutrophil extracellular trap formation (246). The interplay between activated neutrophils and their effect on other immune cells has been also investigated in disease related models (220,247,248). Considering our and other data on MIF's effect on neutrophil activity it could be very likely that there is a correlation between MIF primed neutrophils and macrophage derived pro-inflammatory actions.

5.4 Increased NET formation in MIF-primed neutrophils

In 2004, neutrophil extracellular traps were first described as an extracellular network consisting of double-stranded DNA, histones, myeloperoxidase, neutrophil elastase (NE) and antimicrobial proteins as a biological defense system to kill invading pathogens (172). However, recent studies also show that NET formation is implicated in sterile inflammation and can result in an increased inflammatory response (174,182). Different stimuli and compounds have been found to induce spontaneous and active NETosis (249). Here, I show that MIF alone was not able to induce spontaneous NET formation in neutrophils. Interestingly, I have seen lower levels of extracellular DNA in neutrophils that were pre-treated with MIF and oxMIF compared to control. These results strengthen the effect of MIF on neutrophil survival as shown before in this thesis. In contrast, MIF pre-stimulated neutrophils showed a significantly higher amount of extracellular DNA when NETosis was actively induced by PMA. Enhanced NET formation through primed neutrophils has been reported in several diseases such as rheumatoid arthritis and autoimmune diseases (250,251). Those diseases have also been

implicated with upregulated levels of MIF (35), suggesting a connection between neutrophil activity and MIF. Our data also support our hypothesis that MIF can act as a priming agent for neutrophils, as it does not induce strong activation by itself but accelerate the response of neutrophils to a second activation stimulus.

The role of NETs in sterile inflammation and chronic disease is a new but fast-moving field. For example, NETs were found to play a progressive effect in atherosclerosis and atherothrombosis (182). Studies analyzing human atherosclerotic plaques showed that NETs contributed to plaque disruption through induced apoptosis of endothelial and smooth muscle cells. The specific inhibition of NET release showed potential in plaque stabilization (252,253).

5.5 MIF shows potential in NET degradation

A number of studies suggested that NETs have the potential to act as a biomarker for inflammation and injury, proving an appropriate target for therapeutic approaches. Besides the prevention of NET formation using inhibitors, it could also be beneficial to dissolve formed NET structures as a therapeutic procedure. Studies showed that for example DNase-1 can degrade DNA -containing NETs and was implicated in a decreased disease progression in cystic fibrosis (254). Similar effects were seen in experimental and clinical studies in myocardial I/R injury (255,256). In my thesis, I found that an incubation of NETs with MIF and oxMIF results in decreased detection of extracellular DNA. Although the effect of MIF on NET degradation was only moderate, it could be a hint in its role in disease progression through NET derived tissue damage. Of note, MIF's potential to act as a nuclease was already demonstrated by Wang *et al*. in ischemically stressed neurons. They identified intracellular MIF as a crucial factor in apoptosis-inducing factor (AIF)-mediated cell death trigger through DNA fragmentation (205). Although NET degradation was mainly seen extracellularly, there is also evidence that NETs can be also degraded intracellularly by DNase I (257). Intracellular NET degradation might be beneficial in the reduction and resolution of inflammation, promoting the decreased oxidative activity of neutrophils by reduced NET appearance. MIF is expressed in neutrophil and levels have been shown to be increased in an inflammatory environment (193). This could hypothesize MIF-mediated NET degradation not only extracellularly but also intracellularly, resulting in decreased NET

related tissue damaging and inflammation. However, previous data mainly showed pro-inflammatory actions of MIF, as it can promote neutrophils survival and act as a potential priming agent on neutrophils oxidative activity. Whether MIF's effect on the degradation of NETs can have anti-inflammatory actions, or can result in the effective release of other inflammatory cytokines bound to extracellular neutrophil DNA needs to be further addressed. The effect of MIF isoforms on neutrophil survival and activity is summarized in Table 1 and reveals the potential of strategies blocking MIF's proinflammatory actions.

Table 1 Phenotypic changes induced in neutrophils by the various MIF isoforms. Pre-stimulation of neutrophils with MIF isoforms can have distinct effects on neutrophil activity and results in phenotypic changes. '↑' refers to increased activity compared to non-treated controls, '↓' refers to decreased activity compared to untreated controls, '?' indicates an unknown effect of MIF isoforms.

MIF isoforms	Apoptosis	Chemotaxis	Superoxide Production	HOCl Production	Phagocytosis	NET Formation	NET Degradation
MIF	↓ in the presence of PBMCs; receptor involved: CXCR2	↑	↑ priming agent; accelerated after fMLP and PMA activation	↑ priming agent ; accelerated after zymosan exposure	↑ priming agent ; accelerated after zymosan exposure	not directly induced ↑ priming agent ; accelerated after PMA activation	↑ only minor effect
oxMIF	↓ in the presence of PBMCs; receptor involved: CXCR2	↓	↑ priming agent; accelerated after fMLP and PMA activation	↑ priming agent ; accelerated after zymosan exposure	↑ priming agent ; accelerated after zymosan exposure	not directly induced ↑ priming agent ; accelerated after PMA activation	↑ only minor effect
MIF-2	↓ in the presence of PBMCs; receptor involved: ?	?	?	?	?	?	?
oxMIF-2	↓ in the presence of PBMCs; receptor involved: ?	?	?	?	?	?	?

5.6 MIF and neutrophils co-localize in infarcted area in myocardial I/R injury

Acute myocardial infarction (AMI) is one of the leading causes of death worldwide. Early and effective myocardial reperfusion is one of the most used therapeutic interventions to reduce MI injury and infarct size (217). However, myocardial reperfusion itself can induce oxidative stress that comes along with myocardial death and tissue injury, an event called ischemia reperfusion injury (I/R) (258). Neutrophils have been found to accumulate in the infarcted myocardial area early after reperfusion and are the major origin of ROS (217). This results in acute inflammation and the initiation of myocardial apoptosis (218). The inhibition and depletion of neutrophils or neutrophil-derived enzymes, such as MPO were shown to improve the outcome of I/R injury (259). However, another study using a chronic MI model showed that neutrophil depletion result worsens cardiac function, indicating that neutrophils are also involved in the activation of macrophages which mediate the clearance of apoptotic cells and have a role in cardiac remodeling and healing (131). Within I/R injury, cardiomyocytes were found to secrete MIF and interestingly, it was shown to mediate cardioprotection through the activation of the AMPK signaling cascade and the reduction of oxidative stress (260). However, other studies clarified a more complex function of MIF in I/R injury, indicating that MIF might also have a detrimental effect on the myocardium. MIF deficiency showed decreased expression of pro-inflammatory cytokines, that goes along with decreased infiltration of immune cells such as neutrophils and macrophages (261). Whether MIF is also affecting neutrophil activity *in vivo* in an I/R injury model is still unclear. Here I offer first preliminary data that show co-localization of MIF and neutrophils in the infarcted area of the myocardium. Future studies will be needed to characterize to detailed effect of MIF on neutrophil activity in I/R injury. Additionally, it would be interesting to investigate to potential of neutrophil-derived HOCl production and its impact on modifying MIF.

6 Summary and Outlook

First described in 1966, the atypical cytokine macrophage migration inhibitory factor (MIF) has been shown to play an important role in acute and chronic inflammatory diseases. MIF regulates inflammatory processes by recruiting several immune cell types, by increasing pro-inflammatory cytokine production and by inhibiting apoptosis. MIF has a conserved *N*-terminal proline, which has an atypically low pK_a and is functionally involved in MIF's evolutionarily conserved tautomerase activity. Interestingly, the region around the tautomerase pocket is structurally involved in the binding of MIF to its receptors, whereby the binding of low molecular weight inhibitors directed to the *N*-terminal proline inhibit important biological activities of MIF. In various *in vitro* experiments, we previously showed that neutrophil-derived hypochlorous acid (HOCl) is able to convert the *N*-terminal proline of MIF into a proline imine ('proline-oxidized or oxidized MIF' (oxMIF)), which leads to an inhibition of MIF's tautomerase activity. How this modification influences the various inflammatory activities of MIF is unknown.

During an inflammatory event, neutrophils rapidly infiltrate into the center of inflammation and infection and are known to not only kill invading pathogens, but also promote sterile inflammation. The precise interplay between MIF proteins and neutrophils in sterile inflammation is incompletely understood and the role of neutrophils in promoting oxMIF generation needs further exploration.

This dissertation characterizes the effect of MIF and MIF-2, as well as their oxidized counterparts (oxMIF, oxMIF-2) on various functions of neutrophils like apoptosis, superoxide and HOCl production, as well as phagocytosis and NET formation, in an effort to mechanistically understand the neutrophil/MIF protein interaction network.

In the first part of this thesis, it was shown that not only MIF, but also MIF-2, oxMIF and oxMIF-2 can inhibit the apoptosis of neutrophils in a concentration-dependent manner. However, this effect could only be seen when peripheral blood mononuclear cells (PBMC) were present in the same culture, in which both, lymphocytes and monocytes, promoted MIF-mediated delay of neutrophil apoptosis. All studied MIF isoforms (MIF, oxMIF, MIF-2, oxMIF-2) stimulated the release of various cytokines and chemokines, such as IL-1β, IL-6 or CXCL8 from PBMC cultures. Experiments, in which neutrophils were incubated with the conditioned supernatants from MIF-stimulated PBMCs, also

showed an inhibitory effect on neutrophil apoptosis, indicating that those factors are responsible for the MIF-mediated pro-survival effect. For CXCL8 in particular, a significant increase was detected in supernatants from MIF-stimulated PBMCs. CXCL8-blocking experiments showed a significant reduction of the pro-survival effect from the conditioned supernatants. This suggests that CXCL8 is one of the main mediators promoting the anti-apoptotic effect that MIF has on neutrophils. However, when neutrophils were treated with CXCL8 alone, only minor anti-apoptotic activity was observed. Surprisingly, when neutrophils were co-stimulated with MIF and CXCL8, the pro-survival effect on neutrophils was similar to the effect of the PBMC-conditioned supernatants, indicating a synergistic effect of MIF and CXCL8.

The biological activity of MIF is mediated through binding to its cell surface receptors CD74-CD44 and the CXC chemokine receptors CXCR2 and CXCR4. By specifically blocking those receptors, I demonstrated that the pro-survival effect of MIF was mediated through the surface receptor CXCR2, which is present on both cell types, PBMCs and neutrophils. Interestingly, CXCL8 and MIF share this receptor, suggesting that the inhibition of the pro-survival effect on CXCR2 may be due to blocked binding of CXCL8. Supporting these findings, I was able to show that oxidation of the *N*-terminal proline did not affect the binding to CXCR2. Together, the data suggests that stimulation of PBMCs with MIF, MIF-2 and their oxidized isoforms induce the releases a divergent cytokine pattern. Future studies are needed to determine whether MIF/oxMIF and MIF-2/oxMIF-2 activate different signaling pathways.

In the next part of this thesis, I investigated the role of MIF and oxMIF as priming agents on neutrophil oxidative and phagocytic activity, as well as NET formation. Neutrophils are known as major producers of reactive oxygen species (ROS), a process called oxidative burst. I showed that pre-incubation of neutrophils with MIF results in an accelerated production of superoxide, when neutrophils were further activated with phorbol 12-myristate 13-acetate (PMA) or *N*-Formylmethionyl-leucyl-phenylalanine (fMLP). This effect was independent of the surface receptor CXCR2 but could be blocked by a specific pharmacologic MIF inhibitor (4-IPP). However, the *N*-terminal proline oxidation by HOCl prevented 4-IPP binding, indicating that in a neutrophil-governed oxidative microenvironment, inhibitors targeting the *N*-terminus of MIF might fail to block its pro-inflammatory actions. Additionally, pre-incubation with MIF and oxMIF resulted in accelerated intracellular HOCl production and increased numbers of

phagosomes within neutrophils exposed to zymosan particles. However, the ability of neutrophils to phagocytose zymosan particles did not increase in total, but the uptake rate was accelerated. Moreover, MIF and oxMIF did not induce NETosis themselves, but are able to increase the response of neutrophils towards PMA-induced NETosis. Taken together, those findings supported the potential of MIF as a neutrophil-priming agent, providing an interesting novel basis for the development of neutrophil-targeting therapeutic strategies.

Independently from its potential as a priming agent, I found that MIF and oxMIF are able to degrade DNA in preformed NETs. This activity relates to the recently uncovered surprising MIF nuclease function as demonstrated by others in hypoxically stressed neurons. However, compared to the DNA degradation rate by a conventional DNase, the NET-derived DNA-degrading activity of MIF appeared to be low. Future studies will therefore have to address the physiologic relevance of this activity.

Additionally, in preliminary experiments, I was able to show that in a myocardial I/R injury model, neutrophils and MIF co-localize, which could facilitate the impact of MIF on neutrophil function. Together, I demonstrated that MIF and its oxidized counterpart could play an important role in priming neutrophil activity, which can be further increased by its ability to delay neutrophil apoptosis.

6.1 Summary (German)

Das atypische Zytokin *macrophage migration inhibitory factor* (MIF) wurde erstmals 1966 beschrieben (1). Seither wurde in verschiedenen Studien gezeigt, dass MIF in Entzündungserkrankungen, wie Rheumatoide Arthritis, Mukoviszidose, Asthma, Colitis, Sepsis, Atherosklerose und Myokardinfarkt, sowie Krebs eine entscheidende Rolle einnimmt (26,48,191,262-266). MIF ist in der Lage inflammatorische Prozesse durch die Rekrutierung von Immunzellen, die Verstärkung von Zytokin-Produktionen und der Inhibierung von Apoptose zu regulieren (19,25,26,30,192). MIF besitzt ein konserviertes *N*-terminales Prolin, welches einen untypisch niedrigen pK_a-Wert besitzt und eine tragende Rolle in der evolutionär konservierten Tautomeraseaktivität des Proteins einnimmt (13,15). Interessanterweise ist die Region um das Tautomerasezentrum jedoch strukturell an der Bindung von MIF an seinen Rezeptoren CD74 und CXCR4 beteiligt, wobei die Bindung von niedermolekularen Inhibitoren an das *N*-terminale Prolin entsprechend wichtige biologische Aktivitäten von MIF inhibiert (19,21,79). In verschiedenen *in vitro*-Experimenten konnten wir in Vorarbeiten zeigen, dass die durch neutrophile Granulozyten produzierte hypochlorige Säure (HOCl) in der Lage sind, das *N*-terminale Prolin in MIF in ein Prolin-Imin umzuwandeln, welches zu einer Inhibierung der Tautomeraseaktivität führt (86). Neutrophile sind die ersten Zellen, die während entzündlicher Prozesse in das Entzündungs- und Infektionszentrum einwandern. Dort können sie nicht nur eindringende Krankheitserreger beseitigen, sondern auch sterile Entzündungen fördern (106-109).

Im Rahmen dieser Arbeit erfolgte eine nähere Charakterisierung des Einflusses von MIF und MIF-2 auf verschiedene Funktionen von Neutrophilen Granulozyten, sowie dessen Rolle auf die Inhibierung der spontanen Apoptose von Neutrophilen. Es konnte gezeigt werden, dass nicht nur MIF, sondern auch das Schwesterprotein MIF-2 die Apoptose von Neutrophilen Granulozyten verzögern kann. Allerdings kann dieser Effekt nur gezeigt werden, wenn mononukleären Zellen des peripheren Blutes (PBMC) in der gleichen Kultur vorhanden sind. Ebenso wurde gezeigt, dass MIF und MIF-2 die Ausschüttung verschiedener Zytokine, wie IL-1β, IL-6 oder CXCL8, durch PBMCs anregen. Experimente, in denen Neutrophile nur mit den Überständen von zuvor stimulierten PBMCs inkubiert wurden, zeigten auch inhibitorische Effekte auf die Apoptose. Besonders für CXCL8 konnte ein signifikanter Anstieg in den Überständen von MIF

stimulierten PBMCs nachgewiesen werden. Folgend konnte gezeigt werden, dass durch CXCL8 blockierende Antikörper die Apoptose verzögernde Wirkung der Überstände stark zurück geht. Dies lies vermuten, dass CXCL8 der Hauptmediator dieser anti-apoptotischen Wirkung ist. CXCL8 allein zeigte nur wenig anti-apoptotischen Wirkung, allerdings konnte gezeigt werden, dass MIF und CXCL8 zusammen einen ähnlichen Effekt zeigen, wie die Überstände von MIF stimulierten PBMCs. Dies deutet auf einen synergistischen Effekt von MIF und CXCL8 hin.

Die biologische Aktivität von MIF wird durch die Bindung an die Zelloberflächenrezeptoren CD74-CD44 und die CXC Chemokinrezeptoren CXCR2 und CXCR4 bedingt (25,52,54,55). Durch die spezifische Blockierung dieser Rezeptoren wurde gezeigt, der MIF-Rezeptor CXCR2 eine entscheidende Rolle in der anti-apoptotischen Wirkung von MIF spielt. ELISA-Daten legen nahe, dass die Stimulation von PBMCs mit oxMIF andere Zytokine freisetzt. Zukünftige Studien sind jedoch erforderlich, um festzustellen, ob MIF und oxMIF unterschiedliche Signalwege aktivieren.

Im nächsten Teil dieser Arbeit wurde die Rolle von MIF und oxMIF als Priming-Agent für die oxidative und phagozytische Aktivität von Neutrophilen sowie für die Bildung NETs untersucht. Neutrophile sind als Hauptproduzenten reaktiver Sauerstoffspezies bekannt, darunter Superoxidradikale, Wasserstoffperoxid und Hypochlorige Säure (164) - ein Prozess, der als „oxidative burst“ bezeichnet wird. Vorinkubation von Neutrophilen mit MIF führte zu einer beschleunigten Produktion von Superoxid, wenn Neutrophile mit PMA oder fMLP aktiviert wurden. Dieser Effekt war unabhängig vom Oberflächenrezeptor CXCR2, konnte jedoch durch einen spezifischen MIF-Inhibitor (4-IPP) blockiert werden. Die *N*-terminale Prolinoxidation durch HOCl verhinderte jedoch die 4-IPP-Bindung, was darauf hinweist, dass in einer oxidativen Mikroumgebung Inhibitoren, die auf den *N*-Terminus von MIF abzielen, möglicherweise ihre Wirkung verlieren. Ebenso konnte gezeigt werden, dass Vorinkubation mit MIF und oxMIF zu einer beschleunigten intrazellulären Produktion von Hypochloriger Säure und einer erhöhten Anzahl von Phagosomen in Neutrophilen führte. Die Fähigkeit von Neutrophilen, Zymosan-Partikel zu phagozytieren, nahm jedoch insgesamt nicht zu, wurde aber beschleunigt. Darüber hinaus konnte gezeigt werden, dass MIF und oxMIF selbst keine NETose induzierten können, aber die Reaktion von Neutrophilen auf PMA-induzierte NETose erhöhen. Zusammengenommen unterstützen diese Ergebnisse das

Potenzial von MIF als Priming-Agent für Neutrophile und bilden die Grundlage für die Entwicklung neuer Therapiestrategien. In Pilotexperimenten konnte ich auch zeigen, dass in einem krankheitsrelevanten *in vivo*-Model, beispielsweise im Myokardinfarkt, Neutrophile und MIF kolokalisieren, was einen möglichen Einfluss von MIF auf die Funktion von Neutrophilen unterstreicht.

Unabhängig von seinem Potenzial als Priming-Agent habe ich gezeigt, dass MIF und oxMIF in der Lage sind, DNA in vorgeformten NETs abzubauen. Dieser geringfügige Effekt ist jedoch nicht mit dem DNA-Abbau durch DNase vergleichbar, könnte jedoch das Potenzial einer neuen Funktion von MIF-Proteinen aufzeigen.

Zusammengefasst, habe ich gezeigt, dass MIF und oxMIF eine wichtige Rolle bei der Aktivierung der Neutrophilen spielen können, was durch die Verzögerung die Apoptose von Neutrophilen, weiter gesteigert werden kann.

7 Figures

8 Tables

9 Acknowledgement

First of all, I would like to thank my supervisor Univ. Prof. Dr. Jürgen Bernhagen for giving me the opportunity to work on this extremely interesting and versatile project. I would like to thank him for his excellent support, enthusiasm and supervision during my doctoral studies. His constant willingness for constructive and scientific discussions was essential and extremely helpful for the accomplishment of this work.

I especially would like to thank Dr. Nina Dickerhof for her indefatigable support and numerous discussions, that have contributed to the success of this work. I am very grateful for her invaluable supervision, practical teaching and guidance. It was a pleasure to work with her on this fascinating topic. Thank you for also showing me the indescribable beauty of a fascinating country that I will always call a home because of you.

I would like to thank Prof. Dr. Mark Hampton for his outstanding supervision and the opportunity to work in his lab in Christchurch, New Zealand. Through his expertise in the field of neutrophil research, I learned a lot about those fascinating cells. A big thank you goes to Dr. Heather Parker, Dr. Louisa Ashby, Dr. Leon Smith and Prof. Dr. Tony Kettle for your support, advices and great atmosphere, that made my stay in New Zealand unforgettable.

A special thanks goes to the DAAD for giving me the financial support and the opportunity to do a research year abroad in New Zealand at the University of Otago.

Thank you to Leon Zwissler, who contributed to the neutrophil preparations and some of the functional bioassays, and to Priscila Bourilhón, who performed the yeast receptor binding assay and helped with many of my questions. I would particularly like to thank Simona Gerra, who was a great help in expressing and purifying the MIF proteins needed for this study.

I would also like to thank the nurses of the Nicholls Clinical Research Centre in Christchurch and the ambulance of the ISD in Munich for their help taking blood samples from healthy volunteers.

Thanks to PD Dr. Ulrike Hendgen-Cotta, Univ.-Prof. Dr. med. Tienush Rassaf and the team of the Department of Cardiology and Angiology at the University Hospital in Essen for providing me the myocardial infarct tissue slides and our collaborative efforts.

I would also like to thank the members of the Bernhagen and Hampton lab for their great support and help at any time and, for the very nice and funny atmosphere that also went beyond work.

I thank Christine Krammer for proofreading my work and for helpful suggestions and numerous discussions. Through work, but also beyond, you have become one of my best friends.

A very special thanks goes to my parents for their unlimited support and for giving me the opportunity to make my occupational career and these studies possible. I would also like to thank my sister for our unique connection and her endless encouragement.

I would like to extend this special thank and say thank you to Karsten Behrend, my best friend, partner and love of my life. Thank you for your never-ending support and patience. You inspired me every single day.

10 References

1. David, J. R. (1966) Delayed hypersensitivity in vitro: its mediation by cell-free substances formed by lymphoid cell-antigen interaction. *Proc Natl Acad Sci U S A* **56**, 72-77

2. Bloom, B. R., and Bennett, B. (1966) Mechanism of a reaction in vitro associated with delayed-type hypersensitivity. *Science* **153**, 80-82

3. Bernhagen, J., Mitchell, R. A., Calandra, T., Voelter, W., Cerami, A., and Bucala, R. (1994) Purification, bioactivity, and secondary structure analysis of mouse and human macrophage migration inhibitory factor (MIF). *Biochemistry* **33**, 14144-14155

4. Sun, H. W., Bernhagen, J., Bucala, R., and Lolis, E. (1996) Crystal structure at 2.6-A resolution of human macrophage migration inhibitory factor. *Proc Natl Acad Sci U S A* **93**, 5191-5196

5. Suzuki, M., Sugimoto, H., Nakagawa, A., Tanaka, I., Nishihira, J., and Sakai, M. (1996) Crystal structure of the macrophage migration inhibitory factor from rat liver. *Nat Struct Biol* **3**, 259-266

6. Muhlhahn, P., Bernhagen, J., Czisch, M., Georgescu, J., Renner, C., Ross, A., Bucala, R., and Holak, T. A. (1996) NMR characterization of structure, backbone dynamics, and glutathione binding of the human macrophage migration inhibitory factor (MIF). *Protein Sci* **5**, 2095-2103

7. Sugimoto, H., Suzuki, M., Nakagawa, A., Tanaka, I., and Nishihira, J. (1996) Crystal structure of macrophage migration inhibitory factor from human lymphocyte at 2.1 A resolution. *FEBS Lett* **389**, 145-148

8. Sun, H. W., Swope, M., Cinquina, C., Bedarkar, S., Bernhagen, J., Bucala, R., and Lolis, E. (1996) The subunit structure of human macrophage migration inhibitory factor: evidence for a trimer. *Protein Eng* **9**, 631-635

9. Mischke, R., Kleemann, R., Brunner, H., and Bernhagen, J. (1998) Cross-linking and mutational analysis of the oligomerization state of the cytokine macrophage migration inhibitory factor (MIF). *FEBS Lett* **427**, 85-90

10. Kleemann, R., Kapurniotu, A., Frank, R. W., Gessner, A., Mischke, R., Flieger, O., Juttner, S., Brunner, H., and Bernhagen, J. (1998) Disulfide analysis reveals a role for macrophage migration inhibitory factor (MIF) as thiol-protein oxidoreductase. *J Mol Biol* **280**, 85-102

11. Kleemann, R., Mischke, R., Kapurniotu, A., Brunner, H., and Bernhagen, J. (1998) Specific reduction of insulin disulfides by macrophage migration inhibitory factor (MIF) with glutathione and dihydrolipoamide: potential role in cellular redox processes. *FEBS Lett* **430**, 191-196

12. Thiele, M., and Bernhagen, J. (2005) Link between macrophage migration inhibitory factor and cellular redox regulation. *Antioxid Redox Signal* **7**, 1234-1248

13. Swope, M., Sun, H. W., Blake, P. R., and Lolis, E. (1998) Direct link between cytokine activity and a catalytic site for macrophage migration inhibitory factor. *EMBO J* **17**, 3534-3541

14. Merk, M., Zierow, S., Leng, L., Das, R., Du, X., Schulte, W., Fan, J., Lue, H., Chen, Y., Xiong, H., Chagnon, F., Bernhagen, J., Lolis, E., Mor, G., Lesur, O., and Bucala, R. (2011) The D-dopachrome tautomerase (DDT) gene product is a cytokine and functional homolog of macrophage migration inhibitory factor (MIF). *Proc Natl Acad Sci U S A* **108**, E577-585

15. Rosengren, E., Aman, P., Thelin, S., Hansson, C., Ahlfors, S., Bjork, P., Jacobsson, L., and Rorsman, H. (1997) The macrophage migration inhibitory factor MIF is a phenylpyruvate tautomerase. *FEBS Lett* **417**, 85-88

16. Rosengren, E., Bucala, R., Aman, P., Jacobsson, L., Odh, G., Metz, C. N., and Rorsman, H. (1996) The immunoregulatory mediator macrophage migration inhibitory factor (MIF) catalyzes a tautomerization reaction. *Mol Med* **2**, 143-149

17. Senter, P. D., Al-Abed, Y., Metz, C. N., Benigni, F., Mitchell, R. A., Chesney, J., Han, J., Gartner, C. G., Nelson, S. D., Todaro, G. J., and Bucala, R. (2002) Inhibition of macrophage migration inhibitory factor (MIF) tautomerase and biological activities by acetaminophen metabolites. *Proc Natl Acad Sci U S A* **99**, 144-149

18. Dios, A., Mitchell, R. A., Aljabari, B., Lubetsky, J., O'Connor, K., Liao, H., Senter, P. D., Manogue, K. R., Lolis, E., Metz, C., Bucala, R., Callaway, D. J., and Al-Abed, Y. (2002) Inhibition of MIF bioactivity by rational design of pharmacological inhibitors of MIF tautomerase activity. *J Med Chem* **45**, 2410-2416

19. Rajasekaran, D., Zierow, S., Syed, M., Bucala, R., Bhandari, V., and Lolis, E. J. (2014) Targeting distinct tautomerase sites of D-DT and MIF with a single molecule for inhibition of neutrophil lung recruitment. *FASEB J* **28**, 4961-4971

20. Al-Abed, Y., and VanPatten, S. (2011) MIF as a disease target: ISO-1 as a proof-of-concept therapeutic. *Future Med Chem* **3**, 45-63

21. Ouertatani-Sakouhi, H., El-Turk, F., Fauvet, B., Cho, M. K., Pinar Karpinar, D., Le Roy, D., Dewor, M., Roger, T., Bernhagen, J., Calandra, T., Zweckstetter, M., and Lashuel, H. A. (2010) Identification and characterization of novel classes of macrophage migration inhibitory factor (MIF) inhibitors with distinct mechanisms of action. *J Biol Chem* **285**, 26581-26598

22. Lubetsky, J. B., Dios, A., Han, J., Aljabari, B., Ruzsicska, B., Mitchell, R., Lolis, E., and Al-Abed, Y. (2002) The tautomerase active site of macrophage migration inhibitory factor is a potential target for discovery of novel anti-inflammatory agents. *J Biol Chem* **277**, 24976-24982

23. Petrovsky, N., Socha, L., Silva, D., Grossman, A. B., Metz, C., and Bucala, R. (2003) Macrophage migration inhibitory factor exhibits a pronounced circadian rhythm relevant to its role as a glucocorticoid counter-regulator. *Immunol Cell Biol* **81**, 137-143

24. Baugh, J. A., Chitnis, S., Donnelly, S. C., Monteiro, J., Lin, X., Plant, B. J., Wolfe, F., Gregersen, P. K., and Bucala, R. (2002) A functional promoter polymorphism in the macrophage migration inhibitory factor (MIF) gene associated with disease severity in rheumatoid arthritis. *Genes Immun* **3**, 170-176

25. Bernhagen, J., Krohn, R., Lue, H., Gregory, J. L., Zernecke, A., Koenen, R. R., Dewor, M., Georgiev, I., Schober, A., Leng, L., Kooistra, T., Fingerle-Rowson, G., Ghezzi, P., Kleemann, R., McColl, S. R., Bucala, R., Hickey, M. J., and Weber, C. (2007) MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med* **13**, 587-596

26. Calandra, T., and Roger, T. (2003) Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol* **3**, 791-800

27. Onodera, S., Nishihira, J., Iwabuchi, K., Koyama, Y., Yoshida, K., Tanaka, S., and Minami, A. (2002) Macrophage migration inhibitory factor up-regulates matrix metalloproteinase-9 and -13 in rat osteoblasts. Relevance to intracellular signaling pathways. *J Biol Chem* **277**, 7865-7874

28. Onodera, S., Nishihira, J., Koyama, Y., Majima, T., Aoki, Y., Ichiyama, H., Ishibashi, T., and Minami, A. (2004) Macrophage migration inhibitory factor up-regulates the expression of interleukin-8 messenger RNA in synovial fibroblasts of rheumatoid arthritis patients: common transcriptional regulatory mechanism between interleukin-8 and interleukin-1beta. *Arthritis Rheum* **50**, 1437-1447

29. Onodera, S., Kaneda, K., Mizue, Y., Koyama, Y., Fujinaga, M., and Nishihira, J. (2000) Macrophage migration inhibitory factor up-regulates expression of matrix metalloproteinases in synovial fibroblasts of rheumatoid arthritis. *J Biol Chem* **275**, 444-450

30. Mitchell, R. A., Liao, H., Chesney, J., Fingerle-Rowson, G., Baugh, J., David, J., and Bucala, R. (2002) Macrophage migration inhibitory factor (MIF) sustains macrophage proinflammatory function by inhibiting p53: regulatory role in the innate immune response. *Proc Natl Acad Sci U S A* **99**, 345-350

31. Lolis, E. (2001) Glucocorticoid counter regulation: macrophage migration inhibitory factor as a target for drug discovery. *Curr Opin Pharmacol* **1**, 662-668

32. Mitchell, R. A., Metz, C. N., Peng, T., and Bucala, R. (1999) Sustained mitogen-activated protein kinase (MAPK) and cytoplasmic phospholipase A2 activation by macrophage migration inhibitory factor (MIF). Regulatory role in cell proliferation and glucocorticoid action. *J Biol Chem* **274**, 18100-18106

33. Calandra, T., Bernhagen, J., Metz, C. N., Spiegel, L. A., Bacher, M., Donnelly, T., Cerami, A., and Bucala, R. (1995) MIF as a glucocorticoid-induced modulator of cytokine production. *Nature* **377**, 68-71

34. Baugh, J. A., and Bucala, R. (2002) Macrophage migration inhibitory factor. *Crit Care Med* **30**, S27-s35

35. Lue, H., Kleemann, R., Calandra, T., Roger, T., and Bernhagen, J. (2002) Macrophage migration inhibitory factor (MIF): mechanisms of action and role in disease. *Microbes Infect* **4**, 449-460

36. Calandra, T., Bernhagen, J., Mitchell, R. A., and Bucala, R. (1994) The macrophage is an important and previously unrecognized source of macrophage migration inhibitory factor. *J Exp Med* **179**, 1895-1902

37. Fingerle-Rowson, G., Koch, P., Bikoff, R., Lin, X., Metz, C. N., Dhabhar, F. S., Meinhardt, A., and Bucala, R. (2003) Regulation of macrophage migration inhibitory factor expression by glucocorticoids in vivo. *Am J Pathol* **162**, 47-56

38. Bacher, M., Meinhardt, A., Lan, H. Y., Mu, W., Metz, C. N., Chesney, J. A., Calandra, T., Gemsa, D., Donnelly, T., Atkins, R. C., and Bucala, R. (1997) Migration inhibitory factor expression in experimentally induced endotoxemia. *Am J Pathol* **150**, 235-246

39. Roger, T., David, J., Glauser, M. P., and Calandra, T. (2001) MIF regulates innate immune responses through modulation of Toll-like receptor 4. *Nature* **414**, 920-924

40. Calandra, T., Echtenacher, B., Roy, D. L., Pugin, J., Metz, C. N., Hultner, L., Heumann, D., Mannel, D., Bucala, R., and Glauser, M. P. (2000) Protection from septic shock by neutralization of macrophage migration inhibitory factor. *Nat Med* **6**, 164-170

41. Bozza, F. A., Gomes, R. N., Japiassu, A. M., Soares, M., Castro-Faria-Neto, H. C., Bozza, P. T., and Bozza, M. T. (2004) Macrophage migration inhibitory factor levels correlate with fatal outcome in sepsis. *Shock* **22**, 309-313

42. Plant, B. J., Gallagher, C. G., Bucala, R., Baugh, J. A., Chappell, S., Morgan, L., O'Connor, C. M., Morgan, K., and Donnelly, S. C. (2005) Cystic fibrosis, disease severity, and a macrophage migration inhibitory factor polymorphism. *Am J Respir Crit Care Med* **172**, 1412-1415

43. Radstake, T. R., Sweep, F. C., Welsing, P., Franke, B., Vermeulen, S. H., Geurts-Moespot, A., Calandra, T., Donn, R., and van Riel, P. L. (2005) Correlation of rheumatoid arthritis severity with the genetic functional variants and circulating levels of macrophage migration inhibitory factor. *Arthritis Rheum* **52**, 3020-3029

44. Llamas-Covarrubias, M. A., Valle, Y., Bucala, R., Navarro-Hernandez, R. E., Palafox-Sanchez, C. A., Padilla-Gutierrez, J. R., Parra-Rojas, I., Bernard-Medina, A. G., Reyes-Castillo, Z., and Munoz-Valle, J. F. (2013) Macrophage migration inhibitory factor (MIF): genetic evidence for participation in early onset and early stage rheumatoid arthritis. *Cytokine* **61**, 759-765

45. Hudson, J. D., Shoaibi, M. A., Maestro, R., Carnero, A., Hannon, G. J., and Beach, D. H. (1999) A proinflammatory cytokine inhibits p53 tumor suppressor activity. *J Exp Med* **190**, 1375-1382

46. Tomiyasu, M., Yoshino, I., Suemitsu, R., Okamoto, T., and Sugimachi, K. (2002) Quantification of macrophage migration inhibitory factor mRNA expression in non-small cell lung cancer tissues and its clinical significance. *Clin Cancer Res* **8**, 3755-3760

47. Pan, J. H., Sukhova, G. K., Yang, J. T., Wang, B., Xie, T., Fu, H., Zhang, Y., Satoskar, A. R., David, J. R., Metz, C. N., Bucala, R., Fang, K., Simon, D. I., Chapman, H. A., Libby, P., and Shi, G. P. (2004) Macrophage migration inhibitory factor deficiency impairs atherosclerosis in low-density lipoprotein receptor-deficient mice. *Circulation* **109**, 3149-3153

48. Zernecke, A., Bernhagen, J., and Weber, C. (2008) Macrophage migration inhibitory factor in cardiovascular disease. *Circulation* **117**, 1594-1602

49. Liehn, E. A., Kanzler, I., Konschalla, S., Kroh, A., Simsekyilmaz, S., Sonmez, T. T., Bucala, R., Bernhagen, J., and Weber, C. (2013) Compartmentalized protective and detrimental effects of endogenous macrophage migration-inhibitory factor mediated by CXCR2 in a mouse model of myocardial ischemia/reperfusion. *Arterioscler Thromb Vasc Biol* **33**, 2180-2186

50. Koga, K., Kenessey, A., Powell, S. R., Sison, C. P., Miller, E. J., and Ojamaa, K. (2011) Macrophage migration inhibitory factor provides cardioprotection during ischemia/reperfusion by reducing oxidative stress. *Antioxid Redox Signal* **14**, 1191-1202

51. Alampour-Rajabi, S., El Bounkari, O., Rot, A., Muller-Newen, G., Bachelerie, F., Gawaz, M., Weber, C., Schober, A., and Bernhagen, J. (2015) MIF interacts with CXCR7 to promote receptor internalization, ERK1/2 and ZAP-70 signaling, and lymphocyte chemotaxis. *FASEB J* **29**, 4497-4511

52. Weber, C., Kraemer, S., Drechsler, M., Lue, H., Koenen, R. R., Kapurniotu, A., Zernecke, A., and Bernhagen, J. (2008) Structural determinants of MIF functions in CXCR2-mediated inflammatory and atherogenic leukocyte recruitment. *Proc Natl Acad Sci U S A* **105**, 16278-16283

53. Tillmann, S., Bernhagen, J., and Noels, H. (2013) Arrest Functions of the MIF Ligand/Receptor Axes in Atherogenesis. *Front Immunol* **4**, 115

54. Binsky, I., Haran, M., Starlets, D., Gore, Y., Lantner, F., Harpaz, N., Leng, L., Goldenberg, D. M., Shvidel, L., Berrebi, A., Bucala, R., and Shachar, I. (2007) IL-8 secreted in a macrophage migration-inhibitory factor- and CD74-dependent manner regulates B cell chronic lymphocytic leukemia survival. *Proc Natl Acad Sci U S A* **104**, 13408-13413

55. Leng, L., Metz, C. N., Fang, Y., Xu, J., Donnelly, S., Baugh, J., Delohery, T., Chen, Y., Mitchell, R. A., and Bucala, R. (2003) MIF signal transduction initiated by binding to CD74. *J Exp Med* **197**, 1467-1476

56. Shi, X., Leng, L., Wang, T., Wang, W., Du, X., Li, J., McDonald, C., Chen, Z., Murphy, J. W., Lolis, E., Noble, P., Knudson, W., and Bucala, R. (2006) CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity* **25**, 595-606

57. Miller, E. J., Li, J., Leng, L., McDonald, C., Atsumi, T., Bucala, R., and Young, L. H. (2008) Macrophage migration inhibitory factor stimulates AMP-activated protein kinase in the ischaemic heart. *Nature* **451**, 578-582

58. Nasser, M. W., Raghuwanshi, S. K., Grant, D. J., Jala, V. R., Rajarathnam, K., and Richardson, R. M. (2009) Differential activation and regulation of CXCR1 and CXCR2 by CXCL8 monomer and dimer. *J Immunol* **183**, 3425-3432

59. Kraemer, S., Lue, H., Zernecke, A., Kapurniotu, A., Andreetto, E., Frank, R., Lennartz, B., Weber, C., and Bernhagen, J. (2011) MIF-chemokine receptor interactions in atherogenesis are dependent on an N-loop-based 2-site binding mechanism. *FASEB J* **25**, 894-906

60. Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J. L., Arenzana-Seisdedos, F., Schwartz, O., Heard, J. M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., and Moser, B. (1996) The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* **382**, 833-835

61. Rajasekaran, D., Groning, S., Schmitz, C., Zierow, S., Drucker, N., Bakou, M., Kohl, K., Mertens, A., Lue, H., Weber, C., Xiao, A., Luker, G., Kapurniotu, A., Lolis, E., and Bernhagen, J. (2016) Macrophage Migration Inhibitory Factor-CXCR4 Receptor Interactions: EVIDENCE FOR PARTIAL ALLOSTERIC AGONISM IN COMPARISON WITH CXCL12 CHEMOKINE. *J Biol Chem* **291**, 15881-15895

62. Lacy, M., Kontos, C., Brandhofer, M., Hille, K., Groning, S., Sinitski, D., Bourilhon, P., Rosenberg, E., Krammer, C., Thavayogarajah, T., Pantouris, G., Bakou, M., Weber, C., Lolis, E., Bernhagen, J., and Kapurniotu, A. (2018) Identification of an Arg-Leu-Arg tripeptide that contributes to the binding interface between the cytokine MIF and the chemokine receptor CXCR4. *Sci Rep* **8**, 5171

63. Schwartz, V., Lue, H., Kraemer, S., Korbiel, J., Krohn, R., Ohl, K., Bucala, R., Weber, C., and Bernhagen, J. (2009) A functional heteromeric MIF receptor formed by CD74 and CXCR4. *FEBS Lett* **583**, 2749-2757

64. Klasen, C., Ohl, K., Sternkopf, M., Shachar, I., Schmitz, C., Heussen, N., Hobeika, E., Levit-Zerdoun, E., Tenbrock, K., Reth, M., Bernhagen, J., and El Bounkari, O. (2014) MIF promotes B cell chemotaxis through the receptors CXCR4 and CD74 and ZAP-70 signaling. *J Immunol* **192**, 5273-5284

65. Odh, G., Hindemith, A., Rosengren, A. M., Rosengren, E., and Rorsman, H. (1993) Isolation of a new tautomerase monitored by the conversion of D-dopachrome to 5,6-dihydroxyindole. *Biochem Biophys Res Commun* **197**, 619-624

66. Sugimoto, H., Taniguchi, M., Nakagawa, A., Tanaka, I., Suzuki, M., and Nishihira, J. (1999) Crystal structure of human D-dopachrome tautomerase, a homologue of macrophage migration inhibitory factor, at 1.54 A resolution. *Biochemistry* **38**, 3268-3279

67. Qi, D., Atsina, K., Qu, L., Hu, X., Wu, X., Xu, B., Piecychna, M., Leng, L., Fingerle-Rowson, G., Zhang, J., Bucala, R., and Young, L. H. (2014) The vestigial enzyme D-dopachrome tautomerase protects the heart against ischemic injury. *J Clin Invest* **124**, 3540-3550

68. Merk, M., Mitchell, R. A., Endres, S., and Bucala, R. (2012) D-dopachrome tautomerase (D-DT or MIF-2): doubling the MIF cytokine family. *Cytokine* **59**, 10-17

69. Sparkes, A., De Baetselier, P., Roelants, K., De Trez, C., Magez, S., Van Ginderachter, J. A., Raes, G., Bucala, R., and Stijlemans, B. (2017) The non-mammalian MIF superfamily. *Immunobiol* **222**, 473-482

70. Michelet, C., Danchin, E. G. J., Jaouannet, M., Bernhagen, J., Panstruga, R., Kogel, K. H., Keller, H., and Coustau, C. (2019) Cross-Kingdom Analysis of Diversity, Evolutionary History, and Site Selection within the Eukaryotic Macrophage Migration Inhibitory Factor Superfamily. *Genes* **10**

71. Panstruga, R., Baumgarten, K., and Bernhagen, J. (2015) Phylogeny and evolution of plant macrophage migration inhibitory factor/D-dopachrome tautomerase-like proteins. *BMC Evol Biol* **15**, 64

72. Sinitski, D., Gruner, K., Brandhofer, M., Kontos, C., Winkler, P., Reinstadler, A., Bourilhon, P., Xiao, Z., Cool, R., Kapurniotu, A., Dekker, F. J., Panstruga, R., and Bernhagen, J. (2020) Cross-kingdom mimicry of the receptor signaling and leukocyte recruitment activity of a human cytokine by its plant orthologs. *J Biol Chem* **295**, 850-867

73. Schindler, L., Dickerhof, N., Hampton, M. B., and Bernhagen, J. (2018) Post-translational regulation of macrophage migration inhibitory factor: Basis for functional fine-tuning. *Redox Biol* **15**, 135-142

74. Watarai, H., Nozawa, R., Tokunaga, A., Yuyama, N., Tomas, M., Hinohara, A., Ishizaka, K., and Ishii, Y. (2000) Posttranslational modification of the glycosylation inhibiting factor (GIF) gene product generates bioactive GIF. *Proc Natl Acad Sci U S A* **97**, 13251-13256

75. Nguyen, M. T., Beck, J., Lue, H., Funfzig, H., Kleemann, R., Koolwijk, P., Kapurniotu, A., and Bernhagen, J. (2003) A 16-residue peptide fragment of macrophage migration inhibitory factor, MIF-(50-65), exhibits redox activity and has MIF-like biological functions. *J Biol Chem* **278**, 33654-33671

76. Luedike, P., Hendgen-Cotta, U. B., Sobierajski, J., Totzeck, M., Reeh, M., Dewor, M., Lue, H., Krisp, C., Wolters, D., Kelm, M., Bernhagen, J., and Rassaf, T. (2012) Cardioprotection through S-nitros(yl)ation of macrophage migration inhibitory factor. *Circulation* **125**, 1880-1889

77. Pohl, J., Hendgen-Cotta, U. B., Rammos, C., Luedike, P., Mull, E., Stoppe, C., Julicher, K., Lue, H., Merx, M. W., Kelm, M., Bernhagen, J., and Rassaf, T. (2016) Targeted intracellular accumulation of macrophage migration inhibitory factor in the reperfused heart mediates cardioprotection. *Thromb Haemost* **115**, 200-212

78. Pantouris, G., Syed, M. A., Fan, C., Rajasekaran, D., Cho, T. Y., Rosenberg, E. M., Jr., Bucala, R., Bhandari, V., and Lolis, E. J. (2015) An Analysis of MIF Structural Features that Control Functional Activation of CD74. *Chem Biol* **22**, 1197-1205

79. Winner, M., Meier, J., Zierow, S., Rendon, B. E., Crichlow, G. V., Riggs, R., Bucala, R., Leng, L., Smith, N., Lolis, E., Trent, J. O., and Mitchell, R. A. (2008) A novel, macrophage migration inhibitory factor suicide substrate inhibits motility and growth of lung cancer cells. *Cancer Res* **68**, 7253-7257

80. Ouertatani-Sakouhi, H., El-Turk, F., Fauvet, B., Roger, T., Le Roy, D., Karpinar, D. P., Leng, L., Bucala, R., Zweckstetter, M., Calandra, T., and Lashuel, H. A. (2009) A new class of isothiocyanate-based irreversible inhibitors of macrophage migration inhibitory factor. *Biochemistry* **48**, 9858-9870

81. Brown, K. K., Blaikie, F. H., Smith, R. A., Tyndall, J. D., Lue, H., Bernhagen, J., Winterbourn, C. C., and Hampton, M. B. (2009) Direct modification of the proinflammatory cytokine macrophage migration inhibitory factor by dietary isothiocyanates. *J Biol Chem* **284**, 32425-32433

82. Cross, J. V., Rady, J. M., Foss, F. W., Lyons, C. E., Macdonald, T. L., and Templeton, D. J. (2009) Nutrient isothiocyanates covalently modify and inhibit the inflammatory cytokine macrophage migration inhibitory factor (MIF). *Biochem J* **423**, 315-321

83. Spencer, E. S., Dale, E. J., Gommans, A. L., Rutledge, M. T., Vo, C. T., Nakatani, Y., Gamble, A. B., Smith, R. A., Wilbanks, S. M., Hampton, M. B., and Tyndall, J. D. (2015) Multiple binding modes of isothiocyanates that inhibit macrophage migration inhibitory factor. *Eur J Med Chem* **93**, 501-510

84. Tyndall, J. D., Lue, H., Rutledge, M. T., Bernhagen, J., Hampton, M. B., and Wilbanks, S. M. (2012) Macrophage migration inhibitory factor covalently complexed with phenethyl isothiocyanate. *Acta Crystallogr. Section F Struct Biol Cryst Commun* **68**, 999-1002

85. Dickerhof, N., Magon, N. J., Tyndall, J. D., Kettle, A. J., and Hampton, M. B. (2014) Potent inhibition of macrophage migration inhibitory factor (MIF) by myeloperoxidase-dependent oxidation of epicatechins. *Biochem J* **462**, 303-314

86. Dickerhof, N., Schindler, L., Bernhagen, J., Kettle, A. J., and Hampton, M. B. (2015) Macrophage migration inhibitory factor (MIF) is rendered enzymatically inactive by myeloperoxidase-derived oxidants but retains its immunomodulatory function. *Free Radic Biol Med* **89**, 498-511

87. Borregaard, N., and Cowland, J. B. (1997) Granules of the human neutrophilic polymorphonuclear leukocyte. *Blood* **89**, 3503-3521

88. Cartwright, G. E., Athens, J. W., and Wintrobe, M. M. (1964) The kinetics of granulopoiesis in normal man. *Blood* **24**, 780-803

89. Bainton, D. F., Ullyot, J. L., and Farquhar, M. G. (1971) The development of neutrophilic polymorphonuclear leukocytes in human bone marrow. *J Exp Med* **134**, 907-934

90. Martin, L. B., Kita, H., Leiferman, K. M., and Gleich, G. J. (1996) Eosinophils in allergy: role in disease, degranulation, and cytokines. *Int Arch Allergy Immunol* **109**, 207-215

91. Wen, T., and Rothenberg, M. E. (2016) The Regulatory Function of Eosinophils. *Microbiol Spectr* **4**

92. Eberle, J. U., and Voehringer, D. (2016) Role of basophils in protective immunity to parasitic infections. *Semin Immunopathol* **38**, 605-613

93. Jagannathan-Bogdan, M., and Zon, L. I. (2013) Hematopoiesis. *Development* **140**, 2463-2467

94. Dancey, J. T., Deubelbeiss, K. A., Harker, L. A., and Finch, C. A. (1976) Neutrophil kinetics in man. *J Clin Invest* **58**, 705-715

95. Summers, C., Rankin, S. M., Condliffe, A. M., Singh, N., Peters, A. M., and Chilvers, E. R. (2010) Neutrophil kinetics in health and disease. *Trends Immunol* **31**, 318-324

96. Nakajima, H., and Ihle, J. N. (2001) Granulocyte colony-stimulating factor regulates myeloid differentiation through CCAAT/enhancer-binding protein epsilon. *Blood* **98**, 897-905

97. Hidalgo, A., Chilvers, E. R., Summers, C., and Koenderman, L. (2019) The Neutrophil Life Cycle. *Trends Immunol* **40**, 584-597

98. Casanova-Acebes, M., Pitaval, C., Weiss, L. A., Nombela-Arrieta, C., Chevre, R., N, A. G., Kunisaki, Y., Zhang, D., van Rooijen, N., Silberstein, L. E., Weber, C., Nagasawa, T., Frenette, P. S., Castrillo, A., and Hidalgo, A. (2013) Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* **153**, 1025-1035

99. Rankin, S. M. (2010) The bone marrow: a site of neutrophil clearance. *J Leukoc Biol* **88**, 241-251

100. Furze, R. C., and Rankin, S. M. (2008) The role of the bone marrow in neutrophil clearance under homeostatic conditions in the mouse. *FASEB J* **22**, 3111-3119

101. Suratt, B. T., Young, S. K., Lieber, J., Nick, J. A., Henson, P. M., and Worthen, G. S. (2001) Neutrophil maturation and activation determine anatomic site of clearance from circulation. *Am J Physiol Lung Cell Mol Physiol* **281**, L913-921

102. Pillay, J., den Braber, I., Vrisekoop, N., Kwast, L. M., de Boer, R. J., Borghans, J. A., Tesselaar, K., and Koenderman, L. (2010) In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* **116**, 625-627

103. Kennedy, A. D., and DeLeo, F. R. (2009) Neutrophil apoptosis and the resolution of infection. *Immunol Res* **43**, 25-61

104. von Gunten, S., and Simon, H. U. (2007) Autophagic-like cell death in neutrophils induced by autoantibodies. *Autophagy* **3**, 67-68

105. Mitroulis, I., Kourtzelis, I., Kambas, K., Rafail, S., Chrysanthopoulou, A., Speletas, M., and Ritis, K. (2010) Regulation of the autophagic machinery in human neutrophils. *Eur J Immunol* **40**, 1461-1472

106. Sampson, A. P. (2000) The role of eosinophils and neutrophils in inflammation. *Clin Exp Allergy* **30 Suppl 1**, 22-27

107. Soehnlein, O., Steffens, S., Hidalgo, A., and Weber, C. (2017) Neutrophils as protagonists and targets in chronic inflammation. *Nat Rev Immunol* **17**, 248-261

108. Engler, R. L., Dahlgren, M. D., Peterson, M. A., Dobbs, A., and Schmid-Schonbein, G. W. (1986) Accumulation of polymorphonuclear leukocytes during 3-h experimental myocardial ischemia. *Am J Physiol* **251**, H93-100

109. Dreyer, W. J., Michael, L. H., West, M. S., Smith, C. W., Rothlein, R., Rossen, R. D., Anderson, D. C., and Entman, M. L. (1991) Neutrophil accumulation in ischemic canine myocardium. Insights into time course, distribution, and mechanism of localization during early reperfusion. *Circulation* **84**, 400-411

110. Segal, A. W. (2005) How neutrophils kill microbes. *Annu Rev Immunol* **23**, 197-223

111. Silvestre-Roig, C., Braster, Q., Ortega-Gomez, A., and Soehnlein, O. (2020) Neutrophils as regulators of cardiovascular inflammation. *Nat Rev. Cardiol* **17**, 327-340

112. Kolaczkowska, E., and Kubes, P. (2013) Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* **13**, 159-175

113. Yang, D., de la Rosa, G., Tewary, P., and Oppenheim, J. J. (2009) Alarmins link neutrophils and dendritic cells. *Trends Immunol* **30**, 531-537

114. Charmoy, M., Brunner-Agten, S., Aebischer, D., Auderset, F., Launois, P., Milon, G., Proudfoot, A. E., and Tacchini-Cottier, F. (2010) Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice. *PLoS Pathog* **6**, e1000755

115. Soehnlein, O., Zernecke, A., Eriksson, E. E., Rothfuchs, A. G., Pham, C. T., Herwald, H., Bidzhekov, K., Rottenberg, M. E., Weber, C., and Lindbom, L. (2008) Neutrophil secretion products pave the way for inflammatory monocytes. *Blood* **112**, 1461-1471

116. Zhou, J., Stohlman, S. A., Hinton, D. R., and Marten, N. W. (2003) Neutrophils promote mononuclear cell infiltration during viral-induced encephalitis. *J Immunol* **170**, 3331-3336

117. Soehnlein, O., Weber, C., and Lindbom, L. (2009) Neutrophil granule proteins tune monocytic cell function. *Trends Immunol* **30**, 538-546

118. Sporri, R., Joller, N., Hilbi, H., and Oxenius, A. (2008) A novel role for neutrophils as critical activators of NK cells. *J Immunol* **181**, 7121-7130

119. Scapini, P., Bazzoni, F., and Cassatella, M. A. (2008) Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. *Immunol Lett* **116**, 1-6

120. Takano, T., Azuma, N., Satoh, M., Toda, A., Hashida, Y., Satoh, R., and Hohdatsu, T. (2009) Neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF) produced by macrophages in cats infected with feline infectious peritonitis virus contribute to the pathogenesis of granulomatous lesions. *Arch Virol* **154**, 775-781

121. Maianski, N. A., Mul, F. P., van Buul, J. D., Roos, D., and Kuijpers, T. W. (2002) Granulocyte colony-stimulating factor inhibits the mitochondria-dependent activation of caspase-3 in neutrophils. *Blood* **99**, 672-679

122. Colotta, F., Re, F., Polentarutti, N., Sozzani, S., and Mantovani, A. (1992) Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. *Blood* **80**, 2012-2020

123. Lee, A., Whyte, M. K., and Haslett, C. (1993) Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J Leukoc Biol* **54**, 283-288

124. Romson, J. L., Hook, B. G., Kunkel, S. L., Abrams, G. D., Schork, M. A., and Lucchesi, B. R. (1983) Reduction of the extent of ischemic myocardial injury by neutrophil depletion in the dog. *Circulation* **67**, 1016-1023

125. Jolly, S. R., Kane, W. J., Hook, B. G., Abrams, G. D., Kunkel, S. L., and Lucchesi, B. R. (1986) Reduction of myocardial infarct size by neutrophil depletion: effect of duration of occlusion. *Am Heart J* **112**, 682-690

126. Ma, X. L., Weyrich, A. S., Lefer, D. J., and Lefer, A. M. (1993) Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium. *Circ Res* **72**, 403-412

127. Tsao, P. S., Ma, X. L., and Lefer, A. M. (1992) Activated neutrophils aggravate endothelial dysfunction after reperfusion of the ischemic feline myocardium. *Am Heart J* **123**, 1464-1471

128. Siminiak, T., Flores, N. A., and Sheridan, D. J. (1995) Neutrophil interactions with endothelium and platelets: possible role in the development of cardiovascular injury. *Eur Heart J* **16**, 160-170

129. Kukielka, G. L., Smith, C. W., LaRosa, G. J., Manning, A. M., Mendoza, L. H., Daly, T. J., Hughes, B. J., Youker, K. A., Hawkins, H. K., Michael, L. H., and et al. (1995) Interleukin-8 gene induction in the myocardium after ischemia and reperfusion in vivo. *J Clin Invest* **95**, 89-103

130. Chaikijurajai, T., and Tang, W. H. W. (2020) Myeloperoxidase: a potential therapeutic target for coronary artery disease. *Expert Opin Ther Targets* **7**, 1-11

131. Horckmans, M., Ring, L., Duchene, J., Santovito, D., Schloss, M. J., Drechsler, M., Weber, C., Soehnlein, O., and Steffens, S. (2017) Neutrophils orchestrate post-myocardial infarction healing by polarizing macrophages towards a reparative phenotype. *Eur Heart J* **38**, 187-197

132. Ferraro, B., Leoni, G., Hinkel, R., Ormanns, S., Paulin, N., Ortega-Gomez, A., Viola, J. R., de Jong, R., Bongiovanni, D., Bozoglu, T., Maas, S. L., D'Amico, M., Kessler, T., Zeller, T., Hristov, M., Reutelingsperger, C., Sager, H. B., Doring, Y., Nahrendorf, M., Kupatt, C., and Soehnlein, O. (2019) Pro-Angiogenic Macrophage Phenotype to Promote Myocardial Repair. *J Am Coll Cardio* **73**, 2990-3002

133. Savill, J. S., Wyllie, A. H., Henson, J. E., Walport, M. J., Henson, P. M., and Haslett, C. (1989) Macrophage phagocytosis of aging neutrophils in inflammation. Programmed cell death in the neutrophil leads to its recognition by macrophages. *Journal Cin Invest* **83**, 865-875

134. Payne, C. M., Glasser, L., Tischler, M. E., Wyckoff, D., Cromey, D., Fiederlein, R., and Bohnert, O. (1994) Programmed cell death of the normal human neutrophil: an in vitro model of senescence. *Microsc Res Tech* **28**, 327-344

135. Whyte, M. K., Meagher, L. C., MacDermot, J., and Haslett, C. (1993) Impairment of function in aging neutrophils is associated with apoptosis. *J Immunol* **150**, 5124-5134

136. Dransfield, I., Stocks, S. C., and Haslett, C. (1995) Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. *Blood* **85**, 3264-3273

137. Maianski, N. A., Maianski, A. N., Kuijpers, T. W., and Roos, D. (2004) Apoptosis of neutrophils. *Acta Haemato* **111**, 56-66

138. Ashkenazi, A., and Dixit, V. M. (1998) Death receptors: signaling and modulation. *Science* **281**, 1305-1308

139. Cory, S., and Adams, J. M. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Can* **2**, 647-656

140. Tait, S. W., and Green, D. R. (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. *Nat Reviews Mol Cell Biol* **11**, 621-632

141. El Kebir, D., and Filep, J. G. (2010) Role of neutrophil apoptosis in the resolution of inflammation. *ScientificWorldJournal* **10**, 1731-1748

142. El Kebir, D., and Filep, J. G. (2013) Modulation of Neutrophil Apoptosis and the Resolution of Inflammation through β2 Integrins. *Front Immunol* **4**, 60

143. Nathan, C., and Ding, A. (2010) Nonresolving inflammation. *Cell* **140**, 871-882

144. McKeon, D. J., Condliffe, A. M., Cowburn, A. S., Cadwallader, K. C., Farahi, N., Bilton, D., and Chilvers, E. R. (2008) Prolonged survival of neutrophils from patients with Delta F508 CFTR mutations. *Thorax* **63**, 660-661

145. Ertel, W., Keel, M., Infanger, M., Ungethum, U., Steckholzer, U., and Trentz, O. (1998) Circulating mediators in serum of injured patients with septic complications inhibit neutrophil apoptosis through up-regulation of protein-tyrosine phosphorylation. *J Trauma* **44**, 767-775; discussion 775-766

146. Jia, S. H., Li, Y., Parodo, J., Kapus, A., Fan, L., Rotstein, O. D., and Marshall, J. C. (2004) Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. *J Clin Invest* **113**, 1318-1327

147. Garlichs, C. D., Eskafi, S., Cicha, I., Schmeisser, A., Walzog, B., Raaz, D., Stumpf, C., Yilmaz, A., Bremer, J., Ludwig, J., and Daniel, W. G. (2004) Delay of neutrophil apoptosis in acute coronary syndromes. *J Leuko Biol* **75**, 828-835

148. Wong, S. H., Francis, N., Chahal, H., Raza, K., Salmon, M., Scheel-Toellner, D., and Lord, J. M. (2009) Lactoferrin is a survival factor for neutrophils in rheumatoid synovial fluid. *Rheumatology (Oxford)* **48**, 39-44

149. Bratton, D. L., and Henson, P. M. (2011) Neutrophil clearance: when the party is over, clean-up begins. *Trends Immunol* **32**, 350-357

150. Walmsley, S. R., Print, C., Farahi, N., Peyssonnaux, C., Johnson, R. S., Cramer, T., Sobolewski, A., Condliffe, A. M., Cowburn, A. S., Johnson, N., and Chilvers, E. R. (2005) Hypoxia-induced neutrophil survival is mediated by HIF-1alpha-dependent NF-kappaB activity. *J Exp Med* **201**, 105-115

151. Lindemans, C. A., Coffer, P. J., Schellens, I. M., de Graaff, P. M., Kimpen, J. L., and Koenderman, L. (2006) Respiratory syncytial virus inhibits granulocyte apoptosis through a phosphatidylinositol 3-kinase and NF-kappaB-dependent mechanism. *J Immunol* **176**, 5529-5537

152. van Raam, B. J., Drewniak, A., Groenewold, V., van den Berg, T. K., and Kuijpers, T. W. (2008) Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. *Blood* **112**, 2046-2054

153. Klein, J. B., Rane, M. J., Scherzer, J. A., Coxon, P. Y., Kettritz, R., Mathiesen, J. M., Buridi, A., and McLeish, K. R. (2000) Granulocyte-macrophage colony-stimulating factor delays neutrophil constitutive apoptosis through phosphoinositide 3-kinase and extracellular signal-regulated kinase pathways. *J Immunol* **164**, 4286-4291

154. van den Berg, J. M., Weyer, S., Weening, J. J., Roos, D., and Kuijpers, T. W. (2001) Divergent effects of tumor necrosis factor alpha on apoptosis of human neutrophils. *J Leuko Biol* **69**, 467-473

155. Murray, J., Barbara, J. A., Dunkley, S. A., Lopez, A. F., Van Ostade, X., Condliffe, A. M., Dransfield, I., Haslett, C., and Chilvers, E. R. (1997) Regulation of neutrophil apoptosis by tumor necrosis factor-alpha: requirement for TNFR55 and TNFR75 for induction of apoptosis in vitro. *Blood* **90**, 2772-2783

156. Ottonello, L., Cutolo, M., Frumento, G., Arduino, N., Bertolotto, M., Mancini, M., Sottofattori, E., and Dallegri, F. (2002) Synovial fluid from patients with rheumatoid arthritis inhibits neutrophil apoptosis: role of adenosine and proinflammatory cytokines. *Rheumatology (Oxford)* **41**, 1249-1260

157. Kettritz, R., Gaido, M. L., Haller, H., Luft, F. C., Jennette, C. J., and Falk, R. J. (1998) Interleukin-8 delays spontaneous and tumor necrosis factor-alpha-mediated apoptosis of human neutrophils. *Kidney Int* **53**, 84-91

158. Nguyen, G. T., Green, E. R., and Mecsas, J. (2017) Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Front Cell Infect Microbiol* **7**, 373

159. Chanock, S. J., el Benna, J., Smith, R. M., and Babior, B. M. (1994) The respiratory burst oxidase. *J Biol Chem* **269**, 24519-24522

160. Wientjes, F. B., and Segal, A. W. (1995) NADPH oxidase and the respiratory burst. *Semin Cell Biol* **6**, 357-365

161. Weiss, S. J., and LoBuglio, A. F. (1982) Phagocyte-generated oxygen metabolites and cellular injury. *Lab Invest* **47**, 5-18

162. Kettle, A. J., and Winterbourn, C. C. (1997) Myeloperoxidase: a key regulator of neutrophil oxidant production. *Redox Rep* **3**, 3-15

163. Harrison, J. E., and Schultz, J. (1976) Studies on the chlorinating activity of myeloperoxidase. *J Biol Chem* **251**, 1371-1374

164. Winterbourn, C. C., Hampton, M. B., Livesey, J. H., and Kettle, A. J. (2006) Modeling the reactions of superoxide and myeloperoxidase in the neutrophil phagosome: implications for microbial killing. *J Biol Chem* **281**, 39860-39869

165. Hawkins, C. L., Pattison, D. I., and Davies, M. J. (2003) Hypochlorite-induced oxidation of amino acids, peptides and proteins. *Amino Acids* **25**, 259-274

166. Miralda, I., Uriarte, S. M., and McLeish, K. R. (2017) Multiple Phenotypic Changes Define Neutrophil Priming. *Front Cell Infect Microbiol* **7**, 217

167. Hallett, M. B., and Lloyds, D. (1995) Neutrophil priming: the cellular signals that say 'amber' but not 'green'. *Immunol Today* **16**, 264-268

168. Elbim, C., Chollet-Martin, S., Bailly, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1993) Priming of polymorphonuclear neutrophils by tumor necrosis factor alpha in whole blood: identification of two polymorphonuclear neutrophil subpopulations in response to formyl-peptides. *Blood* **82**, 633-640

169. Elbim, C., Bailly, S., Chollet-Martin, S., Hakim, J., and Gougerot-Pocidalo, M. A. (1994) Differential priming effects of proinflammatory cytokines on human neutrophil oxidative burst in response to bacterial N-formyl peptides. *Infect Immun* **62**, 2195-2201

170. Fuchs, T. A., Abed, U., Goosmann, C., Hurwitz, R., Schulze, I., Wahn, V., Weinrauch, Y., Brinkmann, V., and Zychlinsky, A. (2007) Novel cell death program leads to neutrophil extracellular traps. *J Cell Biol* **176**, 231-241

171. Daniels, R. H., Finnen, M. J., Hill, M. E., and Lackie, J. M. (1992) Recombinant human monocyte IL-8 primes NADPH-oxidase and phospholipase A2 activation in human neutrophils. *Immunology* **75**, 157-163

172. Brinkmann, V., Reichard, U., Goosmann, C., Fauler, B., Uhlemann, Y., Weiss, D. S., Weinrauch, Y., and Zychlinsky, A. (2004) Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535

173. Urban, C. F., Reichard, U., Brinkmann, V., and Zychlinsky, A. (2006) Neutrophil extracellular traps capture and kill Candida albicans yeast and hyphal forms. *Cell Microbiol* **8**, 668-676

174. Papayannopoulos, V. (2018) Neutrophil extracellular traps in immunity and disease. *Nat Rev Immunol* **18**, 134-147

175. Parker, H., Dragunow, M., Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (2012) Requirements for NADPH oxidase and myeloperoxidase in neutrophil extracellular trap formation differ depending on the stimulus. *J Leuko Biol* **92**, 841-849

176. Kenny, E. F., Herzig, A., Kruger, R., Muth, A., Mondal, S., Thompson, P. R., Brinkmann, V., Bernuth, H. V., and Zychlinsky, A. (2017) Diverse stimuli engage different neutrophil extracellular trap pathways. *eLife* **6**

177. Keshari, R. S., Jyoti, A., Dubey, M., Kothari, N., Kohli, M., Bogra, J., Barthwal, M. K., and Dikshit, M. (2012) Cytokines induced neutrophil extracellular traps formation: implication for the inflammatory disease condition. *PloS one* **7**, e48111

178. Alfaro, C., Teijeira, A., Onate, C., Perez, G., Sanmamed, M. F., Andueza, M. P., Alignani, D., Labiano, S., Azpilikueta, A., Rodriguez-Paulete, A., Garasa, S., Fusco, J. P., Aznar, A., Inoges, S., De Pizzol, M., Allegretti, M., Medina-Echeverz, J., Berraondo, P., Perez-Gracia, J. L., and Melero, I. (2016) Tumor-Produced Interleukin-8 Attracts Human Myeloid-Derived Suppressor Cells and Elicits Extrusion of Neutrophil Extracellular Traps (NETs). *Clin Cancer Res* **22**, 3924-3936

179. Cacciotto, C., Cubeddu, T., Addis, M. F., Anfossi, A. G., Tedde, V., Tore, G., Carta, T., Rocca, S., Chessa, B., Pittau, M., and Alberti, A. (2016) Mycoplasma lipoproteins are major determinants of neutrophil extracellular trap formation. *Cell Microbiol* **18**, 1751-1762

180. Remijsen, Q., Vanden Berghe, T., Wirawan, E., Asselbergh, B., Parthoens, E., De Rycke, R., Noppen, S., Delforge, M., Willems, J., and Vandenabeele, P. (2011) Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. *Cell Res* **21**, 290-304

181. Clark, S. R., Ma, A. C., Tavener, S. A., McDonald, B., Goodarzi, Z., Kelly, M. M., Patel, K. D., Chakrabarti, S., McAvoy, E., Sinclair, G. D., Keys, E. M., Allen-Vercoe, E., Devinney, R., Doig, C. J., Green, F. H., and Kubes, P. (2007) Platelet TLR4 activates neutrophil extracellular traps to ensnare bacteria in septic blood. *Nat Med* **13**, 463-469

182. Doring, Y., Libby, P., and Soehnlein, O. (2020) Neutrophil Extracellular Traps Participate in Cardiovascular Diseases: Recent Experimental and Clinical Insights. *Circ Res* **126**, 1228-1241

183. Schauer, C., Janko, C., Munoz, L. E., Zhao, Y., Kienhofer, D., Frey, B., Lell, M., Manger, B., Rech, J., Naschberger, E., Holmdahl, R., Krenn, V., Harrer, T., Jeremic, I., Bilyy, R., Schett, G., Hoffmann, M., and Herrmann, M. (2014) Aggregated neutrophil extracellular traps limit inflammation by degrading cytokines and chemokines. *Nat Med* **20**, 511-517

184. de Buhr, N., Stehr, M., Neumann, A., Naim, H. Y., Valentin-Weigand, P., von Kockritz-Blickwede, M., and Baums, C. G. (2015) Identification of a novel DNase of Streptococcus suis (EndAsuis) important for neutrophil extracellular trap degradation during exponential growth. *Microbiology* **161**, 838-850

185. Buchanan, J. T., Simpson, A. J., Aziz, R. K., Liu, G. Y., Kristian, S. A., Kotb, M., Feramisco, J., and Nizet, V. (2006) DNase expression allows the pathogen group A Streptococcus to escape killing in neutrophil extracellular traps. *Curr Biol* **16**, 396-400

186. Berends, E. T., Horswill, A. R., Haste, N. M., Monestier, M., Nizet, V., and von Kockritz-Blickwede, M. (2010) Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. *J Innate Immun* **2**, 576-586

187. Lee, K. H., Kronbichler, A., Park, D. D., Park, Y., Moon, H., Kim, H., Choi, J. H., Choi, Y., Shim, S., Lyu, I. S., Yun, B. H., Han, Y., Lee, D., Lee, S. Y., Yoo, B. H., Lee, K. H., Kim, T. L., Kim, H., Shim, J. S., Nam, W., So, H., Choi, S., Lee, S., and Shin, J. I. (2017) Neutrophil extracellular traps (NETs) in autoimmune diseases: A comprehensive review. *Autoimmun Rev* **16**, 1160-1173

188. Kang, I., and Bucala, R. (2019) The immunobiology of MIF: function, genetics and prospects for precision medicine. *Nat Rev Rheum* **15**, 427-437

189. Santos, L. L., Fan, H., Hall, P., Ngo, D., Mackay, C. R., Fingerle-Rowson, G., Bucala, R., Hickey, M. J., and Morand, E. F. (2011) Macrophage migration inhibitory factor regulates neutrophil chemotactic responses in inflammatory arthritis in mice. *Arthritis Rheum* **63**, 960-970

190. Pellowe, A. S., Sauler, M., Hou, Y., Merola, J., Liu, R., Calderon, B., Lauridsen, H. M., Harris, M. R., Leng, L., Zhang, Y., Tilstam, P. V., Pober, J. S., Bucala, R., Lee, P. J., and Gonzalez, A. L. (2019) Endothelial cell-secreted MIF reduces pericyte contractility and enhances neutrophil extravasation. *FASEB J* **33**, 2171-2186

191. Dwyer, M., Shan, Q., D'Ortona, S., Maurer, R., Mitchell, R., Olesen, H., Thiel, S., Huebner, J., and Gadjeva, M. (2014) Cystic fibrosis sputum DNA has NETosis characteristics and neutrophil extracellular trap release is regulated by macrophage migration-inhibitory factor. *J Innate Immun* **6**, 765-779

192. Baumann, R., Casaulta, C., Simon, D., Conus, S., Yousefi, S., and Simon, H. U. (2003) Macrophage migration inhibitory factor delays apoptosis in neutrophils by inhibiting the mitochondria-dependent death pathway. *FASEB J* **17**, 2221-2230

193. Daryadel, A., Grifone, R. F., Simon, H. U., and Yousefi, S. (2006) Apoptotic neutrophils release macrophage migration inhibitory factor upon stimulation with tumor necrosis factor-alpha. *J Biol Chem* **281**, 27653-27661

194. Roth, S., Solbach, W., and Laskay, T. (2016) IL-16 and MIF: messengers beyond neutrophil cell death. *Cell Death Dis* **7**, e2049

195. Schneider, C. A., Rasband, W. S., and Eliceiri, K. W. (2012) NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* **9**, 671-675

196. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682

197. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-254

198. Knox, W. E., and Pitt, B. M. (1957) Enzymic catalysis of the keto-enol tautomerization of phenylpyruvic acids. *J Biol Chem* **225**, 675-688

199. Varinelli, L., Caccia, D., Volpi, C. C., Caccia, C., De Bortoli, M., Taverna, E., Gualeni, A. V., Leoni, V., Gloghini, A., Manenti, G., and Bongarzone, I. (2015) 4-IPP, a selective MIF inhibitor, causes mitotic catastrophe in thyroid carcinomas. *Endocr Relat Cancer* **22**, 759-775

200. Brock, S. E., Rendon, B. E., Xin, D., Yaddanapudi, K., and Mitchell, R. A. (2014) MIF family members cooperatively inhibit p53 expression and activity. *PloS one* **9**, e99795

201. Chen, X., Lee, K. A., Ha, E. M., Lee, K. M., Seo, Y. Y., Choi, H. K., Kim, H. N., Kim, M. J., Cho, C. S., Lee, S. Y., Lee, W. J., and Yoon, J. (2011) A specific and sensitive method for detection of hypochlorous acid for the imaging of microbe-induced HOCl production. *Chem Commun (Camb)* **47**, 4373-4375

202. Albrett, A. M., Ashby, L. V., Dickerhof, N., Kettle, A. J., and Winterbourn, C. C. (2018) Heterogeneity of hypochlorous acid production in individual neutrophil phagosomes revealed by a rhodamine-based probe. *J Biol Chem* **293**, 15715-15724

203. Chen, X., Lee, K. A., Ren, X., Ryu, J. C., Kim, G., Ryu, J. H., Lee, W. J., and Yoon, J. (2016) Synthesis of a highly HOCl-selective fluorescent probe and its use for imaging HOCl in cells and organisms. *Nat Protoc* **11**, 1219-1228

204. Gupta, A. K., Hasler, P., Holzgreve, W., Gebhardt, S., and Hahn, S. (2005) Induction of neutrophil extracellular DNA lattices by placental microparticles and IL-8 and their presence in preeclampsia. *Hum Immunol* **66**, 1146-1154

205. Wang, Y., An, R., Umanah, G. K., Park, H., Nambiar, K., Eacker, S. M., Kim, B., Bao, L., Harraz, M. M., Chang, C., Chen, R., Wang, J. E., Kam, T. I., Jeong, J. S., Xie, Z., Neifert, S., Qian, J., Andrabi, S. A., Blackshaw, S., Zhu, H., Song, H., Ming, G. L., Dawson, V. L., and Dawson, T. M. (2016) A nuclease that mediates cell death induced by DNA damage and poly(ADP-ribose) polymerase-1. *Science* **354**

206. de Buhr, N., and von Köckritz-Blickwede, M. (2016) How Neutrophil Extracellular Traps Become Visible. *J Immunol Res* **2016**, 4604713

207. Van Weemen, B. K., and Schuurs, A. H. (1971) Immunoassay using antigen-enzyme conjugates. *FEBS Lett* **15**, 232-236

208. Engvall, E., and Perlmann, P. (1971) Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871-874

209. Sachpatzidis, A., Benton, B. K., Manfredi, J. P., Wang, H., Hamilton, A., Dohlman, H. G., and Lolis, E. (2003) Identification of allosteric peptide agonists of CXCR4. *J Biol Chem* **278**, 896-907

210. Brach, M. A., deVos, S., Gruss, H. J., and Herrmann, F. (1992) Prolongation of survival of human polymorphonuclear neutrophils by granulocyte-macrophage colony-stimulating factor is caused by inhibition of programmed cell death. *Blood* **80**, 2920-2924

211. Rajarathnam, K., Schnoor, M., Richardson, R. M., and Rajagopal, S. (2019) How do chemokines navigate neutrophils to the target site: Dissecting the structural mechanisms and signaling pathways. *Cell Signal* **54**, 69-80

212. White, J. R., Lee, J. M., Young, P. R., Hertzberg, R. P., Jurewicz, A. J., Chaikin, M. A., Widdowson, K., Foley, J. J., Martin, L. D., Griswold, D. E., and Sarau, H. M. (1998) Identification of a potent, selective non-peptide CXCR2 antagonist that inhibits interleukin-8-induced neutrophil migration. *J Biol Chem* **273**, 10095-10098

213. Karlsson, A., Nixon, J. B., and McPhail, L. C. (2000) Phorbol myristate acetate induces neutrophil NADPH-oxidase activity by two separate signal transduction pathways: dependent or independent of phosphatidylinositol 3-kinase. *J Leukoc Biol* **67**, 396-404

214. Caudrillier, A., Kessenbrock, K., Gilliss, B. M., Nguyen, J. X., Marques, M. B., Monestier, M., Toy, P., Werb, Z., and Looney, M. R. (2012) Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J Clin Invest* **122**, 2661-2671

215. Pressler, T. (2008) Review of recombinant human deoxyribonuclease (rhDNase) in the management of patients with cystic fibrosis. *Biologics* **2**, 611-617

216. Martínez-Alemán, S. R., Campos-García, L., Palma-Nicolas, J. P., Hernández-Bello, R., González, G. M., and Sánchez-González, A. (2017) Understanding the Entanglement: Neutrophil Extracellular Traps (NETs) in Cystic Fibrosis. *Front Cell Infect Microbiol* **7**, 104

217. Hausenloy, D. J., and Yellon, D. M. (2013) Myocardial ischemia-reperfusion injury: a neglected therapeutic target. *J Clin Invest* **123**, 92-100

218. Vinten-Johansen, J. (2004) Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. *Cardiovasc Res* **61**, 481-497

219. Ley, K., Laudanna, C., Cybulsky, M. I., and Nourshargh, S. (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* **7**, 678-689

220. Silva, M. T. (2010) When two is better than one: macrophages and neutrophils work in concert in innate immunity as complementary and cooperative partners of a myeloid phagocyte system. *J Leukoc Biol* **87**, 93-106

221. Wright, H. L., Moots, R. J., Bucknall, R. C., and Edwards, S. W. (2010) Neutrophil function in inflammation and inflammatory diseases. *Rheumatology* **49**, 1618-1631

222. Amulic, B., Cazalet, C., Hayes, G. L., Metzler, K. D., and Zychlinsky, A. (2012) Neutrophil function: from mechanisms to disease. *Annual Rev Immunol* **30**, 459-489

223. Schinagl, A., Thiele, M., Douillard, P., Volkel, D., Kenner, L., Kazemi, Z., Freissmuth, M., Scheiflinger, F., and Kerschbaumer, R. J. (2016) Oxidized macrophage migration inhibitory factor is a potential new tissue marker and drug target in cancer. *Oncotarget* **7**, 73486-73496

224. Apostolakis, S., Vogiatzi, K., Amanatidou, V., and Spandidos, D. A. (2009) Interleukin 8 and cardiovascular disease. *Cardiovasc Res* **84**, 353-360

225. Kany, S., Vollrath, J. T., and Relja, B. (2019) Cytokines in Inflammatory Disease. *Int J Mol Sci* **20**

226. Biffl, W. L., Moore, E. E., Moore, F. A., and Barnett, C. C., Jr. (1995) Interleukin-6 suppression of neutrophil apoptosis is neutrophil concentration dependent. *J Leukoc Biol* **58**, 582-584

227. Doring, Y., Drechsler, M., Soehnlein, O., and Weber, C. (2015) Neutrophils in atherosclerosis: from mice to man. *Arterioscler Thromb Vasc Biol* **35**, 288-295

228. Drechsler, M., Megens, R. T., van Zandvoort, M., Weber, C., and Soehnlein, O. (2010) Hyperlipidemia-triggered neutrophilia promotes early atherosclerosis. *Circulation* **122**, 1837-1845

229. Khera, N., and Rajput, S. (2017) Therapeutic Potential of Small Molecule Inhibitors. *J Cell Biochem* **118**, 959-961

230. Vethanayagam, R. R., Almyroudis, N. G., Grimm, M. J., Lewandowski, D. C., Pham, C. T., Blackwell, T. S., Petraitiene, R., Petraitis, V., Walsh, T. J., Urban, C. F., and Segal, B. H. (2011) Role of NADPH oxidase versus neutrophil proteases in antimicrobial host defense. *PloS one* **6**, e28149

231. Ellson, C. D., Davidson, K., Ferguson, G. J., O'Connor, R., Stephens, L. R., and Hawkins, P. T. (2006) Neutrophils from p40phox-/- mice exhibit severe defects in NADPH oxidase regulation and oxidant-dependent bacterial killing. *J Exp Med* **203**, 1927-1937

232. Giustarini, D., Dalle-Donne, I., Tsikas, D., and Rossi, R. (2009) Oxidative stress and human diseases: Origin, link, measurement, mechanisms, and biomarkers. *Crit Rev Clin Lab Sci* **46**, 241-281

233. Lobo, V., Patil, A., Phatak, A., and Chandra, N. (2010) Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacogn Rev* **4**, 118-126

234. Ighodaro, O. M., and Akinloye, O. A. (2018) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid. *Alexandria J Med* **54**, 287-293

235. Condliffe, A. M., Kitchen, E., and Chilvers, E. R. (1998) Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci (Lond)* **94**, 461-471

236. Genestra, M. (2007) Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Cell Signal* **19**, 1807-1819

237. Vogt, K. L., Summers, C., Chilvers, E. R., and Condliffe, A. M. (2018) Priming and de-priming of neutrophil responses in vitro and in vivo. *Eur J Clin Invest* **48**, e12967

238. Jones, S. A., Wolf, M., Qin, S., Mackay, C. R., and Baggiolini, M. (1996) Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. *Proc Natl Acad Sci U S A* **93**, 6682-6686

239. Moser, B., Dewald, B., Barella, L., Schumacher, C., Baggiolini, M., and Clark-Lewis, I. (1993) Interleukin-8 antagonists generated by N-terminal modification. *J Biol Chem* **268**, 7125-7128

240. Hampton, M. B., Kettle, A. J., and Winterbourn, C. C. (1998) Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. *Blood* **92**, 3007-3017

241. Winterbourn, C. C., and Kettle, A. J. (2013) Redox reactions and microbial killing in the neutrophil phagosome. *Antiox Redox Signal* **18**, 642-660

242. Klebanoff, S. J., Kettle, A. J., Rosen, H., Winterbourn, C. C., and Nauseef, W. M. (2013) Myeloperoxidase: a front-line defender against phagocytosed microorganisms. *J Leukoc Biol* **93**, 185-198

243. Poon, I. K., Lucas, C. D., Rossi, A. G., and Ravichandran, K. S. (2014) Apoptotic cell clearance: basic biology and therapeutic potential. *Nat Rev Immunol* **14**, 166-180

244. Weiss, S. J., Klein, R., Slivka, A., and Wei, M. (1982) Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *J Clin Invest* **70**, 598-607

245. Foote, C. S., Goyne, T. E., and Lehrer, R. I. (1983) Assessment of chlorination by human neutrophils. *Nature* **301**, 715-716

246. Rayner, B. S., Zhang, Y., Brown, B. E., Reyes, L., Cogger, V. C., and Hawkins, C. L. (2018) Role of hypochlorous acid (HOCl) and other inflammatory mediators in the induction of macrophage extracellular trap formation. *Free Radic Biol Med* **129**, 25-34

247. Silva, M. T., and Correia-Neves, M. (2012) Neutrophils and macrophages: the main partners of phagocyte cell systems. *Front Immunol* **3**, 174

248. Prame Kumar, K., Nicholls, A. J., and Wong, C. H. Y. (2018) Partners in crime: neutrophils and monocytes/macrophages in inflammation and disease. *Cell Tissue Res* **371**, 551-565

249. Petretto, A., Bruschi, M., Pratesi, F., Croia, C., Candiano, G., Ghiggeri, G., and Migliorini, P. (2019) Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. *PloS one* **14**, e0218946

250. Sur Chowdhury, C., Giaglis, S., Walker, U. A., Buser, A., Hahn, S., and Hasler, P. (2014) Enhanced neutrophil extracellular trap generation in rheumatoid arthritis: analysis of underlying signal transduction pathways and potential diagnostic utility. *Arthr Res Ther* **16**, R122

251. Kessenbrock, K., Krumbholz, M., Schönermarck, U., Back, W., Gross, W. L., Werb, Z., Gröne, H. J., Brinkmann, V., and Jenne, D. E. (2009) Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* **15**, 623-625

252. Quillard, T., Araújo, H. A., Franck, G., Shvartz, E., Sukhova, G., and Libby, P. (2015) TLR2 and neutrophils potentiate endothelial stress, apoptosis and detachment: implications for superficial erosion. *Eur Heart J* **36**, 1394-1404

253. Franck, G., Mawson, T. L., Folco, E. J., Molinaro, R., Ruvkun, V., Engelbertsen, D., Liu, X., Tesmenitsky, Y., Shvartz, E., Sukhova, G. K., Michel, J. B., Nicoletti, A., Lichtman, A., Wagner, D., Croce, K. J., and Libby, P. (2018) Roles of PAD4 and NETosis in Experimental Atherosclerosis and Arterial Injury: Implications for Superficial Erosion. *Circ Res* **123**, 33-42

254. Khan, M. A., Ali, Z. S., Sweezey, N., Grasemann, H., and Palaniyar, N. (2019) Progression of Cystic Fibrosis Lung Disease from Childhood to Adulthood: Neutrophils, Neutrophil Extracellular Trap (NET) Formation, and NET Degradation. *Genes* **10**

255. Ge, L., Zhou, X., Ji, W. J., Lu, R. Y., Zhang, Y., Zhang, Y. D., Ma, Y. Q., Zhao, J. H., and Li, Y. M. (2015) Neutrophil extracellular traps in ischemia-reperfusion injury-induced myocardial no-reflow: therapeutic potential of DNase-based reperfusion strategy. *Am J Physiol* **308**, H500-509

256. Gaul, D. S., Stein, S., and Matter, C. M. (2017) Neutrophils in cardiovascular disease. *Eur Heart J* **38**, 1702-1704

257. Lazzaretto, B., and Fadeel, B. (2019) Intra- and Extracellular Degradation of Neutrophil Extracellular Traps by Macrophages and Dendritic Cells. *J Immunol* **203**, 2276-2290

258. Yellon, D. M., and Hausenloy, D. J. (2007) Myocardial reperfusion injury. *N Engl J Med* **357**, 1121-1135

259. Puhl, S. L., and Steffens, S. (2019) Neutrophils in Post-myocardial Infarction Inflammation: Damage vs. Resolution? *Front Cardiovasc Med* **6**, 25

260. Rassaf, T., Weber, C., and Bernhagen, J. (2014) Macrophage migration inhibitory factor in myocardial ischaemia/reperfusion injury. *Cardiovasc Res* **102**, 321-328

261. Gao, X. M., Liu, Y., White, D., Su, Y., Drew, B. G., Bruce, C. R., Kiriazis, H., Xu, Q., Jennings, N., Bobik, A., Febbraio, M. A., Kingwell, B. A., Bucala, R., Fingerle-Rowson, G., Dart, A. M., Morand, E. F., and Du, X. J. (2011) Deletion of macrophage migration inhibitory factor protects the heart from severe ischemia-reperfusion injury: a predominant role of anti-inflammation. *J Mol Cell Cardiol* **50**, 991-999

262. Arjona, A., Foellmer, H. G., Town, T., Leng, L., McDonald, C., Wang, T., Wong, S. J., Montgomery, R. R., Fikrig, E., and Bucala, R. (2007) Abrogation of macrophage migration inhibitory factor decreases West Nile virus lethality by limiting viral neuroinvasion. *J Clin Invest* **117**, 3059-3066

263. Leech, M., Metz, C., Hall, P., Hutchinson, P., Gianis, K., Smith, M., Weedon, H., Holdsworth, S. R., Bucala, R., and Morand, E. F. (1999) Macrophage migration inhibitory factor in rheumatoid arthritis: evidence of proinflammatory function and regulation by glucocorticoids. *Arthritis Rheum* **42**, 1601-1608

264. Rossi, A. G., Haslett, C., Hirani, N., Greening, A. P., Rahman, I., Metz, C. N., Bucala, R., and Donnelly, S. C. (1998) Human circulating eosinophils secrete macrophage migration inhibitory factor (MIF). Potential role in asthma. *J Clin Invest* **101**, 2869-2874

265. Morand, E. F., Leech, M., and Bernhagen, J. (2006) MIF: a new cytokine link between rheumatoid arthritis and atherosclerosis. *Nat Rev* **5**, 399-410

266. Bernhagen, J., Calandra, T., Mitchell, R. A., Martin, S. B., Tracey, K. J., Voelter, W., Manogue, K. R., Cerami, A., and Bucala, R. (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature* **365**, 756-759