Elucidation of thiol-protein oxidoreductase activity of the cytokine macrophage migration inhibitory factor (MIF) by biochemical redox and site-specific mutagenesis analysis

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Brief Contents

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***, deceased*

• Abbreviations

aa	Amino acid (s)
ACTH	Adenocorticotropic hormone
ADF	Adult T-cell leukemia-derived factor
Amp	Ampicillin
AN	Acetonitrile
AP-1	Activator protein 1
APS	Ammonium persulphate (peroxodisulphate)
ARDS	Acute respiratory distress syndrome
(k)Bp	(kilo) Base pair
BSA	Bovine serum albumine
cam	Camptothecine
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CD	Circular dichroism spectroscopy
cDNA	Complementary DNA
CDTA	Bis 1, 2 diaminocyclohexane N, N, N´, N´ tetraacetic acid
CHMI	5-Carboxymethyl-2-hydroxymuconate isomerase
C-terminal	Carboxy-terminal
CXXC	Cys-Xaa-Xaa-Cys motif
ddH_2O	Distilled water
DMEM	Dulbecco´s modified Eagle´s Minimum Essential Medium
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleic acid
dNTP	Desoxyribonucleoside triphosphate
dox	Doxicycline
DsbA	periplasmatic *E. coli* DsbA
DTH	Delayed-type hypersensitivity
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK1/2	Extracellular signal-regulated kinases
FCS	Fetal Calf Serum
FPLC	Fast protein liquid chromatography
GC-OR	Glucocorticoid overriding

Abbreviations

GdnHCl	Guanidinium hydrochloride
GIF	Glycosylation inhibiting factor
Grx	Glutaredoxin
GSH/GSSG	Reduced/oxidized glutathione
GST	Glutathione-S-transferase
h	Hour (s)
HED	Bis-(2-hydroxyethyl)-disulfide
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
huMIF	Human MIF
IFN-γ	Interferon-γ
IL	Interleukin
IPTG	Isopropyl-1-thio-β-D-galactoside
JAB1	Jun-activation domain-binding protein 1
JNK	c-Jun N-terminal Kinase
kDa	Kilodalton
LB	Luria Bertani Medium
LPS	Lipopolysaccharid
M	Molar
MALDI-MS	Matrix-assisted laser desorption ionization-MS
MAPK	Mitogen-activated protein kinase
MCS	Multiple Cloning Site
MIF	Macrophage migration inhibitory factor
min	Minute (s)
Mr	Molecular weight
NADPH	β-Nicotinamide adenine dinucleotide phosphate (reduced form)
NaPP	Sodium phosphate buffer
ND	Not determined
NFκB	Nuclear factor kappa B
NMR	Nuclear magnetic resonance
NP-40	Nonidet P-40
N-terminal	Amino-terminal
$OD_{x\ nm}$	Optical density at x nm

Abbreviations

o/n	Overnight
ONPG	O-Nitrophenyl β-D-galactopyranoside
OT	4-Oxalocrotonate tautomerase
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripherial blood monocyte
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PMA	Phorbolester
PNGase	Peptide N-glycosidare
POD	Peroxidase
RA	Rheumatoid arthritis
rMIF	Recombinant human MIF
rpm	Revolutions per minute
RP-HPLC	Reverse phase-HPLC
RT	Room temperature
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulfate
[θ]	Mean molar residue ellipticity
TBE	TrisHCl-boric-EDTA-buffer
TBS	Tris-buffered saline
TE	TrisHCl-EDTA buffer
TFA	Triflouroacetic acid
TNF	Tumor necrosis factor
TPOR(s)	Thiol-protein oxidoreductase(s)
Tris	Tris-(hydroxymethyl)-aminomethane
Trx	Thioredoxin
U	Unit (Enzymeinheit)
UV	Ultraviolet
v/v	Volume/volume
wt	Wildtype
v/v	Weight/volume

Contents

1 Summary

It was the aim of this work to investigate and establish potential connections between the biochemical redox properties of MIF and the role of the redox activity of MIF for its biological functions and effects on cellular signaling. In particular, I wished to determine in detail the biochemical redox properties of MIF such as its redox potential and to investigate potential links on MIF-dependent JAB1 effects and apoptosis-regulation through MIF. A quantification of the redox capacity of MIF therefore stood at the beginning of these investigations. When I began, a redox potential for MIF had not been determined, because no assay systems had been available to distinguish and separate the oxidized and reduced species of MIF. Oxidized and reduced MIF species had only been detected in a non-quantitative manner by circular dichroism spectroscopy and ESI-based liquid chromatography mass spectroscopy.

I therefore first attempted to establish an experimental system to distinguish and preparatively separate the oxidized and reduced MIF species. A HPLC-based system was deemed most suitable to fulfil this goal, as the oxidized and reduced species of some other thiol-protein oxidoreductases (TPORs) had been successfully separated by HPLC. Covalent stabilization of the oxidized versus reduced forms of MIF by side chain-thiol modification were ruled out as an approach as previous studies had shown that S-alkylated MIF was poorly soluble. Despite a number of experimental variants such as the choice of the reductant, properties of the gradient and variation of the reduction protocol were tried, no reproducible and significant separation of oxidized and reduced MIF species was obtained. Oxidized MIF usually eluted about 0.3 min later than reduced MIF, but there was a lot of variability in the elution profiles obtained, such that no preparative separation was possible.

To be able to get some information about the redox potency of MIF, I then pursued an approach based on partial sequence MIF-derived peptides. This approach was chosen as it had been shown for other TPORs that partial sequence peptides could retain some of the redox properties of the full-length parent proteins. MIF(50-65) and corresponding control peptides were elected. Oxidized and reduced peptide could be nicely distinguished and separated using the established HPLC procedure. Using CD spectroscopy, MIF(50-65) was found to form a β-turn conformation following disulfide-mediated cyclization; the peptide had catalytic oxidoreductase activity as it reduced insulin disulfides *in vitro* compared to MIF, whereas no MIF-like tautomerase activity was observed.

Next, equilibrium measurements and determination of the redox potential of the peptide was performed. Equilibrium between the oxidized and reduced peptide was not reached before 20 h of incubation with glutathione redox buffer and cysteine/cystine mixtures. MIF(50-65)

had a redox potential E'_0 of - 0.257 ± 0.003 V, which is a very electronegative potential compared to other redox factors. Depending on the parameters of the reference redox pair, the peptide and was able to quickly form both mono- and di-mixed disulfides with glutathione and cysteine. Mixed disulfide formation with cysteine was faster than with glutathione.

As the peptide shared with MIF the catalytic activity *in vitro*, I postulated that it may also share some of the biological activities of MIF. In order to evaluate this potential activity, I wished to utilize a well-known and MIF-specific biological assay. Glucocorticoid overriding (GC-OR) by MIF had been shown to represent a surprising and unique property of MIF, which distinguishes MIF from other cytokines. However, available assay formats were variable as they were based on the use of primary donor monocytes with donor-dependent variations frequently observed. As such an assay was not suitable for optimizing purposes for molecules with an as yet unknown activity profile, I first attempted to establish a more robust cell line-based GC-OR assay. Preliminary data indicated that the THP-1 cell line could be suitable. I successfully established a THP-1 cell-based GC-OR assay in 2 different formats, i.e. a TNF-ELISA read-out system and a NFκB reporter gene read-out, with rMIF showing dose-dependent and bell-shaped curve-like GC-OR activity.

I found that peptide MIF(50-65), at a concentration of 3.3 µM, showed glucocorticoid overriding activity that was comparable to the effect observed for rMIF at 0.16 nM. MIF(50-65) activity was found to be active in both the TNF ELISA and the reporter gene formats of the assay. The observed lower potency of the peptide as compared to full-length MIF was within the range usually seen for activity differences between peptides and proteins. So therefore, I obtained the surprising result that the 16-meric MIF-derived peptide not only shared with MIF the catalytic activity in vitro, but also exhibited significant biological activity of the full-length protein.

To investigate whether the peptide also exhibited MIF-like activity with respects to other cellular effects and, in particular, whether MIF(50-65) could also influence intracellular pathways related to the recently identified MIF-JAB1-regulated functions, I tested whether MIF(50-65) was able to regulate the levels of the cell cycle inhibitor $p27^{Kip1}$. Intriguingly, MIF(50-65) was able to stabilize p27 levels as previously seen for MIF and did so at a concentration of around 1 µM. For comparison, optimal MIF effects on p27 stabilization are observed at concentrations from 100 nM to 1 µM. Of note, biotinylated MIF(50-65) had a comparable activity, indicating that biotinylated peptide may be used for future pull-down studies to identify proteins binding to the peptide in the cell.

To date, only a limited number of the molecular components involved in MIF-mediated signaling have been identified. In addition to JAB1, these have been proteins linked to MIF-mediated inhibition of p53-dependent apoptosis. Thus, I next investigated potential links between the redox activity of MIF and its inhibitory effects on apoptosis. As the redox activity of MIF had been shown to at least partly depend on the presence of the Cys-60 residue of MIF, I first investigated whether there were differences between wtMIF and C60SMIF in the apoptosis-inhibiting effect of MIF following induction of apoptosis by various stimuli. My results, using several different stimulation conditions and cell types, showed that inhibition of apoptosis by MIF was dependent on the presence of Cys-60, provided endogenously overexpressed C60SMIF was used. In contrast, when exogenous rC60SMIF was applied, the variant exhibited a full apoptosis-inhibiting effect, indicating that the redox activity of MIF is only linked to its apoptosis-inhibiting activity, when MIF acts through intracellular signaling pathways. It could not be addressed in this thesis, whether it is the MIF-JAB1 pathway that is affected under these circumstances.

To further address the link between the redox activity of MIF and its apoptosis-inhibiting effect, a cellular system was established in which apoptosis was induced through mild oxidative stress. I demonstrated that under such conditions, MIF was able to inhibit apoptosis. Follow-up work by others in the lab now indicates that rescue of cells by MIF from pro-oxidative stress-induced apoptosis is also, at least in part dependent on residue Cys-60, as overexpression of the mutant failed to confer protection from apoptosis. Of note, my data also demonstrated that MIF was able to inhibit pro-oxidative stress-induced apoptosis in leukocytes, indicating that these mechanisms could be important for the immuno-regulatory effects of MIF. Interestingly, inhibition by MIF of pro-oxidative stress-induced apoptosis correlated with a MIF-induced enhancement of cellular glutathione (GSH) levels and a MIF-induced reduction in the phosphorylation of endogenous c-Jun following thiol starvation.

Together, my findings confirm the notion that MIF is a redox enzyme with an oxidoreductase-type of activity that is based on a CXXC motif as catalytic site. Through the detailed characterization of peptide MIF(50-65), I have obtained important first evidence as to the redox potential of MIF, which is likely to be relatively electronegative, and potential redox reactions that MIF may be involved in the cell such as regulation of the cellular redox pool and formation of possibly transient mixed disulfides with glutathione or cysteine. The observed fast and transient cysteinylation of MIF(50-65) could be an indication that cysteinylation may be a means of MIF modification to modulate its activity. Moreover, MIF(50-65) spanning the redox center of MIF has arisen as a surprisingly small molecule that can mimic important MIF functions. This thesis has therefore served to suggest that MIF(50-

65) could be a template for the design of novel MIF-based peptide drugs. I showed that inhibition of apoptosis and possibly other cellular signaling pathways modulated by MIF are at least in part linked to the catalytic redox activity of MIF.

2 Zusammenfassung

Der Makrophagen-migrationsinhibierende Faktor (MIF) ist ein proinflammatorisches Zytokin mit enzymatischer Aktivität. Obwohl eine Vielzahl biologischer Aktivitäten beschrieben wurden, ist der molekulare Mechanismus von MIF weitgehend unbekannt. Ziel dieser Arbeit war es, mögliche Zusammenhänge zwischen den biochemischen Redox-Eigenschaften von MIF und den biologischen Eigenschaften sowie der Signaltransduktion – MIF-abhängige Effekte von JAB1 und Regulation der Apoptose durch MIF – zu untersuchen.

Zu Beginn der Untersuchungen sollte das Redoxpotential von MIF quantifiziert werden. Oxidiertes und reduziertes MIF wurden bisher nur nicht-quantitativ durch CD-Spektroskopie und ESI-LC-Massenspektroskopie detektiert. Daher versuchte ich zuerst, ein Experimentalsystem zur Identifizierung und präparativen Trennung oxidierter und reduzierter MIF-Spezies zu etablieren. Ein HPLC-basiertes System schien das geeignetste zu sein, da bereits oxidierte und reduzierte Spezies anderer Thiol-Protein-Oxidoreduktasen (TPOR) erfolgreich mittels HPLC getrennt werden konnten. Obwohl eine Vielzahl experimenteller Variationen wie Wahl des Reduktionsmittels, verschiedene Gradienten oder Reduktions-protokolle angewendet wurden, war keine reproduzierbare und signifikante Separation der oxidierten und reduzierten MIF-Spezies möglich. Oxidiertes MIF eluierte üblicherweise ca. 0.3 min nach reduzierten MIF, wegen der großen Variation der Elutionsprofile war jedoch keine präparative Trennung der beiden Spezies möglich.

Um weitere Informationen über das Redoxpotential von MIF zu erhalten, habe ich einen Ansatz verwendet, der auf der Verwendung partieller MIF-Sequenz-basierter Peptide beruht. Auch für diesen Ansatz wurde erfolgreich für andere TPOR gezeigt, dass partielle Sequenz-Peptide Redoxeigenschaften der Mutterproteine aufweisen können. MIF(50-65) und entsprechende Kontrollpeptide wurden ausgewählt. Mit der etablierten HPLC-Prozedur waren oxidierte und reduzierte Spezies gut voneinander unterscheidbar und separierbar. Mittels CD-Spektroskopie konnte ich zeigen, dass MIF(50-65) nach Disulfid-vermittelter Cyklisierung β-Faltblatt-Konformation einnimmt. Das Peptid zeigte außerdem Oxidoreduktase-Aktivität, da es im Vergleich zu MIF *in vitro* ebenfalls Insulin reduzierte; eine MIF-ähnliche Tautomerase-Aktivität konnte jedoch nicht gezeigt werden.

Messungen der Gleichgewichtsreaktion und des Redoxpotentials der Peptide ergaben, dass sich das Gleichgewicht zwischen oxidiertem und reduziertem Peptid erst nach 20 h einstellte, wenn Glutathion-Redoxpuffer und Cystein/Cystin-Gemische verwendet wurden. MIF(50-65) besitzt ein Redoxpotential von E'o = - 0.257 ± 0.003 V, abhängig von den Parametern des Redoxpaares, und ist in der Lage, einfach- und doppeltgemischte Disulfide mit Glutathion

und Cystein zu bilden. Die Bildung gemischter Disulfide mit Cystein erfolgte schneller als mit Glutathion.

Da das Peptid wie MIF eine katalytische Aktivität *in vitro* aufwies, habe ich postuliert, dass es möglicherweise auch MIF-artige biologische Aktivität aufweist. Um dies zu untersuchen, wurde ein MIF-spezifischer biologischer Assay verwendet, der die *Glucocorticoid overriding*-Aktivität (GC-OR) ausnutzt. Dafür etablierte ich einen zuverlässigen, THP-1-basierten GC-OR-Test, der über zwei Messverfahren – den TNF-ELISA und einen NFkB-Reportergen-Assay – ausgewertet werden kann. Wie erwartet, zeigte rMIF eine konzentrationsabhängige, glockenförmige GC-OR-Aktivität. Bei einer Konzentration von 3.3 µM zeigte MIF(50-65) GC-OR-Aktivität, die vergleichbar mit der von rMIF bei 0.16 nM war, wobei MIF(50-65) sowohl im TNF-ELISA als auch im Reportergen-Format aktiv war. Der beobachtete Unterschied zwischen der Aktivität des Peptids und dem Wildtyp-Protein lag im Bereich der üblicherweise beobachteten Unterschiede zwischen Peptiden und Proteinen. Ich konnte also das überraschende Ergebnis beobachten, dass ein 16 Aminosäuren langes, von MIF abstammendes Peptid, signifikante biologische Aktivität des Wildtyp-Proteins besitzt.

Um zu untersuchen, ob das Peptid auch andere MIF-ähnliche Aktivitäten und zelluläre Effekte ausübt, speziell ob MIF(50-65) intrazelluläre Signalwege beeinflusst, die im Zusammenhang mit den kürzlich entdeckten MIF-JAB1-Funktionen stehen, testete ich, ob MIF(50-65) den Zellzyklusinhibitor $p27^{Kip1}$ reguliert. Interessanterweise war MIF(50-65) bei einer Konzentration von 1 µM in der Lage, p27 in ähnlicher Weise wie MIF zu stabilisieren. Zum Vergleich: optimale MIF-Effekte auf die Stabilisierung von p27 wurden bei 0.1-1 µM beobachtet. Bemerkenswert ist außerdem, dass biotinyliertes MIF(50-65) vergleichbare Aktivität besitzt. Damit könnte es zukünftig für Präzipitationsstudien zur Identifizierung intrazellulärer peptidbindender Proteine verwendet werden.

Bislang wurden nur wenige molekulare Komponenten identifiziert, die an MIF-vermittelten Signaltransduktionswegen beteiligt sind. Neben dem cytosolischen Protein JAB1 sind dies vor allem Proteine, die in Zusammenhang mit der MIF-vermittelten Inhibition p53-abhängiger Apoptose gebracht werden. Daher untersuchte ich als nächstes, ob Verbindungen zwischen der Redoxaktivität von MIF und der Apoptose-Inhibition existieren. Für die Redox-Aktivität von MIF wurde bereits früher beobachtet, dass sie, zumindest teilweise, von der Anwesenheit des Cys-60-Restes abhängt. Unter Verwendung der Zelllinien und den Stimuli konnte ich zeigen, dass die MIF-vermittelte Apoptose-Inhibition von der Existenz des Cys-60-Rests abhängt, wenn endogenes MIF bzw. überexprimiertes C60SMIF verwendet wurde. Im Gegensatz dazu wurde beobachtet, dass bei Zugabe von exogenem MIF oder C60SMIF die

Cys-Mutante ebenfalls Apoptose-Inhibition zeigte. Die Redox- und die Apoptose-inhibierende Aktivität von MIF scheinen daher nur im Zusammenhang zu stehen, wenn MIF über intrazelluläre Signalwege wirkt. In dieser Arbeit war es nicht möglich, festzustellen, ob unter den gewählten Bedingungen ein Zusammenhang zum MIF-JAB1-Signalweg bestand.

In einem anderen zellulären System mit HL-60 und HtTAM Zellen, bei dem Apoptose durch milden oxidativen Stress induziert wurde, konnte ich ebenfalls zeigen, dass MIF in der Lage ist, Apoptose zu inhibieren. Folgearbeiten aus unserem Labor weisen nun darauf hin, dass der Schutz von Zellen vor pro-oxidativem Stress-induzierter Apoptose wenigstens teilweise von Cys-60 abhängt, da die Expression der Mutante keinen Schutz vor Apoptose bietet. Meine Daten zeigen ausserdem, dass MIF in der Lage ist, vor pro-oxidativem Stress-induzierter Apoptose in Leukozyten zu schützen, was darauf hinweist, dass dieser Mechanismus wichtig für die immunregulatorischen Effekte von MIF sein könnte. Interessanterweise korrelliert dieser Effekt ausserdem mit einer MIF-induzierten Erhöhung der zellulären Glutathion-Konzentration und einer Reduzierung der Phosphorylierung von endogenem c-Jun nach Thiol-Depletion.

Zusammengefasst bestätigen meine Daten, dass MIF ein Redoxenzym ist, dessen Oxidoreduktase-Aktivität auf einem CXXC-Motiv als katalytischem Zentrum basiert. Ich konnte zeigen, dass die Inhibition der Apoptose, möglicherweise auch die Modulation anderer Signalwege durch MIF, mindestens teilweise im Zusammenhang mit der Redoxaktivität stehen. Durch die detaillierte Charakterisierung des Peptids MIF(50-65), konnte ich erste Beweise sammeln, dass MIF eine Rolle bei der Regulation des zellulären Redoxpools und möglicherweise auch bei der Bildung gemischter Disulfide mit Glutathion und Cystein spielt. Die beobachtete schnelle und transiente Cysteinylierung von MIF(50-65) könnte darauf hin weisen, dass Cysteinylierung eine Möglichkeit darstellt, die MIF-Aktivität zu modulieren. Darüber hinaus ist MIF(50-65) ein überraschend kleines Molekül, das wichtige MIF-Funktionen nachahmen kann. MIF(50-65) könnte daher als Templat für die Entwicklung neuartiger, MIF-basierter Peptid-Wirkstoffe dienen.

3 Introduction

3.1 MIF as cytokine and hormone

3.1.1 History

Macrophage migration inhibitory factor (MIF) was first discovered more than 30 years ago to be a T lymphocytes product which inhibited the random migration of guinea pig peritoneal macrophages (Bloom & Bennett, 1966; David, 1966). Over the years, MIF activity was associated with delayed-type hypersensitivity (DTH) and was shown to be produced by lectin-activated T cells (Bloom & Bennett, 1966; David, 1966, Nathan, 1971). Nathan and co-workers showed that MIF altered macrophage functions, such as adherence and phagocytosis (Nathan *et al.,* 1971; Nathan *et al.,* 1973).

Although MIF was one of the first cytokines discovered, at the beginning of this study, its molecular mode of action had not been fully explained, in particular because a classical cytokine receptor had not been identified. Throughout the last decade, other pro-inflammatory factors such as TNF or INF-γ or IL-1 emerged, and became of major interest as their signaling pathways could be elucidated rather rapidly compared to MIF. Research focused on cytokines with more defined mechanisms, which were found to regulate pivotal cellular actions such as the cell cycle and cell death while MIF research got halted. A new area in MIF research began with its cloning in 1989 (Weiser *et al*., 1989) and 1993 (Bernhagen *et al*., 1993) which subsequently allowed for the production of recombinant material (Bernhagen *et al*., 1994). After the development of a method to correctly refold MIF (Bernhagen *et al*., 1994), bioactive recombinant MIF was available in large amounts and the MIF protein could be investigated extensively. Since MIF was demonstrated to be distinct from previously discovered cytokines with similar migration inhibitory activity it was unclear what the precise relationship between the 12.5-kDa MIF protein and the various migration inhibitory assays that had been applied over the years. The structural properties of MIF have recently been reviewed (Bernhagen *et al.,* 1998). It is worthwhile of mentioning, however, that certain structural characteristics of MIF have not yet been resolved. For example, it is unclear whether the MIF trimer, which is the entity of MIF that was found in all 4 published crystallographic studies, is physiologically occuring and the relevant oligomerization state of MIF. In fact, several studies have suggested that it could rather be a dimer or monomer that mediates MIF action (Tomura *et al*., 1999; Mischke *et al*., 1998; Mühlhahn *et al.,* 1996).

3.1.2 MIF as a T cell cytokine

MIF was first described in 1966 by two independent reports of Bloom & Bennett and David (Bloom & Bennett, 1966; David, 1966). Both groups studied the basis of delayed-type hypersensitivity reactions and found that crude extracts of sensitized, e.g. lectin-activated, T-cells, which contained MIF inhibited the random migration of guinea pig peritoneal macrophages *in vitro*. Bernhagen and colleagues found that in the tuberculin-induced delayed-type hypersensitivity response in the mouse, the macrophage rather than the T-lymphocyte is the predominant source of MIF-protein and mRNA expression (Bernhagen *et al.,* 1996).

Bacher and Bucala showed that MIF plays an important regulatory role in the activation of T cells induced by mitogenic or antigenic stimuli. Activated T cells produce MIF and neutralizing anti-MIF antibodies inhibits T cell proliferation and IL-2, and suppress antigen-driven T cell activation and antibody (Bacher *et al*., 1996). By ELISA, the MIF protein content in these cells was determined to be 4 fg/cell and 170 fg/cell in primary cells and T cell lines, respectively. This is similar to the values observed in primary monocytes (2 fg/cell) and macrophage cell lines (120 fg/cell). T cell activation by specific antigens, mitogens or anti-CD3 antibody has been found to lead to increased MIF mRNA expression and secretion of MIF protein, indicating that MIF production is also an important feature of T cell activation (Bacher *et al.,* 1996).

3.1.3 MIF as a pituitary hormon and macrophage cytokine

Macrophage migration inhibitory factor (MIF) is one of the first cytokines discovered as a secretary product of corticotropic pituitary cells (Bernhagen *et al*., 1993). Pituitary-derived MIF enters the circulation after infectious or stressful stimuli and appears to act to counter-regulate glucocorticoid suppression of cytokine production. MIF is localized to granules present exclusively in adrenocorticotroic hormone (ACTH)-and thyrotropic hormone (TSH)-secreting cells (Bernhagen *et al.,* 1993, Nishino *et al*., 1995). MIF protein accounts for about 0.05 % of the total pituitary protein content (Bernhagen *et al*., 1993). Pituitary MIF significantly contributes to the MIF that is measured in serum after LPS administration. Moreover, serum concentrations of MIF were measured after LPS administration and were found to be similar to the concentrations observed for classical pituitary hormones (Bernhagen *et al*., 1993).

Analysis of monocyte/macrophage expression of MIF demonstrated that MIF was found in these cells as well (Calandra *et al.,* 1994). Monocyte/macrophage MIF is released upon stimulation with various pro-inflammatory stimuli, such as LPS, TSST-1, streptococcal

pyrogenic exotoxin A, malaria pigment (hemazoin), and the cytokine TNF and interferon-γ (Calandra *et al.,* 1994). The production of MIF in response to increasing concentrations of the bacterial toxins follows a bell-shaped curve, decreasing at high concentration of LPS or TSST-1. The fact that MIF production is turned off at high concentrations of bacterial product may be an important protective mechanism of the host to prevent the detrimental effects of excessive amounts of MIF release. Bernhagen et al. identified MIF as a major secreted protein released by anterior pituitary cells in response to LPS stimulation (Bernhagen *et al*., 1993). These studies also showed that the macrophage is both an important source and an important target of MIF *in vivo*. MIF-induced TNF secretion by macrophages may act to synergize with interferon-γ to induce macrophage nitric oxide production (Bernhagen *et al*., 1993; Calandra *et al.,* 1994). The pivotal role of MIF to trigger inflammatory reactions was expanded by the observation that activation of immune cells in response to gram-positive stimuli such as the toxic syndrome protein (TSST-1) occured in a MIF-mediated fashion (Calandra *et al*., 1998). In all tested septic shock model involving live bacteria or bacterial toxins, MIF generally was found to potentate lethality, while anti-MIF-antibody conferred full protection (Bernhagen *et al.,* 1995; Calandra *et al*., 1998; Bozza *et al*., 1999)
Together, these data suggest that MIF may take part in a peripheral amplification loop that is aimed at launching a fast and effective macrophage response to infection and tissue invasion via mutual stimulatory feedbacks of the participating macrophage cytokines (Bernhagen *et al*., 1998).

3.1.4 MIF as a gene product of other cells and tissues

Over the years, studies have shown that MIF is not only produced by activated T-cells and macrophages, but also by endothelial and epithelial cells of several organs, including the lung, kidney, liver (Donnelly *et al.,* 1997a; Imamura *et al*., 1996; Suzuki *et al.,* 1996b), brain (Nishibori *et al.,* 1996; Nishibori *et al*., 1997), skin (Shimizu *et al*., 1996) and retina (Matsuda *et al*., 1997). In rat brain, MIF protein and MIF mRNA were recently discovered in the epithelial cells of the choroid plexus and in ependymal cells as well as astrocyte-like cells (Ogata *et al.,* 1998). They found that neuronal cells as well as glial cells have the potency to produce MIF (Suzuki *et al*., 1999). Subsequently, it was shown that MIF is expressed in a variety of cells and tissues, including the liver (Bacher *et al*., 1997). They found that MIF is an important mediator of the inflammatory response in alcoholic liver disease and potential therapeutic target (Amin *et al*., 2001). Together, these data suggested that MIF is a ubiquitous molecule that is involved in the regulation of the immune, endocrine and nervous system (Bacher *et al*., 1997). MIF might play a role in insulin release and MIF is also expressed constitutively in adipocytes and its expression is dependent on glucose and

insulin levels *in vitro* (Sakaue *et al*. 1999), It has therefore been suggested that release of MIF into the circulation occurs from performed intracellular stores (Bernhagen *et al*., 1993; Bernhagen *et al.,* 1998; Benigni *et al.,* 2000). MIF may play a role in the mechanism of insulin resistance that is often observed in obesity and type II diabetes. Also current MIF research has focussed on MIF expression and function in nervous and endocrine tissues (Rowson and Bucala, 2001). MIF´s role and expression in the myocardium has been reported by Takahashi (Takahashi *et al*., 2001). Although a receptor for MIF has not been identified yet, it is now recognized that MIF is constitutively expressed in a variety of tissues and cells. Moreover, it was also found that potential MIF membrane receptors can not be widely expressed and it has been suggested that the cellular uptake of MIF occurs by nonreceptor-mediated endocytosis rather than penetration (Kleemann *et al.*, 2002). Burger-Kentischer et al. suggested that MIF is produced abundantly by various cells in all types of human atherosclerotic lesions and may play an important role in early plaque development and advanced complicated lesions. MIF-JAB1 complexes could serve critical regulatory functions in atherosclerotic lesion evoltion (Burger-Kentischer *et al*., 2002).

3.1.5 The role of MIF in disease

Numerous studies were performed that aimed to investigate the role of MIF in various disease conditions. MIF is a cytokine with a critical role in several inflammatory conditions (Calandra *et al.,* 2000; Bernhagen *et al.,* 1998; Bozza *et al*., 1999) but also displays endocrine (Calandra *et al*., 1995; Bernhagen *et al*., 1993) and enzymatic functions (Bernhagen *et al*., 1998; Bozza *et al*., 1999; Kleemann *et al*., 1998a; Rosengren *et al*., 1996). MIF has been implicated as a mediator of septic shock (Calandra *et al*., 2000; Bernhagen *et al*., 1998; Bozza *et al*., 1999; Bernhagen *et al*., 1993), rheumatoid arthritis (Mikulowska *et al.,* 1997), acute respiratory distress syndrome (ARDS) (Donnelly *et al.,* 1997a; Donnelly *et al*., 1997b; Donnelly *et al*., 1999), and several cell mediated immune conditions (Bernhagen *et al*., 1996; Lan *et al*., 1997; Lan *et al*., 1998). MIF is a unique cytokine which has a potential role as an antagonist of glucocorticoid action; MIF overrides glucocorticoid-mediated immunosuppression and can counter-regulate the effects of glucocorticoids on inflammation and immunity. MIF-based strategies are of relevance for the development of novel therapeutical approaches for various inflammatory and immune diseases and cancer. Currently, only antibody-based MIF therapeutic strategies are being developed. Recently, Lue and co-workers, in a review article, have attempted to correlate current knowledge on the molecular pathways of MIF activity with its functions in immunity and disease (Lue *et al.,* 2002).

3.2 MIF structure

The MIF gene was found to be small (< 1kb) and to consist of three exons that are separated by two small introns of 100-200 bp as described in detail in (Bozza *et al*., 1995; Kozak *et al*., 1995; Mitchell *et al*., 1995; Paralkar & Wistow, 1994). Currently it is not clear, how transcription of human MIF is regulated and only little is known about the promoter region of the MIF gene and the stability of the mRNA of MIF as reviewed in (Bernhagen *et al*., 1998). More is known about the translated 115 aa protein MIF, the N-terminal Met1 of which is posttranslationally removed. Briefly, MIF does not have a N-terminal or an apparent internal signal sequence that could direct MIF export through the endoplasmic reticulum (Bernhagen *et al.,* 1994; Weiser *et al*., 1989) and evidence accumulated that MIF is secreted via a non-classical pathway (Flieger, Ph.D thesis 2002). MIF is not posttranslationally glycosylated as mass spectrometric analyses and PNGase digesting studies of MIF from various tissues have shown (Bernhagen *et al*., 1994; Zeng *et al*., 1993). The molecular mass of reduced human MIF was determined 12346 Da. Its structure was solved by X-ray crystallography (Sugimoto *et al*., 1996; Sun *et al*., 1996a) and the reported structures differ only in the C-terminal region. The MIF structure is unique among cytokines, and no significant sequence homologies have been found between MIF and known proteins. The MIF monomer however somewhat resembles the IL-8 dimer in that two α-helices pack against a four-stranded β-sheet, but the arrangement and the topology of the secondary structure of MIF have been found entirely different from that of IL-8 (Sun *et al*., 1996a). Although all crystal structure analyses had been performed with reduced recombinant MIF reported MIF trimers, recent crosslinking studies and gel filtration analysis with MIF demonstrated that MIF exists in an equilibrium of monomers, dimers and trimers in physiological solutions (Mischke *et al*., 1997). It is currently being investigated which oligomerization state is required to exert biological activity or whether the MIF monomer is already bioactive. The monomer has a $\beta\alpha\beta\beta\beta\alpha\beta$ motif, with the β-elements β1, β2, β4, and β5 forming the central sheet (Figure 1A). In the MIF trimer as depicted in Figure 1B, the β-sheets form a barrel-like structure with a central channel and are surrounded by the α-helices.

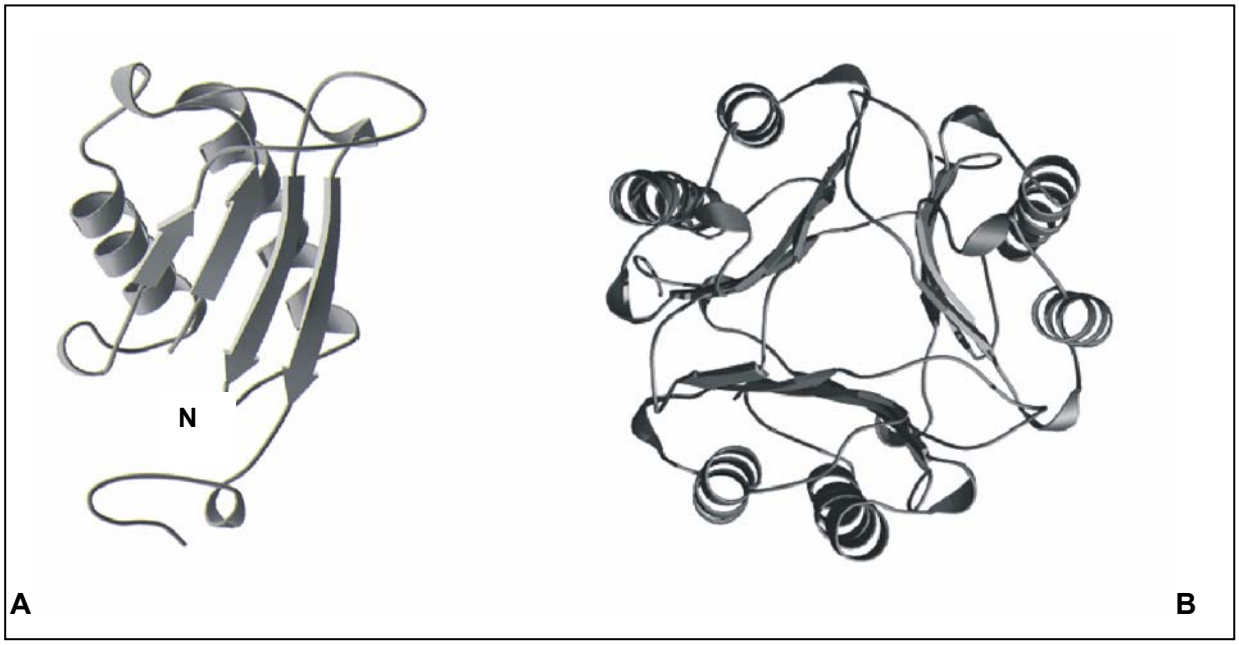

Figure 1: Structure of human MIF. **A:** Ribbon structure of the MIF monomer. **B:** Architecture of the MIF trimer (Kraulis, 1991).

So far, a physiological role for this channel or pore could not be identified (R. Mischke, personal communication). MIF was found to have an architectural similarity with almost identical subunit topology and similar trimeric packing with the *E. coli* enzyme 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI). This observation was further investigated by Rosengren (Rosengren *et al*., 1996), since MIF exhibits tautomerase activity, which is based on the presence of Pro2 as a catalytic base in analogy to CHMI.

3.3 MIF as an enzym

MIF is different from cytokines in that it catalyses chemical reactions. Recent studies have shown that MIF has enzymatic activities. MIF exhibits a tautomerase/isomerase activity (Rosengren *et al*., 1996) and an enzymatic thiol-protein oxidoreductase activity (Kleemann *et a*l., 1998a).

3.3.1 MIF as tautomerase

MIF was shown to catalyze a tautomerization reaction, specifically the conversion of the non-naturally occurring D-isomer of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) to dihydroxyindole carboxylic acid (DHICA) (Rosengren *et al*., 1996). D-dopachrome

tautomerase was discovered during the study of melanin biosynthesis. This enzyme was found to have an amino acid sequence that is highly homologous with that of MIF. MIF also was identified as a phenylpyruvate tautomerase with phenylpyruvate and p-hydroxyphenylpyruvate as natural substrates (Rosengren *et al.,* 1997).

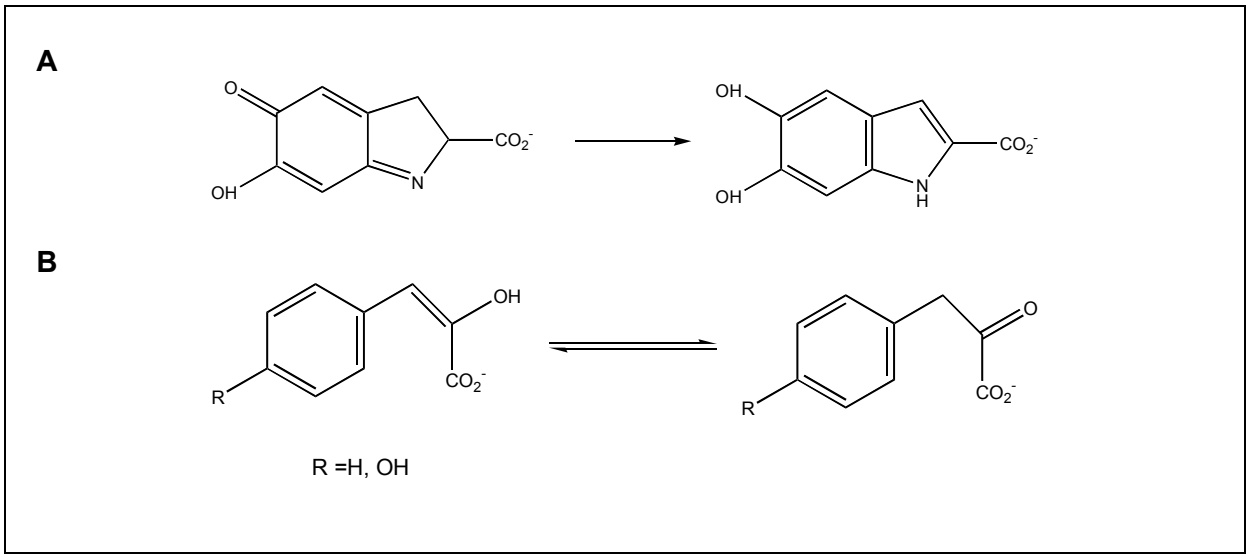

Figure 2: MIF-catalysed tautomerase reaction. **A** D-Dopachrome-tautomerase reaction (Rosengren *et al*., 1996); **B** Phenylpyruvate-tautomerase reaction (Rosengren *et al*., 1997).

The studies showed that the N-terminal proline of MIF is required for the D-dopachrome tautomerase and the phenylpyruvate tautomerase activities (Swope *et al*., 1998a, Lubetsky *et al*., 1999, Stamp *et al*., 1998, Kleemann *et al*., 2000b). Interest in the relation of the biological activities of MIF with its tautomerase activities increased; however, their association has not been clarified and natural ligands for MIF have not yet been identified. D-dopachrome is not a physiological molecule, and phenylpyruvate and p-hydroxyphenylpyruvate are thought not to be the physiological substrates because of their separate localization from MIF and the kinetic parameters for the tautomerase reaction (Rosengren *et al*., 1997). On the other hand, it has recently been shown that dopachrome analogues inhibit the tautomerase avtivity of MIF (Zhang and Bucala*,* 1999), they discovered small molecules, tryptophan derivatives that inhibit the tautomerase activity of MIF (Al-Abed *et al*., 2000), they also found that several MIF residues near the N-terminal proline are perturbed upon addition of S-hexylglutathione and effected by p-hydroxyphenylpyruvate, a substrate for the phenylpyruvate tautomerase activity of MIF (Lubetsky *et al*., 1999; Swope *et al*., 1998b). Jung and colleagues also demonstrated that the proliferation-associated gene (PAG) could inhibit the D-dopachrome tautomerase activity of MIF (Jung *et al*., 2001). A

phenylpyruvate tautomerase inhibitor, (E)-2-fluoro-p-hydroxycinnamate, inhibits the tautomerase reaction of MIF and it has recently been shown that dopachrome analogues inhibit the tautomerase activity of MIF as reported by Taylor and co-workers (Taylor *et al.,* 1999). A potent tautomerase inhibitor is expected to be a validation tool which can clarify the role of the enzymatic activity of MIF and the relation between its biological and enzymatic activity, and there is a possibility that a tautomerase inhibitor may once be used for MIF-related disease. Orita et al. has identified 14 tautomerase inhibitors of MIF whose Ki values area in the range of 0.038 - 7.4 mM and also determined the crystal structure of a MIF complex with one of the hit compounds and also shown that MIF has a hydrophilic surface and a second hydrophobic surface at the rim of the active site, it is expected that a specific potent inhibitor for MIF could be designed using the interaction with this region (Orita *et al.,* 2001). More recently, Senter and colleagues showed that acetaminophen metabolites and several related chemotypes irreversibly inhibit both the enzymatic and the biological activities of MIF (Senter *et al*., 2002).

3.3.2 MIF as a thiol-protein oxidoreductase

The molecular mechanism of action of macrophage migration inhibitory factor (MIF), a cytokine with a critical role in the immune and inflammatory response, has not yet been identified.

Thiol-protein oxidoreductase (TPOR) activity has been investigated widely for proteins of the thioredoxin superfamily (Aslund *et al.,* 1997; Holmgren, 1985b; Nelson & Creighton ,1994; Walker *et al*., 1995) and activity studies have been routinely performed using either the HED transhydrogenase assay as originally devised for glutaredoxin (Holmgren, 1985a) or the insulin reduction assay as originally devised for thioredoxin (Trx) (Chandler & Varandani, 1975; Holmgren, 1979b).

For MIF, Bernhagen and colleagues had shown that a cystine bond is located between Cys57 and Cys60, suggesting that oxidoreductase action of MIF could be similar to that of TPORs, i.e. to be dependent on a CXXC dithiol-disulfide mechanism (Kleemann *et al*., 1998a, Kleemann *et al*., 1998b; Kleemann *et al*., 1999). MIF has been reported to possess a TPOR activity (Kleemann *et al*., 1998a), which involves the reduction of insulin and 2-hydroxyethyldisulfide. The widely conserved Cys-Ala-Leu-Cys (CALC) motif of MIF was shown to form an intramolecular disulfide bond and oxidoreductase activity was found to be dependent on the presence of the cysteines in this region and certain MIF-mediated immune processes are due to the cysteine-mediated redox mechanism. Kleemann and colleagues also confirmed that the activity of MIF was dependent on the CALC motif in the central region of the MIF sequence. They also provided evidence for a role of the CALC motif in the

oxidoreductase and cytokine activities of MIF (Kleemann *et al*., 1999). Takahashi and colleagues also showed that MIF may function in the myocardium as redox-sensitive cytokine (Takahashi *et al.,* 2001).

A possible correlation between the enzymatic oxidoreductase and tautomerase activities of MIF has only been investigated in past. The tautomerase activity is dependent on the presence of the basic Pro2 residue (Bendrat *et al*., 1997; Stamp *et al.,* 1998; Lubetsky *et al*., 1999; Taylor *et al*., 1999; Kleemann *et al.,* 2000b) and the oxidoreductase activity is mediated by the central CALC motif (Kleemann *et al*., 1998a, Kleemann *et al.,* 1998b; Kleemann *et al.,* 1999). A link between the catalytic activities of MIF and its immunologic functions has not been unequivocally established. Prior studies indicating that native MIF contained both oxidized and reduced MIF molecular forms (Bernhagen *et al.,* 1993; Bernhagen *et al*., 1994; Calandra *et al*., 1995), suggest that there is an equilibrium between these two species at physiological conditions. However, a redox potential for MIF has not yet been determined, because no assay systems have been available to quantitatively distinguish the oxidized and reduced species of MIF. The determination of the redox potential of MIF and the MIF-derived peptide was one major aim of this thesis to further define the essential role of the oxidoreductase activity for the immunological function of MIF.

3.4 The role of MIF in cell proliferation and cancer

Cellular redox state is regulated by cellular thiols, including glutathione (GSH) and thioredoxin (Trx). Holmgren had show that Trx is a small ubiquitous multifunctional protein having a redox-active disulfide/dithiol within its active site sequence, -Cys-Gly-Pro-Cys- (Holmgren, 1985b). Intracellular Trx plays an important role in the regulation of protein-nucleic acids interactions through the redox regulation of its cysteine residues (Hirota *et al.,* 1997; Akamatsu *et al*., 1997). Trx also shows a cytokine-like extracellular activity to promote cells growth (Wakasugi *et al*., 1990; Silberstein *et al*., 1993; Rosen *et al*., 1995) and protect cells against oxidative stress (Nakamura *et al*., 1994; Matsuda *et al*., 1991; Yokomise *et al*., 1997). Previous work by others had shown that Trx by a enzymatic redox mechanism plays an important role in the regulation of oxidative stress, cell proliferation, and apoptosis (Hayashi *et al*., 1993). They also found the mechanisms and the role of Trx in stimulating cancer cell growth, as well as Trx offers a target for the development of drugs to treat and prevent cancer (Powis *et al.,* 2000).

MIF is released from T cells and from macrophages (Calandra *et al*., 1997). Historically, activated T cells were considered to be the source of MIF and monocyte/macrophage populations to be the target of its migration inhibitory effects. More recent studies have established that monocytes/macrophages, in fact, are the primary site of MIF production after

exposure of the host to bacterial endo- and exotoxins (LPS, toxic shock syndrome toxin-1, and streptococcal pyrogenic exotoxoin), malaria pigment (hemozoin), or cytokines (TNF and INF-γ; refs. Calandra *et al*., 1994; Calandra *et al*., 1998). Once released, MIF modulates the expression of proinflammatory mediators by macrophages, and is an important component of T cell activation (Calandra *et al*., 1994; Bacher *et al*., 1996).

For MIF, first evidence has been collected that there may be an interplay between cellular redox effects and MIF action (Kleemann *et al*., 1998b). A number of recent studies imply that MIF could be centrally involved in processes regulating cell proliferation and tumor angiogenesis (Takahashi *et al*., 1998; Chesney *et al*., 1999; Shimizu *et al*., 1999; del Vecchio *et al*., 2000; Ogawa *et al*., 2000; Kleemann *et al*., 2000a). Although a link between the catalytic properties of MIF and its immunoregulator activities has not yet been unanimously derived (Bendrat *et al*., 1997; Hermanowski *et al.,* 1999), it is likely that MIF-mediated "enzymatic signaling" may critically contribute to at least some of the effects of MIF, as, for example, the enzyme-dead mutant C60SMIF does not exhibit certain MIF-like macrophage-activating properties (Swope *et al*., 1998a; Kleemann *et al*., 1998a; Kleemann *et al*., 1999). Through its enzymatic thiol-protein oxidoreductase activity, MIF participates in the regulation of cellular redox stress (Tagaya *et al*., 1989; Tanaka *et al*., 2000). Furthermore, a physiological involvement of MIF in the regulation of the cellular redox state is supported by studies showing that MIF binds to and regulates the PAG (Jung *et al*., 2001) and MIF functions as a redox regulator in the myocardium (Takahashi *et al*., 2001). Potential intracellular effectors of MIF action that have been described to date include components of the MAP kinase pathway (Mitchell *et al*., 1999), the JAB1 (Kleemann *et al*., 2000a; Kleemann *et al*., 2002). The CALC redox motif of MIF is important for at least some of the JAB1-mediated activities of MIF (Kleemann *et al*., 2000a).

The cellular effects of MIF reported include effects on cell proliferation and differentiation, cell migration, and on the gene expression of other inflammatory mediators (Mitchell *et al*., 2000; Lue *et al*., 2002). Depending on the physiological context and the cell type, MIF can stimulate or inhibit cell proliferation (Mitchell *et al*., 2000; Kleemann *et al*., 2000a; Abe *et al*., 2001). MIF down-regulates the expression of the cell cycle regulators p21 and cyclin G, while it stabilizes p27 levels (Kleemann *et al*., 2000a; Hudson *et al*., 1999). MIF enhances the migration of tumor cells (Shimizu *et al*., 1999). MIF induces the gene expression of inflammatory cytokines such as TNF and interleukin (IL)-1β and, depending on the context, can induce or down-regulate inducible nitric oxide synthase (iNOS) and IL-6 (Calandra *et al*.,1995; Bozza *et al*., 1999; Satoskar *et al*., 2001). Together, this indicates that MIF participates in the regulation of cellular activity in a complex and multiple fashion.

Recently, Hudson et al. reported that MIF, as a proinflammatory cytokine released at the sites of inflammation, is capable of functionally inactivating p53, a tumor suppressor that

normally functions to prevent proliferation of cells carrying genotoxic damage, a notion which provided a mechanistic link between inflammation and cancer.

3.5 Monocytes/macrophages and MIF as an antagonist of glucocorticoid action

Monocytes and macrophages belong to the main effector cells of the immune system, because they play a central role in the initiation, development, and outcome of the immune response (Adams *et al*., 1984). Glucocorticoids are potent anti-inflammatory and immunosuppressive agents that act on many cells including monocytes (Fausi *et al*., 1976). They are potent down-regulators of monocyte-secreted proinflammatory mediators including TNF (Han and Beutler, 1990), IL-1β (Lew *et al*., 1998) or IL-6 (Breuninger *et al*., 1993). However, the exact cellular mechanisms by which glucocorticoids exert their anti-inflammatory capacity in monocytes are only uncompletely understood. Monocytes have been shown to undergo rapid apoptosis in culture unless stimulated with LPS or with proinflammatory cytokines such as TNF or IL-1β, which enhance monocyte surviral by bypassing the initiation of apoptosis (Mangan and Wahl, 1991; Mangan *et al.*, 1991). Recent studies dealing with the role of anti-inflammatory cytokines and their suppressive effects on monocytes demonstrated that IL-4 as well as IL-10 are able to induce apoptosis in monocytes (Estaquier and Ameisen, 1997; Mangan *et al.,* 1992). Therefore, it was postulated that the regulatory role of anti-inflammatory cytokines in the development of inflammatory reactions also involves the regulation of monocyte apoptosis.

MIF was discovered as a mediator that acts as an endogenous counter-regulator of glucocorticoid action within the immune system. These studies showed that MIF expression by monocytes/macrophages and T cells is induced rather than suppressed by glucocorticoids (Calandra *et al*., 1995). MIF release from pituitary cells is also stimulated by glucocorticoid hormone (Calandra *et al*., 1998). MIF secretion by macrophages occurs at low physiological concentrations of glucocorticoids and decreases at higher (non-physiological) concentrations (Calandra *et al.,* 1995). When MIF is added together with dexamethasone, it can overcome, in a dose-dependent fashion, glucocorticoid inhibition of monocyte TNF, IL-1β, IL-6, and IL-8 secretion. When human monocytes were stimulated with LPS in the presence of dexamethasone (2×10^{-10} M) and control IgG, TNF production was inhibited by only 9.4 %, compared with 32.7 % for monocytes stimulated with LPS in the presence of dexamethasone (2×10^{-10} M) and neutralizing anti MIF IgG (Calandra *et al*., 1995). The balance between the glucocorticoid and MIF concentrations was found to be the main determinant of cytokine production in this *in vitro* system. The ability of MIF to override steroid action was confirmed by *in vivo* experiments in a mouse model of endotoxic shock. MIF, when administered

together with glucocorticoids 2 h before a lethal dose of LPS, was demonstrated to override glucocorticoid inhibition of LPS lethality (Calandra *et al*., 1995). Mitchell et al. demonstrated induction of apoptosis by glucocorticoid in human monocytes, the well-known anti-inflammatory and immunosuppressive effects of glucocorticoid on monocytes and macrophages are mediated by the induction of apoptosis.

MIF is induced by glucocorticoids and has the unique ability to override the anti-inflammatory and immunosuppressive effects of glucocorticoids on macrophages and T cells (Calandra *et al*., 1995; Bacher *et al*., 1996; Mitchell *et al.,* 1999). Thus, MIF and glucocorticoids function as a physiological counter-regulatory dyad that control the host inflammatory and immune response. Interestingly, this could be a connection between glucocorticoids and the enzymatic activity of MIF. The glucocorticoid receptor which has been thought to be regulated in its steroid-binding activity by oxidation/reduction of a vicinally spaced pair of cysteine residues (Simons and Pratt, 1995) could potentially be inactivated by a MIF-mediated redox event in which GSH and could the corresponding reductant.

Although the catalytic centers responsible for the tautomerase and oxidoredutase activities have been well studied, a clear-cut correlation between these catalytic properties and the physiologic and pathophysiologic functions of MIF is not yet established (Kleemann *et al.,* 1998a; Kleemann *et al*., 1998b; Kleemann *et al.,* 1999; Kleemann *et al*., 2000b; Bendrat *et al.,* 1997; Swope *et al*., 1998b; Hermanowski *et al.,* 1999). Among the several structure function correlations drawn, it only seems conclusive that the glucocorticoid counter-regulating activity of MIF is associated with its redox rather than tautomerase activity (Bendrat *et al*., 1997; Kleemann *et al*., 1999; Kleemann *et al*., 2000b).

3.6 Molecular mechanism of MIF action

In contrast to the broad knowledge gathered about the biological functions of MIF, relatively little is known about its mechanism(s) of action. Cytokines usually signal through receptors located on the plasma membrane of a target cell. For MIF, such a membrane receptor has not yet been found. Recently, our lab identified JAB1, as an intracellular receptor protein for MIF (Kleemann *et al*., 2000a). Other mechanistic studies have implied the ERK1/2 mitogen-activated protein kinase (MAPK) and activator protein-1 (AP-1) pathways in MIF-mediated signaling (Kleemann *et al*., 2000a; Mitchell *et al*., 1999; Onodera *et al*., 2000). As mentioned, under certain conditions or for certain target cells, MIF signaling may be accomplished by enzymatic conversion of target proteins and/or small molecule substrates.

3.6.1 MIF receptor

The identification of a membrane receptor for MIF has been reported in one study (Berenson *et al*., 1996). However, the proposed receptor was a macrophage ganglioside necessary for cell migration and not in any molecular relation to the protein known as MIF today (Weiser *et al*., 1989). The study should thus be considered descriptive.

Cytokine receptors are usually identified a few years after the cloning of the corresponding cytokine (Thomson, 1998). For MIF, cloning was achieved in 1989 (Weiser *et al.,* 1989), but no receptor has been identified, nor cloned or characterized biochemically since. As MIF is a critical mediator of septic shock, it was attempted to isolate a MIF receptor as a soluble, potentially shed, entity from urine samples of sepsis patients, this approach was unsuccessful (J. Bernhagen, S. Donnelly, and R. Bucala, unpublished). Nevertheless, there are at least two observations that indicate that a membrane receptor for MIF may exist. First, Ishizaka and co-workers have reported the identification, though not cloning, of an “uptake receptor” for the protein glycosylation inhibition factor (GIF) (Mikayama *et al*., 1993), on certain human T suppressor cells (Sugie *et al.,* 1997). Although a functional homology between MIF and GIF has been controversial (Mikayama *et al*., 1993; Liu *et al*., 1994), it is interesting to note that GIF is highly similar in structure to MIF (Mikayama *et al*., 1993; Kato *et al*., 1996). Secondly, an intriguing model of MIF signaling has been proposed by Bucala (Bucala, 2000), which argues for receptor-based signaling pathways modulated by MIF and which reconvenes many of the biochemical and biological activity features of MIF. In fact, modulation of typical signaling pathways by MIF has now been demonstrated by several laboratories, suggesting that a MIF receptor may be induced under certain conditions (see below).

3.6.2 MIF-modulated signaling pathways and role of MIF in apoptosis

The pleiotropic immunologic and endocrine regulatory profile exerted by MIF suggests that its actions may be connected with complex signal transduction pathways. However, a fast and comprehensive elucidation of how MIF modulates cellular signal transduction cascades has been strongly hampered by the lack of a cloned cytokine membrane receptor for MIF. Nevertheless, MIF-mediated signaling that is consistent with the involvement of typical receptor-based signaling (Bucala, 2000) has recently been reported for some pathways.

Mitchell and colleagues showed that both recombinant human MIF (rMIF) exogenously added to cells or endogenously released and acting in an apparent autocrine fashion, can stimulate the proliferation of quiescent fibroblasts (Mitchell *et al.,* 1999). This response was associated with a sustained phosphorylation and activation of the p44/p42 extracellular

signal-regulated (ERK1/2) subfamily of mitogen-activated protein kinases (MAPK). Activation of ERK led to the phosphorylation and activation of cytoplasmic phospholipase A2 (cPLA2), which then induced the release of arachidonic acid. This effect was later confirmed by Sampey (Sampey *et al*., 2001). cPLA2 is a critical component of the proinflammatory cascade (Hayakava *et al.,* 1993), and its enzymatic product, arachidonic acid, is known to activate the c-Jun N-terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathway (Cui *et al.,* 1997). Additionally, MIF was shown to override the suppressive effect of glucocorticoids on tumor necrosis factor (TNF)-induced arachidonic acid release. Glucocorticoid inhibition of TNF translation is known to be dependent on the blocking of JNK/SAPK activation (Swantek *et al*., 1997). Of note, the ability of MIF to override the glucocorticoid-mediated suppression of arachidonate release provides the first direct mechanistic explanation for the ability of MIF to counter-regulate the immunosuppressive activities of glucocorticoids.

MIF is also involved in calcium-dependent signal transduction. In rat testis, MIF was found to be secreted by Leydig cells and to accumulate in considerable amounts in the interstitial fluid. MIF then affected peritubular myoid cell function in a calcium-dependent manner. Wennemuth and colleagues showed that rMIF evoked a transient increase in calcium levels in peritubular cells but not in Sertoli cells from dissociated rat testis (Wennemuth *et al.,* 2000). The authors conclude that calcium is mobilized from endoplasmic reticulum stores and serves as a second messenger during MIF-mediated signal transduction in testis.

MIF action is associated with the pathogenesis of rheumatoid arthritis (Santos *et al*, 2001; Mikulowska *et al*., 1997; Leech *et al.,* 1998; Sampey *et al*., 2001). Degradation of extracellular matrix components by neutral matrix metalloproteinases (MMPs) is often seen as a typical pathological feature of rheumatoid arthritis. Onodera et al. (Onodera *et a*l., 2000) demonstrated that MIF up-regulates mRNAs of MMP-1 and MMP-3 in cultured synovial fibroblasts obtained from RA patients. No particular signaling pathway was identified to be directly modulated by MIF, but it was suggested that up-regulation of MMP gene transcription by MIF may be mediated via a complex signaling system involving protein kinase C, tyosine kinase and AP-1.

As mentioned, these MIF effects could well be based on an interaction between MIF and a receptor on the surface of a target cell. Bucala proposed that it may be especially MIF at low physiological concentrations that would act to regulate such pathways (Bucala, 2000). Although no particular signaling pathway has been demonstrated to be associated with the glucocorticoid overriding activity of MIF as reported in reviews by Lue (Lue *et al*., 2002), the low nanogram concentrations of MIF needed for this effect to occur and the fact that modulation of signal transduction events by MIF is observed at low MIF concentrations indicate that this major function of MIF could be mediated by one of the above signaling

pathways and thus by a receptor mechanism. However, no direct experimental evidence has yet been obtained for such a scenario and other reports imply a role for the enzymatic activity of MIF in MIF-mediated glucocorticoid overriding (Kleemann *et al*., 1999).

Recently an intriguing signaling mechanism of MIF was unravelled that apparently bypasses the need for a cell membrane receptor. MIF was found to interact with an intracellular protein, the transcriptional coactivator and cell cycle regulator JAB1 and to modulate several cellular signaling events through JAB1 (Kleemann *et al*., 2000a). The recent identification of an interaction of JAB1/CSN5 with the tumor suppressor p53 (Bech-Otschir *et al.,* 2001) offers an intriguing molecular connection between MIF-mediated signaling, the COP9 signalosome and MIF-modulated cell proliferation effects, and could provide an important molecular explanation for the observed potent roles of MIF in inflammation and tumorigenesis.

Apoptosis is a key cellular event, contributing, among many other processes, to the degree of immune activation and inflammation under various conditions. Recently, Hudson and colleagues showed that MIF participates in the regulation of apoptosis. They reported that either ectopically expressed MIF or exogenously added rMIF could overcome p53-induced growth arrest, senescence and apoptosis by suppressing p53-dependent transcriptional activation (Hudson *et al.,* 1999). On the other hand, MIF has also been shown to promote TNF and nitric oxide (NO) production in murine monocytes and macrophages (Bernhagen *et al.,* 1994) and TNF and NO induction by MIF appears to be associated with MIF-induced killing of *Leishmania major* by macrophages (Jüttner *et al*., 1998; Xu *et al*., 1998). Nitric oxide in turn can potently induce macrophage apoptosis via a p53-dependent mechanism (Messmer *et al.,* 1996). As MIF can negatively regulate p53 activity and should thus be able to also protect macrophages from NO-induced apoptosis, MIF may normally act to protect macrophages from the destructive machinery these cells use to kill invading microorganisms. Upon invasion however, it appears that MIF would act to enhance or amplify the proinflammatory cascade launched by the host for efficient defense. The effects of MIF on NO-induced apoptosis will need to be studied in more detail to clarify the role of MIF in these signaling events. Such studies should also involve other prominent signaling pathways, i.e. the mitogen-activated extracellular signal-regulated kinases (MEKs), ERKs, JNK/SAPK, and p38 kinase, which are all associated with the regulation of p53-dependent apoptosis and which can be induced by stimuli such as Cisplatin or UV radiation (Hong *et al*., 1999; Persons *et al*., 2000; She *et al*., 2000). A relationship between MIF, the MAPK cascade and p53-dependent apoptosis has not been addressed. Recently, Mitchell et al. reported that MIF is required for the maintenance of activation-induced p53 induction and macrophage apoptosis (Mitchell *et al*., 2002). This finding confirmed the role of MIF in the innate immune response. However, identification of a specific interaction between p53 and JAB1 (Bech-Otshir *et al*., 2001) and MIF and JAB1 (Kleemann *et al*., 2000a) could provide a connection

between apoptotic processes, the regulation of cellular stress by MIF and the JAB1- and p53-dependent pathways of MIF action. Overall, MIF functions include effects on cell proliferation and differentiation, cell migration and on the gene expression of other inflammatory mediators (Mitchell *et al*., 2000; Lue *et al*., 2002). MIF also can stimulate or inhibit cell proliferation (Mittchell *et al*., 2000; Kleemann *et al*., 2000a; Abe *et al*., 2001).

3.6.3 Interaction of MIF and JAB1

Considering that MIF is constitutively expressed in many cells (Bernhagen *et al.,* 1998; Wennemuth *et al*., 2000) and exhibits catalytic properties reminiscent of certain cellular enzymes (Kleemann *et al*., 1998a; Rosengren *et al*., 1996), it was reasoned that MIF interacts with intracellular proteins (Lue *et al*., 2002). The two hybrid screening (THS) method is a powerful approach to identify such interactions *in vivo*. When a human MIF cDNA was screened against a human brain cDNA library in a THS, several clones expressing proteins that appeared to interact specifically with MIF were isolated. Sequencing of one such clone led to the identification of the coactivator JAB1 (Kleemann *et al*., 2000a; Claret *et al*., 1996; Bounpheng *et al*., 2000). JAB1 was initially discovered as a coactivator of AP-1 transcriptional activity (Claret *et al*., 1996), but several other functions for JAB1 such as regulation of $p27^{Kip1}$ degradation, binding to glucocorticoid and progesterone receptors, binding of integrin protein LFA-1, interaction with p53, and its role as a signalosome component have since been discovered (Tomoda *et al.,* 1999; Seeger *et al*., 1998; Wei *et al*., 1999; Chauchereau *et al*., 2000; Bech-Otschir *et al*., 2001; Chamovitz *et al*., 2001).
In the THS, they also identified two other sequences that appeared to specifically interact with MIF, but work is still in progress to verify and characterize these interactions in detail (J. Bernhagen, unpublished). MIF-JAB1/CSN5 interaction was verified to be also highly specific at the protein level by coimmunoprecipitation studies in several systems, including cell-free and cellular systems under native, non-overexpressed, conditions. Of note, MIF-JAB1/CSN5 interaction can occur following uptake of MIF into target cells by non-receptor-mediated pathways, offering a simple signaling route for MIF (Kleemann *et al*., 2000a).
Subsequent functional studies showed that MIF can antagonize several JAB1/CSN5-based cellular effects. MIF inhibits JAB1/CSN5-mediated AP-1 activity and reduces JNK activity stimulated by JAB1/CSN5. While JAB1/CSN5 rescues fibroblasts from G1 growth arrest, MIF was found to counteract this effect, resulting in a net growth-inhibitory effect on fibroblasts. This effect is JAB1/CSN5-dependent and appears to occur through stabilization of $p27^{Kip1}$ by MIF (Kleemann *et al.,* 2000a). As mentioned above, MIF appears to have a proliferation-enhancing effect on quiescent fibroblasts (Mitchell *et al.,* 1999). Bucala suggests that higher MIF concentrations, that are typically associated with some of the enzymatic activities of MIF

and its direct stimulation of TNF and NO in macrophages, could be responsible for non-receptor-based JAB1/CSN5-mediated MIF signaling, while receptor-mediated MIF signaling may mainly occur at low concentrations of MIF (Bucala, 2000). They have observed functional differences of MIF over the entire concentration range that could be consistent with a model where stimulatory MIF effects mainly occur in quiescent cellular systems, while cellular effects under prestimulated conditions could generally be counter-regulated by MIF (Calandra *et al.,* 1995; Kleemann *et al.,* 2000; Bernhagen *et al.,* 1994; H. Lue and J. Bernhagen, unpublished).

3.7 Aim of the thesis

The cytokine MIF exhibits important immunological functions and is unusual in showing enzymatic properties. Two enzymatic activities have been reported to be catalyzed by MIF. One of these activities is a thiol-protein oxidoreductase activity and this activity is dependent on the CALC motif in the central region of the MIF sequence. Renatured MIF contains both oxidized and reduced MIF species and an intramolecular disulfide with catalytic redox potential can form at the CXXC site.

There had been evidence that the catalytic oxidoreductase activity of MIF is important for some of its immunological functions. However, the precise biochemical parameters of the oxidoreductase activity of MIF had been unresolved. In particular, oxidized and reduced MIF species had not been available at a preparative scale and the redox potency, i.e. the redox potential of MIF, was unknown. Moreover, there had been no studies directly addressing potential links between the redox activity of MIF and the reported molecular pathways that MIF is associated with during cellular signalling.

It was therefore one aim of this thesis to characterize the redox potency of MIF more precisely. Following separation of oxidized and reduced MIF, the redox potential was to be determined. Because it turned out that the oxidized and reduced species of full-length MIF were almost impossible to separate from each other, partial sequence MIF-derived peptides were analyzed to learn more about the redox properties of MIF. This approach seemed promising, as it had been shown for other thiol-protein oxidoreductase that such partial sequence peptides, showed a lot of the activities of the corresponding full-length proteins.

MIF(50-65) was chosen and it was an aim of the thesis to broadly characterize the redox and biological properties of this peptide. This included separation of oxidized and reduced species, determination of the redox potential and redox reactivity, conformational studies and an investigation of potential MIF-like biological activities of the peptide.

As during this thesis, JAB1 was discovered as an intracellular MIF binding protein, and because binding could be competed for with MIF(50-65), I also studied whether the peptide could influence MIF-JAB1-based biological effects.

In addition to the MIF-JAB1 pathway, p53-dependent apoptosis-regulation by MIF has been a second cellular MIF-regulated pathway with molecular components identified. I therefore addressed the possibility, whether apoptosis-regulation through MIF was linked to redox regulation and the redox activity of MIF. To investigate this issue, I wished to establish a cellular system of pro-oxidative stress-induced apoptosis.

In summary, it was therefore the overall aim of this thesis to establish and determine molecular parameters that could serve to link the catalytic redox activity of MIF and its cellular functions.

4 Materials and Methods

4.1 Bacterial clones, plasmids and cell lines

4.1.1 Bacterial clones

Competent *E. coli* DH5α™ were bought from Gibco BRL, competent *Epicurian coli* ® BL21/DE3 were bought from Stratagene. The clones were stored at - 80°C until they were used. After successful transformation, glycerol stocks were made (15 % glycerol) and the glycerol stocks stored at - 80°C.

***E. coli*-strain**	**Genotype/Phenotype**	**Reference**
BL21/DE3	F^- *dcm ompT hsdS*($r_B^-m_B^-$) *gal* λ(DE3)	Stratagene, Heidelberg, Germany
DH5α	F^-Φ80d*lac*ZΔM15Δ(*lac*ZYA-*arg*F)U169 *sup*E44 *deo*R *rec*A1 *end*A1 *hsd*R17($r_K^-m_K^+$) *pho*A λ^-*thi*-1 *gyr*A96 *rel*A1	Gibco, Eggenstein, Germany

4.1.2 Plasmids

The plasmids which were generated for this thesis were created using pMIF which was cloned by Bernhagen *et al*., 1994.

Plasmid	Relevant genotype	Resistance	Reference
pET11b	T7-Promotor region	Amp	Novagen/Calbiochem, Heidenberg, Germany
pUHD 10-3	PhCMV*-1, MCS	Amp	Gossen/Bujard, ZMBH Heidelberg
pUHG 16-3	PhCMV*-1, lacZ	Amp	Gossen/Bujard, ZMBH Heidelberg
pUHD 16-1	lacZ-transfections efficiency	Amp	Gossen/Bujard, ZMBH Heidelberg
pUHD 15-1 neo	tTA G418	Amp	Gossen/Bujard, ZMBH Heidelberg
pMIF	huMIF in pET 11b	Amp	Bernhagen *et al.,* 1994
PC60SMIF	huC60SMIF in pET 11b	Amp	Kleemann *et al.,* 1998
pMIFRP	huMIF in pUHD 10-3	Amp	Andrea Braun, Diplomarbeit, 1998
PC60SMIFRP	huC60SMIF in pUHD 10-3	Amp	this work

4.1.3 Cell lines

Cell line	Characterization	Reference
HeLa	Human cervix carcinoma adherent	H. Bujard, ZMBH Heidelberg
HtTA	HeLa with transactivator tTA adherent	H. Bujard, ZMBH Heidelberg
HL-60	Human acute myeloid leukemia in suspension	DSMZ, Braunschweig
Kym-1	Human rhabdomyosarcoma adherent	K. Pfizenmaier, IZI Stuttgart
COS-1	African green monkey kidney adherent	DSMZ, Braunschweig
THP-1	Human acute monocytic leukema in suspension	DSMZ, Braunschweig
NIH 3T3	Swiss mouse embryo adherent	DSMZ, Braunschweig
Jurkat	Human Jurkat T-cell in suspension	F.-J. Johannes*, IGB Stuttgart

*, deceased

4.2 Equipments, materials, chemicals, enzymes and primers

4.2.1 Equipments

- Beckman J2-21 Centrifuge — Beckman, München, Germany
- Centrifuge Biofuge fresco — Kendro laboratory products, Hanau, Germany
- CO_2–incubator B5060 EC-C0_2 — Kendro laboratory products, Hanau, Germany
- Drygel slab gel dryer SE 1160 — Hoefer Scientific Instruments, San Fransisco, USA

- DU®-65 spectrophotometer	Beckman Instruments, München, Germany
- ELISA-reader microplate reader 3550	Bio-Rad laboratories GmbH, München, Germany
- Eppendorf centrifuge 5415C	Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany
- FACSCalibur	Becton Dickinson GmbH, Heidelberg, Germany
- Fluorescence microscope	Nikon-Microphot-FXA, Japan
- FPLC chromatography system	Pharmacia, Freiburg, Germany
- French pressure cell press	Colora, Lorch, Germany
- Herasafe laminar flow hood	Kendro laboratory products, Hanau, Germany
- HPLC chromatography system	Beckman, Germany
- Horizontal gel electrophoresis-apparatus Horizon® 11.14	Life Technologies, Eggenstein, Germany
- Jasco J-700 CD-spectropolarimeter	JAPAN Spectroscopic LTD, Tokyo, Japan
- KRATOS compact MALDI III laser-desorptions-mass-spectrometer	Shimadzu Europa GmbH, Duisburg Germany
- LABCONO freeze dry system	UNIEQUIP, Martinsried, Germany
- MinifugeT	Heraeus, Fellbach, Germany
- Mini-PROTEAN II dual slab cell mini electrophoresis system	BIORAD, München, Germany
- Mini trans-blot transfer cell	BIORAD, München, Germany
- Perkin Elmer Cetus 480 DNA thermal cycler	Perkin Elmer Cetus, Norwalk, USA
- pH-meter	Knick electrophoresis measure equipment GmbH & Co., Berlin, Germany
- Phast system	Amersham Biosciences, Freiburg, Germany
- Pipette device pipetman	Gilson, Villiers-le-Bel, France
- Pipette device vacuboy	INTERGRA Biosciences, Fernwald, Germany
- Pipette device pipetboy	INTERGRA Biosciences, Fernwald, Germany
- Pipette device multipipette plus	Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany

- Polaroid MP-4 camera	Photo Hirrlinger, Stuttgart, Germany
- Rosys anthos Lucy 2	Anthos Labotechnology, Germany
- Speckol 1200	Analytik Jena AG, Jena, Germany
- UNIVAPO 100 H	UNIEQUIP, Martinsried, Germany
- UNIVAPO autospin 150 AS	UNIEQUIP, Martinsried, Germany
- Vacuum pump laborport N86 KN18	KNF Neuberger GmbH, Freiburg Germany
- Vortexer Vortex Genie-2	Scientific Industries, Inc. Bohemia NY, USA
- Water bath GFL 1002	Company for labor technology GmbH, Burgwedel, Germany

4.2.2 Materials

- C8-SepPak RP-cartridge	Waters GmbH, Eschborn, Germany
- C18 column	Grom, Herrenberg, Germany
- CENTRI-SEP column	Princeton separations, Adelphia, USA
- Filter Rotrand 0.2 µm sterile	Schleicher & Schuell, Dassel, Germany
- Single-use cuvettes (1 ml)	Greiner Labortechnik, Frickenhausen, Germany
- Eppendorf reaction tubes	Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany
- ELISA 96-well plate Immunolon II flat bottom	Dynatech Corporation, Burlington MA, USA
- Filter 0.45 µm for FPLC-solution	Waters GmbH, Eschborn, Germany
- GeneAmp® PCR-reactions	Roth, Karlsruhe, Germany
- Greiner PP tubes	Greiner Labortechnik, Frickenhausen, Germany
- Greiner cell culture plate	Greiner Labortechnik, Frickenhausen, Germany
- MONO Q FPLC® anion exchanger column	Amersham Biosciences, Freiburg, Germany
- Nitrocellulose membrane	Schleicher & Schuell, Dassel,Germany
- Spectra Por® dialysis membrane, MWCO: 8000 Da	Roth, Karlsruhe, Germany
- Whatman 3 mm paper	Roth, Karlsruhe, Germany

4.2.3 Chemicals

Acetonitril p.a.	Mallinckrodt Baker B.V., Deventer, NL
Acetic acid	Merck, Darmstadt, Germany
Acrylamide/bisacrylamide solution (29%/1%)	BIO-RAD laboratories, München, Germany
Agarose	Life Technologies, Eggenstein, Germany
Ammonium hydrogencarbonate	Sigma-Aldrich, Steinheim, Germany
Ammonium peroxidisulfide	Sigma-Aldrich, Steinheim, Germany
BIOXYTECH GSH-400 colorimetric assay	Calbiochem-Novabiochem, Heidelberg, Germany
BSA	Sigma-Aldrich, Steinheim, Germany
Bromphenol blue	Sigma-Aldrich, Steinheim, Germany
CAPS	Sigma-Aldrich, Steinheim, Germany
Camptothecin	Sigma-Aldrich, Steinheim, Germany
CDTA	Sigma-Aldrich, Steinheim, Germany
Cysteine/L-Cystine	Sigma-Aldrich, Steinheim, Germany
Coomassie-Brilliant-Blue G-350	Serva Feinbiochemica, Heidelberg, Germany
DEAE/Dextran	Promega , USA
Dexamethasone	Sigma-Aldrich, Steinheim, Germany
DMSO	Sigma-Aldrich, Steinheim, Germany
Doxycycline	Sigma-Aldrich, Steinheim, Germany
D-Dopachrome	Sigma-Aldrich, Steinheim, Germany
DTT	Sigma-Aldrich, Steinheim, Germany
DMEM medium	Life Technologies GmbH, Karlsruhe, Germany
EMEM medium	Life Technologies GmbH, Karlsruhe, Germany

EDTA	Sigma-Aldrich, Steinheim, Germany
Ethidium bromide	Sigma-Aldrich, Steinheim, Germany
Glutathione, reduced form	Sigma-Aldrich, Steinheim, Germany
Glutathione, oxidized form	Sigma-Aldrich, Steinheim, Germany
Glycerine	Sigma-Aldrich, Steinheim, Germany
HED	Sigma-Aldrich, Steinheim, Germany
Hydrochloride	Merck, Darmstadt, Germany
IPTG	Life Technologies, Eggenstein, Germany
L-glutamine	Life Technologies GmbH, Karlsruhe, Germany
LPS 0111:B4	Merck, Darmstadt, Germany
Magnesium sulfate	Sigma-Aldrich, Steinheim, Germany
Methanol	Merck, Darmstadt, Germany
β-Mercaptoethanol	Sigma-Aldrich, Steinheim, Germany
Molecular weight standard for SDS-PAGE	Gibco BRL, Eggenstein, Germany
Meta phosphoric acid	Merck KGaA, Damstadt, Germany
NADPH	Sigma-Aldrich, Steinheim, Germany
ONPG	Roche Diagnostics, Mannheim, Germany
2-Propanole	Sigma-Aldrich, Steinheim, Germany
Penicillin/streptomycin	LifeTechnologies GmbH, Karlsruhe, Germany
PhastGel™ silver kit	Amersham Biosciences, Freiburg, Germany
Phenol:chloroform:IAA (25:24:1)	Sigma-Aldrich, Steinheim, Germany
Phosphoric acid	Merck, Darmstadt, Germany
Potassium dihydrogenphosphate dihydrate	Merck, Darmstadt, Germany
Potassium chloride	Sigma-Aldrich, Steinheim, Germany
Propidium iodide	Sigma-Aldrich, Steinheim, Germany

Protein assay (Bradford reagent)	Biorad, München, Germany
RPMI 1640 medium	Life Technologies GmbH, Karlsruhe, Germany
RPMI 1640 without L-glutamine and L-cysteine	Life Technologies GmbH, Karlsruhe, Germany
Sacharose	Sigma-Aldrich, Steinheim, Germany
Sodium acetate	Merck, Darmstadt, Germany
Sodium dihydrogenphosphate monohydrate/ dihydrate	Merck, Darmstadt, Germany / Roth, Karlsruhe, Germany
Sodium dodecysulfate	Merck, Darmstadt, Germany
Sodium chloride	Merck, Darmstadt, Germany
Sodium carbonat	Merck, Darmstadt, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium m-periodate	Sigma-Aldrich, Steinheim, Germany
Sodium phosphate	Roth, Karlsruhe, Germany
Streptavidin peroxidase	Boehringer Mannheim, Germany
Sulfuric acid	Sigma-Aldrich, Steinheim, Germany
Super Signal West Dura extended duration substrate	Pierce/KMF Laborchemie GmbH, St. Augustin, Germany
TEMED	Sigma-Aldrich, Steinheim, Germany/ AMRESCO, Solon, USA
Tri chlorine acetic acid	Merck, Darmstadt, Germany
TFA	Sigma-Aldrich, Steinheim, Germany
Tetramethylbenzidin-solution	Merck, Darmstadt, Germany
Tris	Merck, Darmstadt, Germany
Triton X-100	Sigma-Aldrich, Steinheim, Germany
Tween 20	Sigma-Aldrich, Steinheim, Germany

All other reagents were purchased from Sigma-Aldrich, Steinheim, Germany or Merck, Darmstadt, Germany.

4.2.4 Enzymes, antibodies and molecular biology reagents

Biotinylated anti-human TNF antibody (BAF210)	R & D Systems, Wiesbaden, Germany
Monoclonal anti-human TNF antibody (MAB 610)	R & D Systems, Wiesbaden, Germany
Anti $p27^{Kip1}$ mouse monoclonal antibody (F-8)	Santa Cruz Biotechnology, Heidelberg, Germany
POD-Streptavidin	Santa Cruz Biotechnology, Heidelberg, Germany
Anti-phospho-c-Jun antibody (clone KM-1)	Santa Cruz Biotechnology, Heidelberg, Germany
Anti-c-Jun antibody (clone H-79)	Santa Cruz Biotechnology, Heidelberg, Germany
Anti-actin antibody	Sigma-Aldrich, Steinheim, Germany
Peroxidase-conjugated secondary antibody	Dianova, Hamburg, Germany
Annexin-V-fluos staining kit	Roche, Mannheim, Germany
Cell death detection ELISA	Boehringer Mannheim, Germany
Desoxynucleotide	Life Technologies, Karlsruhe, Germany
Dnase I	Boehringer Mannheim, Germany
DNA ligase	New England Biolabs GmbH, Heidelberg, Germany
Rnase	New England Biolabs GmbH, Heidelberg, Germany
SuperFect-transfection reagent	QIAGEN GmbH, Hilden, Germany
T4 DNA Ligase	New England Biolabs GmbH, Heidelberg, Germany

$Vent_R$® DNA polymerase	New England Biolabs GmbH, Heidelberg, Germany

All other enzymes were purchased from New England Biolabs, Heidelberg, Germany.

4.2.5 Overview of the used primers

All primers were bought from Life Technologies as lyophilized powders, and were resuspended in aqua bidest. To yield a 200 µM stock solution, which was then further diluted to a 10 µM working solution.

Table 1*:* Overview of the used primers for the cloning of pC60SMIFRP.

	Upstream primer [name, sequence]	**Downstream primer [name, sequence]**
pC60SMIFRP	N-Sac II 5‘ GCG CCG CGG ATG CCG ATG TTC ATC GTA AAC AC 3‘	huMIF 356-323 5‘ CGG GAT CCT TAG GCG AAG GTG GAG TTG TTC CAG C 3‘

Table 2: The primers used to sequence plasmid pC60SMIFRP.

	Sequence
Upstream T7-1	TAATACGACTCACTATAGGG
Dowstream T7-2	GCTAGTTATTGCTCAGCGG

4.3 Media, buffer and solutions

4.3.1 Media for bacteria and cell culture

LB-medium

Trypton	10	g
Yeast extract	5	g
NaCl	10	g
H_2O	1	L

Ampicillin: 100 mg/ml in ethanol stored at -20°C
For use diluted 1:1000

Cell culture medium for HtTA: 9.7 g/L EMEM powder and 2.2 g/L $NaHCO_3$, pH 7.0; 10 % FCS
100 µg/ml penicillin
100 µg/ml streptomycin
1 x MEM vitamins
1 x non-essential amino acids
2 mM L-glutamine

Cell culture medium for HL60, Jurkat T and THP-1 cells

RPMI 1640
10 % FCS
100 µg/ml penicillin
100 µg/ml streptomycin
2 mM L-glutamine

Cell culture medium for Kym-1 cells

RPMI 1640
5 % FCS
100 µg/ml penicillin
100 µg/ml streptomycin
2 mM L-glutamine

Cell culture for medium COS-1 cells

Dulbecco´s MEM
10 % FCS
100 µg/ml penicillin
100 µg/ml streptomycin

Cell culture for medium NIH 3T3 cells

Dulbecco´s MEM
10 % FCS
100 µg/ml penicillin
100 µg/ml streptomycin
2 mM L-glutamine

4.3.2 Buffers and solutions

If not indicated differently, all buffers and solutions were prepared in reagent grade water (ddH_2O).

Ca - Mg free medium

NaCl	140	mM
KCl	2.7	mM
Na_2HPO_4	16	mM
KH_2PO_4	1.5	mM
EDTA	0.8	mM

Trypsin/EDTA

Trypsin	0.025 % - 0.25 %
EDTA	0.02 % - 0.2 %
	In Ca - Mg free medium

PBS (phosphate-buffered saline) pH 7.2

NaCl	37	mM
KCl	2.7	mM
K_2HPO_4	1.5	mM
Na_2HPO_4	8.1	mM

TBS (Tris-buffered saline) pH 7.3

Tris-HCl	20	mM
NaCl	150	mM

TE (Tris-EDTA buffer) pH 8.0

Tris-HCl	10	mM
EDTA	0.1	mM

Sodium phosphate buffer

NaH_2PO_4	500	mM

Dox (doxycycline) 1 mg/ml
steril-filtered and stored at 4°C

Cam (camptothecin) 2 mg/ml in DMSO
Steril-filtered and stored at - 20°C

Dex (dexamethasone) 10^{-2} M in ethanol
prepared freshly before use

LPS (lipopolysaccharide) 1 mg/ml

RIPA lysis buffer pH 7.5

Tris-HCl	50	mM
NaCl	150	mM
Igepal CA-630	1	%
Desoxychol acid	0.5	%
SDS	1	%
EDTA	2	mM

Lysis-buffer pH 7.8

Tris-phosphate	25	mM
DTT	2	mM
EDTA	2	mM
Glycerol	10	%
Triton X-100	1	%

ONPG-solution 4 mg/ml 2-Nitrophenylgalactosid in 100 mM sodium phosphate buffer, pH 7.5

Z-buffer

Sodium phosphate buffer pH 7.0	100	mM
KCl	10	mM
$MgSO_4$	1	M

Substrate solution for β-Gal test

For 1 sample

Z-buffer	700	µl
β-Mercaptoethanol	50	mM
ONPG solution	200	µl
	Prepare fresh before use	

Stop solution for β-Gal test

Na_2CO_3	1	M

Agarose gel electrophoresis

10 x TBE-buffer

Tris	89	mM
Boric acid	89	mM
EDTA	2	mM

Agarose gel

1 % (w/v) agarose in 1 x TBE; boil in microwave and stain the polymerized gels with a 0.5 µg/ml ethidium bromide solution.

10 x sample buffer

Phenolblue	0.2	% (w/v)
Xylencyanol FF	0.2	% (w/v)
Orange G	0.2	% (w/v)
Saccharose	60	% (w/v)

Ethidium bromide stock solution

3,8-Diamino-6-ethyl-5-phenyl-phenanthridiumbromide (Stratagene, Heidelberg, Germany) 10 mg/ml in ddH_2O; store at 4°C and do not expose to light.

CD measurements

Dialysis buffer (of the last step of the renaturation procedure)

NaPP	20	mM	pH 7.2

CD denaturation buffer

NaPP	20	mM	pH 7.2
Gdn-HCl	8	M	

FPLC buffer

For purification of rhuMIF with the Mono Q anion exchange column (Amersham Biosciences):

Low salt loading buffer

Tris-HCl	50	mM	pH 7.2
NaCl	150	mM	

High salt elution buffer

Tris-HCl	50	mM	pH 7.5
NaCl	2	M	

HPLC buffer

For purification of MIF(50-65) with the Grom C18 column (Grom, Herrenberg, Germany):

Buffer A	0.058 % TFA in ddH_2O
Buffer B	0.050 % TFA in 90 % acetonitrile

Insulintest

1 mM insulin stock solution	6.12	mg/ml in 50 mM Tris pH 8.0

Working mixture

KPP	100	mM	pH 7.5
EDTA	2	mM	
Insulin	1	mg/ml	

Glutathione	200	mM

Tautomerase assay

Solution (25 mM potassium phosphate, pH 6.0/0.5 mM EDTA)

$K_2HPO_4.3H_2O$	25	mM
KH_2PO_4	25	mM
EDTA	0.5	mM

Preparation of L-Dopa

L-3,4 dihydroxyphenylalanine methyl ester	4	mM	(a)
Sodium m-periodate	8	mM	(b)
	1vol. (a) + 1 vol. (b) mix at room temperature for 5 min, then put on ice.		

Working mixture 25 mM potassium phosphate, pH 6.0/0.5 mM EDTA
100 µl L-Dopa
100 nM MIF or 3.3 µM peptide MIF(50-65)

MIF renaturation

Denaturation buffer

Gdn-HCl	6	M	pH 7.2
NaPP	20	mM	pH 7.2
DTT	10	mM	

Renaturation buffer 1

NaPP	20	mM	pH 7.2
DTT	5	mM	

Renaturation buffer 2

NaPP	20	mM	pH 7.2

Oxidoreductase test

Working mix

Tris	100	mM	pH 8.0
NADPH	0.4	mM	
BSA	0.01	% (w/v)	
EDTA	2	mM	
Glutathione reductase	0.006	mg/ml	

Glutathione	1	mM
HED	14	mM

RNase stock solution

10 mg/ml dissolved in ddH_2O and stored - 20°C.

SepPak hydrophobic reverse phase chromatography

Acetonitrile in ddH_2O	20	% (v/v)
Acetonitrile in ddH_2O	60	% (v/v)
Methanol	100	% (v/v)

SDS-PAGE

Tris-HCl stock solution for separation gel	1.5	M	pH 8.8
Tris-HCl stock solution for stacking gel	0.5	M	pH 6.8

2 x Sample buffer

Tris-HCl	0.125	M	pH 6.8
SDS	4	% (w/v)	
Glycerol	20	% (v/v)	
β-Mercaptoethanol	10	% (v/v)	
Bromphenolblue	0.01	%(w/v)	

SDS solution

SDS	10	%

Staining buffer

Coomassie Blue R-250	1	% (w/v)
Methanol	50	% (v/v)
Acetic acid	10	% (v/v)

Destaining buffer

Methanol	50	% (v/v)
Acetic acid	10	% (v/v)

Storage buffer

Acetic acid	7	% (v/v)
Glycerol	5	% (v/v)

Tank buffer

Tris-HCl	25	mM	pH 8.3
SDS	0.1	% (w/v)	
Glycin	192	mM	

Ammonium persulphate (APS) solution

APS	10	% (w/v)

Separation gel (12 %)

Acrylamide	8	ml
1.5 M Tris-HCl pH 8.8	5	ml
SDS 10 %	0.2	ml
ddH_2O	6.7	ml
APS-solution	0.1	ml
TEMED	0.01	ml

Stacking gel

Acrylamide	1.34	ml
0.5 M Tris-HCl pH 6.8	2.5	ml
SDS 10 %	0.1	ml
ddH_2O	6.0	ml
APS solution	0.05	ml
TEMED	0.01	ml

Tris-EDTA-buffer

TE pH 8.0

Tris-HCl	10	mM	pH 8.0
EDTA	1	mM	

TE pH 7.5

Tris-HCl	10	mM	pH 7.5
EDTA	1	mM	

Western Blot

High salt TBS

Tris-HCl	50	mM	pH 7.5
NaCl	500	mM	

Milk powder blocking buffer

Tris-HCl	50	mM	pH 7.5
NaCl	500	mM	
Milk powder	5	% (w/v)	

High salt-Tween-TBS

Tris-HCl	50	mM	pH 7.5
NaCl	500	mM	
Tween-20	0.05	% (v/v)	

Low salt TBS

Tris-HCl	50	mM	pH 7.5
NaCl	150	mM	

Transfer buffer

CAPS	10	mM	pH 11.0
Methanol	20	% (v/v)	

4.4 Molecular biology methods

4.4.1 Miniprep

QIAprep was used according to QIAGEN to purify a small amount of up to 20 µg of plasmid DNA from a 1-5 ml overnight culture. pET11b/*E. coli* DH5α was transferred into 1-5 ml LB medium and incubated overnight at 37°C, 250 rpm. 2 ml culture medium was centrifuged at room temperature (RT) for 5 min, 14000 rpm. The bacterial pellet was resuspended in 250 µl of buffer P1 and transferred to a microfuge tube. After addition of 250 µl of buffer P2, the tube was inverted gently 4-6 times to mix until the solution became viscous and slightly clear. The viscous lysate was incubated for 5 min and then 350 µl buffer N3 was added and mixed immediately well by inverting 4-6 times. The mixture was centrifuged at RT for 10 min at 14000 rpm.

During centrifugation, the QIAprep columns were placed in 2 ml collection tubes. The supernatants were applied to QIAprep columns by decanting or pipetting. The columns were centrifuged at 14000 rpm for 30 - 60 seconds at RT. The flow-through was discarded.
The QIAprep spin column were washed with 0.5 ml of buffer PB to remove trace nuclease activity.
The QIAprep spin columns were washed with 0.75 ml of buffer PE and centrifuged for 30-60 sec. The flow-through was discarded and centrifuged for 60 seconds to remove the residual wash buffer.
The QIAprep spin columns were placed in clean 1.5 ml microfuge tubes. To elute DNA, 50 µl of buffer EB or ddH_2O were added to the center of each QIAprep column, incubated by standing for 1 min, and centrifuged for 1 min.
The purified plasmid DNA was stored at – 20°C.

4.4.2 Maxiprep

QIAprep was used according to QIAGEN to purify a small amount up of to 200 µg of plasmid DNA from a 100-500 ml overnight culture. pET11b/*E. coli* DH 5α was centrifuged at 20,000 x g for 30 min at 4°C. The bacterial pellet was resuspended in 10 ml of buffer P1 and transferred to a microfuge tube. After addition of 10 ml of buffer P2, the tube was inverted gently 4-6 times until the solution became viscous and slightly clear. The viscous lysate was incubated for 5 min and then 10 ml buffer P3 was added and mixed immediately well by inverting 4-6 times and incubated on ice for 20 min. The mixture was centrifuged at 20,000 x g for 30 min at 4°C.
During centrifugation, a QIAGEN-tip 500 was equilibrated by applying 10 ml buffer QBT, and the column allowed to empty by gravity flow. The supernatants were applied to a QIAGEN-tip and allowed to enter the resin by gravity flow.
The QIAGEN-tip was washed with 1 x 30 ml of buffer QC to remove trace nuclease activity. To elute DNA, 15 ml of buffer QF, and DNA were precipitated with 0.7 volumes of room temperature isopropanol, and was centrifuged immediately at 20,000 x g for 30 min at 4°C. The supernatant was removed carefully. DNA was washed with 5 ml of 80 % ethanol and was redissolved in a suitable volume of buffer.
The purified plasmid DNA was stored at – 20°C.

4.4.3 Purification of plasmid DNA using Jetsorb kit (QIAGEN)

The Jetsorb extraction kit was used to extract DNA from agarose gels according to the manufacturer´s instructions. Purified DNA was resuspended in TE buffer and quantified with a UV-VIS-spectrophotometer at a wavelength 280 nm and stored at – 20°C.

4.4.4 Agarose gel electrophoresis

To analyse and isolate the DNA, agarose gel electrophoresis was carried out according to Sambrook et al. (1989). DNA fragments with a size of up to 0.5 kb were applied to a gel of 0.8 to 1 % agarose. The voltage was kept at 100 V to avoid high temperature. The DNA was stained with ethidium bromide and visualized on a UV light box at 356 nm.

4.4.5 Digestion of DNA with restriction enzymes

1 µg purified DNA was incubated with 1 U restriction enzyme and a corresponding specific buffer for 2 h at 37°C. To assess complete digestion, the fragments were separated by agarose gel electrophoresis.

4.4.6 Cloning of MIF and C60SMIF in the Tet-off response plasmid

The open reading frames for human MIF and the redox mutant C60SMIF were amplified originally from Jurkat H33HJ-JA1 T-cell DNA and cloned into the pET11b expression vector as described previously (Bernhagen *et al*., 1994; Kleemann *et al*., 1998a; Kleemann *et al*., 1999).

The following Tet-off system-related plasmids were used: pUHD15-1neo, a “regulator” plasmid for both the transient and stable transfections containing the tetracycline-controlled transcriptional activator (tTA) gene and a neomycin resistance gene; pUHD10-3, a “response” plasmid for both the transient and stable transfections containing the target gene cloned after the tetracycline-responsive element (TRE) and a minimal cytomegalovirus (CMV) promoter; pUHD16-1, a “response” plasmid for monitoring the efficiency of the transient transfections containing a lacZ reporter gene cloned after a constitutive CMV promoter; pX343, a “response” plasmid containing a hygromycin resistance gene for the selection of the stably transfected clones; pUHG16-3, a “response” plasmid containing a lacZ gene cloned after the TRE element and a minimal cytomegalovirus (CMV) promoter that was used to control for tTA activity in HtTA (see below) cells and for the screening for high switch-on factors of the target gene. These constructs as well as the HtTA cell line were a kind gift

from Prof. H. Bujard (Center for Molecular Biology, ZMBH, Heidelberg, Germany). The construction of these Tet-off system reagents has been described (Gossen *et al*., 1992).
The construction of a human MIF cDNA-bearing tetracycline-responsive plasmid, pMIFRP, for the transient and stable transfection experiments, was performed by PCR cloning. The human MIF sequence was amplified from the pET11b-huMIF plasmid using the *Sac* II restriction site-containing 5´primer: 5´-GCG CCG CGG ATG CCG ATG TTC ATC GTA AAC AC-3´ and the *Bam* HI restriction site-containing 3´-primer: 5´-CGG GAT CCT TAG GCG AAG GTA GAG TTG TTC CAG C-3´. PCR-amplified DNA was ligated into the pUHD 10-3 vector digested with *Sac* II and *Bam* HI. pC60SMIFRP was constructed in an identical manner except that pET11b plasmid bearing the human C60SMIF cDNA sequence (Kleemann *et al*., 1998a) was used as template in the amplification step.

4.4.7 Polymerase chain reaction (PCR)-coupled mutagenesis

First, the template DNA in the presence of a large molar excess of each of two primers and the dNTPs was denatured by heating. Then the mixture was cooled to a temperature for annealing of the primers to their target sequences, after which the annealed primers were extended with a polymerase. The cycle of denaturation, annealing, and polymerization was repeated 35 times. The PCR program used for amplification of MIF mutants is listed in the below tables (Table 3 and Table 4):

Table 3: Components for the PCR reaction.

Components	Volume (µl)
- 10 x Thermopol reaction buffer [100 mM KCl, 100 mM $(NH4)_2SO_4$, 200 mM Tris-HCl pH 8.0, 20 mM $MgSO_4$, 1 % Triton X –100].	10
- dNTP mixture 10 mM.	2
- DNA template 1µg/µl.	2
- 1 st PCR primer 10 mM	5
- 2 nd PCR primer 10 mM	5
- Vent DNA polymerase	1
- Total volume	25
- Mineral oil	50

Table 4: PCR program for the amplification.

	Reaction	Temperature	Time	Cycle
Denaturation		94°C	6 min	1
	Denaturation	96°C	75 sec	
Synthesis	Annealing	50°C	45 sec	35
	Polymerization	72°C	30 sec	
Product storage		4°C	o/n	

4.4.8 Ligation

The amount of DNA fragments was estimated on agarose gels. Purified fragments were mixed in a molar ratio of 1:1 or 1:3 vector/insert with 1 U T4 ligase (NEB) and its specific buffer in a 20 µl reaction. The reaction was incubated at 16°C o/n.

4.4.9 Transformation of plasmid DNA in *E. coli*

The ligation product was transformed in to *E. coli* DH5α. Briefly, after the sample was ligated, it was taken and added to 50 µl *E. coli* DH5α. The mixture was incubated them on ice for 30 min and then at 42°C water bath for 90 sec. 1 ml LB medium without ampicillin was added and the sample was kept at 37°C water bath for 30-60 min. Afterwards, the *E. coli* cells were centrifuged for 10 sec at 14000 rpm at RT. The pellet was resuspended in 100 µl LB medium without ampicillin. The transformed cells were plated with a sterile spreader on the LB/ampicillin agar plates. The plates were incubated at 37°C for 15 h or o/n.
A single colony from the transformation plate in 5 ml LB medium containing ampicillin was shaken overnight at 37°C with 225-250 rpm. A miniprep was performed to isolate the mutation-containing plasmid DNA. Then pC60SMIFRP was cut with *Sac* II and *Bam* HI to check for the insert length of 730 bp. Briefly, 7 µl miniprep DNA, 1 µl *Sac* II, 1 µl *Bam* HI, 1 µl buffer for *Bam* HI were added to each other. The mixture was incubated at 37°C for 2 h, then put on agarose gel. If the mutant had 730 bp length DNA for sequencing was prepared.
The correctness of the sequence was performed by commercially available sequencing service.

4.5 Biochemical methods

4.5.1 Bacterial expression of the mutant proteins in *E. coli* BL 21/DE3

MIF mutants containing pET11b plasmid DNA were prepared and used to transform *E. coli* BL21/DE3. Briefly, transformed *E. coli* BL21/DE3 containing the mutant expression plasmid were grown in LB/Amp at 37°C for about 3-4 h until a optical density of OD_{650} of 0.6-0.9 was reached and 1 mM IPTG was added. After 4 h, the cells were pelleted by centrifugation and were frozen at – 20°C until use (Bernhagen *et al*., 1994).

4.5.2 Purification MIF and MIF mutants

For protein purification, the bacterial pellets were thawed and resuspended in 5 ml of Tris-buffer saline 50 mM Tris-HCl, 150 mM NaCl, pH 7.5. The cells were disrupted by a French Press at 1200 psi. The disrupted cells were centrifuged at 18,000 x g for 30 min (JA 20 rotor) at 4°C. The supernatant was prefiltered through 0.2 µm a membrane filter and subjected to Mono Q anion exchange chromatography. The MIF containing-eluent was subjected to C8-SepPak reverse chromatography, and eluted with 60% (v/v) acetonitrile and lyophilized (Bernhagen *et al*., 1994).

4.5.3 Renaturation of MIF and MIF mutant

For renaturation, MIF was dissolved in renaturation buffer and dialyzed in 1 liter renaturation buffer 1 followed by 3 x 5 liters 20 mM renaturation buffer 2 at 4°C. Renatured MIF was centrifuged at 14000 rpm for 2 min at RT and kept at 4°C until use.

4.5.4 Synthesis and purification of the MIF peptide fragment MIF(50-65)

Synthesis and purification of MIF(50-65) was performed by a procedure essentially as described previously (Kapurniotu *et al*., 1992), except that the purification and oxidation procedure needed to be tailored for the requirements of the redox properties of MIF(50-65). The N-terminus of the peptide was N-acetylated and the C-terminus amidated. For the synthesis of the bis-serine variant of MIF(50-65), serine residues were coupled at positions 57 and 60 instead of the active site cysteines. Biotinylation of MIF(50-65) and C57S/C60S-MIF(50-65) was performed at the N-terminus and included an amidocaproate spacer.

Briefly, synthesis was done by a solid phase peptide synthesis (SPPS) procedure on Rink resin applying the 9-fluorenyl-methoxycarbonyl group (Fmoc) for temporary protection of the α-amino function. Side-chains of trifunctional amino acids were protected with tBu (Ser), Trt (Cys), Trt (His), and Xyz (Glu). Deprotection with simultaneous cleavage from the resin was performed by treatment with 95 % TFA/2.5 % ethandithiol/2.5 % water. Following removal of TFA, the resulting product was dissolved in 10 % acetic acid and extracted with diethylether. The aqueous phase was then lyophilized. Characterization of the side products of the synthesis will be described in detail elsewhere. The obtained crude product, which was present in its reduced form and which had a purity of 90-95 %, was purified by C18 reverse phase HPLC (250 x 8 mm; 100 Å pore size, 7 µm particle size; Grom, Herrenberg, Germany). Elution of the peptides was achieved with a water/acetonitrile gradient (10 % B to 90 % B; 30 min; buffer A: 0.058 % TFA; buffer B: 90 % acetonitrile, 0.05 % TFA; flow rate: 2 ml/min). The identity and purity of the peptides was verified by mass spectrometric analysis and amino acid analysis, where necessary. Following lyophilization, purified reduced peptide was stored at - 20°C until used further. Oxidized forms of the MIF peptide fragment MIF(50-65) were obtained by air-oxidation of the sulfhydryl groups in the presence of 0.1 M ammonium bicarbonate. Briefly, 15 mg reduced MIF(50-65) peptide (either crude product or HPLC-purified reduced peptide) was stirred in 175 ml 0.1 M ammonium bicarbonate for a minimum of 7 h and the solution lyophilized. After lyophilization, the sample was dissolved in 10 % acetic acid and immediately loaded on an analytical C18 reverse phase HPLC column (125 x 4 mm; 100 Å pore size, 5 µm particle size; Grom, Herrenberg, Germany and Maisch, Herrenberg, Germany). Elution was performed with a linear acetonitrile/water gradient as described above at a flow rate of 1ml/min. Absorbance was monitored at 220 nm. The oxidized peptide as well as the mixed disulfide species were collected on dry-ice, deep-frozen and lyophilized. Mutant peptide C57S/C60S-MIF(50-65) and the biotinylated peptides were purified from their corresponding crude synthesis products by HPLC. For the biological assays, the biotinylated derivatives were applied in their reduced forms.

4.5.5 SDS-PAGE

SDS-PAGE was carried out as described by Laemmli (Laemmli 1970) using a BioRad equipment. 12 % PAA gels were applied using the conditions as described in Table 5.

Table 5: SDS - PAGE composition and buffers.

Running gel	2.5 ml lower Tris [1.5 M Tris-HCl, pH 8.8], 100 µl 10 % (m/v) SDS, 4 ml acrylamid [30 % (m/v)], 3.4 ml H_2O, 50 µl 10 % (m/v) APS, 5 µl TEMED.
Stacking gel	2.5 ml upper Tris [0.5 M Tris-HCl, pH 6.8], 100 µl 10 % (m/v) SDS, 1.34 ml acrylamid [30 % (m/v)], 6 ml H_2O, 50 µl 10 % (m/v) APS, 10 µl TEMED.
SDS sample buffer	2.5 ml Tris pH 6.8 0.5 M (0.125 M), 4.0 ml 10 % (m/v) SDS, 2.0 ml glycerol 20 % (v/v), 1.0 ml β-mecaptoethanol 10 % (v/v), 1 mg bromphenol blue 0.01 % (m/v).
Running buffer	3 g/L Tris, 14.4 g/L glycin, 10 ml/L SDS 10 %, pH 8.3

Protein samples were mixed with one volume of the SDS sample buffer and boiled for 10 min at 100°C. A voltage of 8 V/cm was applied to collect the samples in the stacking gel. After the dye front moved into the resolving gel, the voltage was increased to 15 V/cm and the gel was run until the bromphenol blue reached the bottom of the resolving gel. The molecular weight standards were electrophoresed together with the samples.

4.5.6 Western and immunoblotting

The supernatant was applied to a gel and immunoblotting performed as described (Tomoda *et al.,* 1999 and Kleemann *et al.,* 2000a). Briefly, the protein concentration in the cell lysates was determined by the Bradford method (Bradford, 1976). Lysates were adjusted to contain equal protein concentrations and were mixed with 2 x Laemmli SDS-PAGE buffer. Electrophoresis was performed in 12 % SDS-PAGE gels and separated proteins were transferred to a nitrocellulose membrane at 30 V and 90 mA for 16 h-18 h at 4°C or 50 V for 5 h at 4°C in CAPS transfer buffer (10 mM CAPS, 20 % methanol, pH 11.0) using a *Mini Trans-blot Cell System Western Blotting*.

After the transfer, the membranes were blocked with milk powder blocking buffer (10 mM Tris/HCl, 150 mM NaCl, pH 7.4, 5 % milk powder, 0.2 % Tween 20) for 2 h at room temperature and then incubated for 2 h at room temperature (or 16 h at 4°C) with the appropriate antibody diluted 1:1000 in blocking buffer, followed by an incubation with an anti-mouse POD antibody (diluted 1:10000 in blocking buffer) [Santa Cruz, Heidelberg, Germany]

for 2 h at room temperature. Membranes were washed three times in high salt-tween-TBS (10 mM Tris/HCl buffer pH 7.4 containing 500 mM NaCl and 0.05 % Tween 20) and immunoreactive bands were visualized using the Super Signal West Dura Extended Duration Substrate (Pierce/KMF Laborchemie GmbH, St. Augutin, Germany). The intensity of the bands was quantified by densitometry using the Aida 2D software (Raytest Isotopenmessgeräte GmbH, Straubenhart, Germany).

4.5.7 Circular dichroism spectrometric measurements

Near- and far-ultraviolet (UV) circular dichroism (CD) spectropolarimetry was performed essentially as described previously (Kleemann *et al*., 1998a). HPLC-purified oxidized and reduced MIF(50-65) and mutant peptide C57S/C60S-MIF(50-65) were prepared as described above. For CD analysis, peptides were dissolved in 10 mM Tris-HCl buffer (pH 8.0) at a concentration of 1 mM, briefly sonicated, and diluted from their stock solutions with water to a final concentration of 100 µM. Spectra were recorded within 30 min thereafter. For some of the kinetics experiments, reduction of oxidized MIF(50-65) was followed by incubating the working solution of oxidized MIF(50-65) with 1 mM DTT for 10 min to 24 h. CD spectra are presented as a plot of the mean molar ellipticity per residue ([θ], deg cm^2 $dmol^{-1}$) versus the wavelength and represent net spectra, with the spectra of the control buffer solution that was measured in the same cuvette subtracted. Secondary structure analysis of the CD spectra was performed by the computer program LINCOMB together with the data sets of Brahms and Brahms (Brahms and Brahms, 1980).

4.5.8 Reduction of MIF and MIF peptide fragment MIF(50-65)

For MIF, the reaction was initiated by mixing of 20 µg MIF with GSH (5 mM) or DTT (15 mM) or of the ratio GSH/GSSG of 4/1.

For the MIF peptide fragment MIF(50-65), the reaction was initiated by mixing 20-40 µg oxMIF(50-65) with GSH/GSSG at ratios of 50/0.001, 50/1, 50/10, 48/2, 44/6, 46/4, 48/2 and cysteine/cystine at ratios of 42/8, 44/6, 46/4, 48/2. The reactions were incubated at 30°C for different times in a volume of 100 µl. Afterwards, 40 µl of 1 M H_3PO_4 were added to 100 µl of the reaction mixture to stop the reaction by reducing the pH to below 2.

Afterwards, the sample loaded on a Grom C18 reverse phase HPLC column. Elution was performed with linear gradients of acetonitrile in 0.05 % (v/v) TFA at 1ml/min. The absorbance was monitored simultaneously at 220 nm. The column was washed with approximately 2 column volumes of buffer between each run.

4.6 Cell biology methods

4.6.1 Cell Culture

THP-1, HL-60 and human Jurkat T cells were maintained in RPMI 1640 medium and COS-1 cells, NIH3T3 cells were maintained in DMEM medium with 10 % FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine. Kym-1 cells were maintained in RPMI 1640 medium with 5 % FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine. Cells were cultured by routine protocols at 37°C in a humidified incubator with 5 % CO_2.

HtTA cells represent HeLa cells stably transfected with a Tet-off regulator gene cassette (see above). HtTA cells stably expressing the human MIF gene (HtTAM) were generated by transfecting HtTA cells with phuMIFRP using the SuperFect transfection reagent. HtTA cells were transfected with pMIFRP and pX343 that had been linearized with *Ase* I and *Pvu* II, respectively, before transfection. The plasmids were applied at a ratio of pMIFRP/pX343 of 1:10. Cells were incubated overnight, transferred to 10 cm cell culture dishes and incubated for 13 days in the presence of 250 µg/ml hygromycin. From a total of 36 positive clones, 34 were transferred to 24-well plates, expanded and clones carrying the human MIF sequence stably integrated in their genome further selected through G418 and hygromycin treatment as described (Gossen *et al.,* 1992). Three clones were finally obtained and analyzed for their responsiveness to dox and for their MIF expression levels. To determine the efficiency of target gene expression and the switch-on factor of the MIF gene, the cell clones were plated in 3.5 cm dishes at a density of 1 x 10^5 (1 ml medium), incubated for 72 h in presence versus absence of dox (1 µg/ml) and analyzed by Bradford protein assay and MIF ELISA. Clone 13 was used for the apoptosis studies.

HtTA and HtTAM cells were cultured in EMEM medium (Life Technologies) containing 10 % fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin/streptomycin as described previously (Gossen *et al.*, 1992). To remove the cells from the flasks or plates during passaging or transfer, cells were incubated with EDTA-containing calcium-magnesium-free PBS (CMF-PBS) for 2-3 min, but neither scraped nor treated with trypsin.

For experiments applying the HtTAM cells, 1-5 x 10^6 cells were transferred to 75 cm^2 flasks and the cells incubated for 48-72 h in the presence versus absence of 1 µg/ml dox. Cells were lysed in the appropriate lysis buffer and analyzed for β-galactosidase activity, MIF concentration, apoptosis, GSH content, or phospho-c-Jun levels (see below).

Transient transfection experiments were performed differently for each cell line used and the condition applied (see below).

4.6.2 Flow cytometry

HtTAM cells were cultured in normal medium in the presence versus absence of dox for 48 h. Cells were washed with PBS and with TFM medium. 1 µg/ml dox or control solution was added and the cells were cultured in TFM for another 18 h at 37°C. Afterwards, the cell samples were analyzed with the annexin-V-fluos staining kit (Roche, Germany). Briefly, medium was removed from the cells and the cells were washed in PBS. Then, cells were incubated with annexin–V-fluorescein in a HEPES buffer containing propidium iodide (PI). The samples were analyzed on a flow cytometer (FACSCalibur, Becton Dickinson, Heidelberg, Germany). Ten thousand cells from each sample were analyzed, and both histogram and dual-parameter dot plot analyses were performed with WinMDI 2.7 software. Apoptotic cells were characterized by annexin-V fluorescein binds in a Ca^{2+} -dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine (Fadok *et al*., 1992).

4.6.3 Fluorescence microscopy

HtTAM cells were cultured in normal medium in the presence versus absence of dox. Cells were washed with PBS and with TFM medium. 1 µg/ml dox or control solution was added and the cells were cultured in TFM for another 18 h at 37°C. Afterwards, cells were fixed in 5 % formaldehyde, stained with 0.05 mg/ml propidium iodide and examined with a fluorescence microscope. Apoptotic cells, characterized by morphologic changes in PI-stained nuclei (Meyer *et al*., 1984) were counted, along with intact non-apoptotic cell nuclei. Percent apoptotic cells $\pm$ SEM was calculated as follows: number of apoptotic cells/total cells x 100 = percent apoptotic cells.

4.6.4 Transient transfections

The day before transfection, 2-5 x 10^5 cells (depending on the cell type) were seeded in 2 ml of an appropriate growth medium in 3.5 cm dishes. The cell number seeded was to produce 80 % confluence on the day of transfection. On the day of transfection, cells were transfected with 2-3 µg DNA and the superfect transfection reagent (QIAGEN) according to the manufacturer´s instructions.

The cells with the complexes were incubated for 48 h at 37°C and 5 % CO_2 in the presence or absence of 1 µg/ml of dox. After 48 h, the medium containing the remaining complexes was removed from the cells by gentle aspiration, and the cells were washed once with 2 ml of PBS. Fresh cell growth medium (containing serum) was added and the cells were

incubated in the presence or absence of 1µg/ml dox and 2 µg/ml cam for 24 h at 37°C and 5 % CO_2. The cells were harvested and assayed for gene expression, by the cell death ELISA, for β-galactosidase activity or for DNA fragmentation.

4.6.5 Transient transfections and luciferase reporter gene assays

5 x 10^6 THP-1 cells were plated in 100 mm plates in 10 ml of SFM (hybridoma serum-free medium, Life Technologies) the day before transfection. Prior to transfection, THP-1 cells were washed in SFM, pelleted, and resuspended in 800 µl SFM. 4 µg of the plasmid NFκB-luc was mixed with 20 µl of 10 mg/ml DEAE-dextran (Promega). The cells and the DEAE-dextran-plasmid mixture were mixed and placed in a 35 mm plate. After a 30 min incubation period at room temperature, cells were washed twice in SFM and resuspended in the same medium, distributed into a 48-well plate at 2 x 10^5 cells/well and incubated at 37°C and 5 % CO_2. The pSV-β-Galatosidase plasmid was co-transfected as a positive control vector. 24 h after transfection, cells were stimulated with 1µg/ml lipopolysaccharide. Glucocorticoid-treated cultures were exposed to 1 µM dexamethasone (Dex) and rMIF 1 h before LPS and rMIF addition. After 6 h, the cells were harvested in 100 µl lysis solution provided with the β-galactosidase Enzyme Assay System kit (Promega).
Similar triplicate cultures without LPS or Dex served as controls. Lysates were analyzed for firefly luciferase expression (Steady-Glo Luciferase Assay System, Promega) using a Lucy2 luminometer (Anthos). For β-galatosidase activity analysis the β-galactosidase Enzyme Assay System with Reporter Lysis Buffer was used. Normalized transfection efficiencies are indicated by the quotient of relative luciferase units and relative β-galactosidase units. All luciferase reporter gene assays were carried out in triplicates and were performed 3-5 times.

4.6.6 Measurement of glutathione levels

Intracellular glutathione levels were measured in lysates from cells overexpressing human MIF in a Tet-off-dependent manner.
For glutathione determinations from HtTAM cell lysates, 2 x 10^6 cells were plated in 75 cm^3 cell culture flasks and incubated for 72 h at 37°C in the presence versus absence of 1 µg/ml dox. Cells were washed once with PBS and twice with TFM. 1 µg/ml dox or control solution was added and the cells were cultured in TFM for another 18 h at 37°C. Cells treated with 200 µM L-cystine served as a positive control. The cells were harvested and assayed for intracellular GSH by a commercial glutathione assay (BIOXYTECH GSH-400 colorimetric assay). Briefly, the cells were removed from the flasks by incubation in CMF-PBS and 4 x 10^6

cells were resuspended in 5 % freshly prepared metaphosphoric acid (MPA). The suspensions were mixed by rigorous pipetting and subjected to dounce homogenization applying 20 pushes with a tight pestle. The resulting suspension was transferred to a 1.5 ml cup, centrifuged, and the supernatant placed on ice. The GSH content of the acid-soluble supernatant was then measured in triplicate wells of a 96-well plate with 0.025 % (w/v) lubrol according to the manufacturer's instructions. The absorbance was recorded at 1 min intervals for 5 min at 405 nm.

For GSH determinations in Kym-1 cells, 2 x 10^6 cells were plated in 75 cm^3 cell culture flasks, subjected to the transient transfection procedure with pMIFRP and treated with dox as described above. Cells were washed with PBS and TFM and incubated in TFM as described for the HtTAM cells. The quantification of the cellular GSH content was performed as for the HtTAM cells, except that MPA was added directly to the cell layer.

4.6.7 Analysis of the phosphorylation of endogenous c-Jun

For measuring the levels of endogenous phospho-c-Jun, 2 x 10^6 HtTAM cells were plated in 75 cm^3 cell culture flasks and incubated for 72 h at 37°C in the presence versus absence of 1 µg/ml dox. Cells were then removed from the flasks and washed once with PBS and twice with TFM. Cells were resuspended in TFM and 1.5 x 10^6 cells plated in a 3.5 cm dish and incubated for 2-6 h in the presence versus absence of 1 µg/ml dox. Cells were harvested, washed once with PBS and lysed. Lysis was performed in RIPA buffer. The protein concentration in the cell lysates was determined by the Bradford method (Bradford, 1976). Lysates were adjusted to contain equal protein concentrations and were mixed 1:1 with 2 x Laemmli SDS-PAGE buffer. Electrophoresis was performed in 12 % SDS-PAGE gels and separated proteins were transferred to a nitrocellulose membrane. To visualize c-Jun, membranes were probed with a monoclonal anti-phospho-c-Jun antibody (clone KM-1, Santa Cruz Biotechnology, Heidelberg, Germany). For quantification, the blots were reprobed for c-Jun and actin staining. Membranes were stripped with 0.5 % Tween 20/TBS for 1 h at room temperature, washed, and stained with an anti-c-Jun (clone H-79, Santa Cruz) or anti-actin (Sigma) antibody. The intensity of the phospho-c-Jun, c-Jun and actin bands was quantified by densitometry using the Aida 2D software (Raytest Isotopenmessgeräte GmbH, Straubenhart, Germany).

4.6.8 $p27^{Kip1}$ assay

Transfections were performed in serum-starved NIH3T3 cells. Briefly, 0.25 x 10^6 NIH 3T3 cells were plated in a 60 mm plate in Dulbecco´s MEM medium supplemented with 10 %

FCS and antibiotics (100 U/ml penicillin/streptomycin, 2 mM L-glutamine) at 37°C in 100 % humidity with 5 % CO_2. After 6 h of incubation, cells were washed with PBS and incubated in Dulbecco´s MEM medium supplemented with 1 % FCS and antibiotics (100 U/ml penicillin/streptomycin, 2 mM L-glutamine) at 37°C in 100 % humidity with 5 % CO_2 o/n.
Then, NIH 3T3 cells were treated with different concentrations of rMIF and MIF peptide fragment MIF(50-65) for 24 h at a density of 0.25 x 10^6/6 cm cell culture plate with duplicates performed for each transfection and incubated at 37°C and 5 % CO_2 in medium containing 10 % FCS. Afterwards, the cells were harvested and lysis was performed in RIPA buffer.
The supernatant was applied to a gel and immunoblotting performed as described (see above).

4.7 Immunology methods

4.7.1 TNF ELISA protocol

Solutions required:

Wash Buffer	0.05 % Tween 20 in PBS, pH 7.4
Diluent	0.1 % BSA, 0.05 % Tween 20 in Tris-bufferd saline pH 7.3 (20 mM Tris base, 150 mM NaCl).
Substrate solution	1:1 mixture of color reagent A (H_2O_2) and color reagent B (Tetramethylbenzidine).
Stop solution	1 M H_2SO_4

Plate preparation:

- 100 µl/well of the capture antibody (diluted to the appropriate concentration in PBS) were transferred to an ELISA plate. The plate was scaled and incubated overnight at room temperature.
- Aspirate each well and wash with wash buffer, repeating the process two times or a total of 3 washes. Wash by filling each well with wash buffer using a squirt bottle, multi channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance.
- Block plates by adding 300 ml of PBS containing 1 % BSA, 5 % sucrose to each well. Incubate at room temperature for a minimum of 1 h.
- Repeat the aspiration/wash as above. The plates are now ready for sample addition. Alternatively, the blocking buffer was aspirated as above and the plates can be dried under vaccum. When sealed with dessicant, the plates can be stored at 4° - 8°C for at least 2 months.

Assay procedure:

- Add 100 µl/well of sample or standards in an appropriate diluent, per well. Mix by gently tapping the plate frame for 1 min, cover the plate with an adhesive stripe and incubate 2 h at RT.
- Repeat the aspiration/wash as above.
- Add 100 µl/well of the biotinylated detection antibody, diluted in the approriate diluent, to each well. Cover the plate with a new adhesive strip and incubate 2 h at RT.
- Repeat the aspiration/wash as above.
- Add 100 µl/well streptavidin-POD (1 :10 000) to each well. Cover the plate and incubate for 20 minutes at RT.
- Repeat the aspiration/wash as in step of plate preparation.
- Add 100 µl/well of substrate solution to each well. Incubate for 20-30 minutes at RT. Avoid placing the plate in direct light.
- Add 50 µl of stop solution to each well.
- Determine the optical density of each well within 30 min, using a microtiter plate reader set to 459 nm. The wavelength correction was set to 540 nm. This subtraction will correct for optical imperfection in the plate.

4.7.2 huMIF ELISA

The cellular MIF content following Tet-off-induced overexpression was measured by a commercial ELISA assay from R&D Systems (Wiesbaden, Germany). The cell lysates to be subjected to the ELISA determinations were obtained applying the Tris-phosphate/CDTA/Triton lysis solution (see β-galactosidase assay) and lysates were diluted appropriately in the incubation buffer of the ELISA kit. The protocol to assay huMIF was essentially the same as for TNF, except that for the huMIF ELISA, another antibody was used.

4.7.3 Apoptosis assays with Kym-1 cells

Apoptosis assays following transient transfection of Kym-1 cells with the pMIFRP and pC60SMIFRP plasmids were generally performed according to the following protocol: 2-5 x 10^5 cells were seeded in 3.5 cm dishes (2 ml culture medium) the day before transfection. At the time of transfection, the cells had reached approximately 80 % confluence. Transfections were performed with 2-3 µg of the corresponding plasmid according to the SuperFect protocol. Afterwards, the cells were incubated for 48 h in the presence or absence of 1 µg/ml

of dox. Then, the medium containing the remaining DNA-SuperFect complexes was removed from the cells by gentle aspiration and the cells were washed with 2 ml PBS.
For culture stress-induced apoptosis, cells were harvested thereafter and lysed for subsequent analysis. For camptothecine-induced apoptosis studies, fresh cell culture medium, containing serum (full medium; for TFM see below), 1 µg/ml dox or control solution and 2 µg/ml cam or control solution was added to the washed cells and the cells were incubated for another 24 h before harvesting and analysis.
For probing the effects of rMIF and rC60SMIF on apoptosis in Kym-1 cells, the cells were plated at a density of 2 x 10^6 in 3.5 cm culture dishes and rested for 24 h. rMIF or rC60SMIF were added at the indicated concentrations and the cells incubated for 48 h. 2 µg/ml cam or control solution was added and cells incubated for another 24 h before harvesting and analysis.

4.7.4 Apoptosis of HL-60 cells in full medium

HL-60 promyeloblasts were used to compare the effects of rMIF and rC60SMIF on apoptosis induced by cam treatment and to study the effect of rMIF on thiol starvation-induced apoptosis. For the cam-induced apoptosis experiments, HL-60 cells were cultured in RPMI 1640 medium supplemented with 10 % FCS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at a density of 2 x 10^5. The recombinant MIF proteins were added and the cells incubated for 24 h. 2 µg/ml cam or control buffer was added and the cells incubated for another 24 h. Apoptosis was then assessed by the cell death ELISA.

4.7.5 Apoptosis of HL60 cells in thiol-free medium

For apoptosis experiments in TFM, HL-60 cells were grown in full medium (FM) as described above. Before treatment, the cells were washed once with PBS and twice with TFM and were incubated in TFM for 6 h. After another wash with TFM, 200 µM L-cystine, 2 µM β-ME or rMIF at the indicated concentrations was added and the cells incubated for 20 h. Apoptosis was then assessed by the cell death ELISA.

4.7.6 Apoptosis assays with HtTAM cells

Apoptosis in HtTAM cells was induced by cam or thiol starvation. For cam-induced apoptosis experiments, cells were cultured in the presence versus absence of dox (see above) and treated with cam (0.1-1 µg/ml) for 18 h and cell lysates analyzed by the cell death ELISA. For

thiol starvation-induced apoptosis studies in HtTAM cells, cells were cultured in the presence versus absence of dox as described above, washed extensively in TFM (see above) and cultured in TFM for up to 18 h. Apoptosis was then assessed by the cell death ELISA or by DNA fragmentation.

4.7.7 UV stress-induced apoptosis in Jurkat T cells

Ultraviolet light (UV) stress-induced apoptosis was studied in Jurkat T cells. Cells were incubated with or without rMIF for 16 h and subsequently treated with UV light for 1 or 6 h using a Stratalinker (Stratagene, Heidelberg, Germany; 3.6 Joule/cm^2) as the source for the UV light.

4.7.8 β-galactosidase assay

Cell lysis was achieved by adding a buffer containing 25 mM Tris-phosphate buffer, pH 7.8, 2 mM DTT, 2 mM bis-1, 2-diaminocyclohexane-N, N, N', N' tetraacetic acid (CDTA), 10 % glycerol, and 1 % Triton X-100. To determine the β-galactosidase activity, 10 µl of each lysate was mixed with 900 µl substrate solution, containing 100 mM sodium phosphate buffer, pH 7.0, 10 mM potassium chloride, 1 mM magnesium sulfate, 50 mM 2-mercaptoethanol, and 4 mg/ml ONPG in a 1.5 ml tube. The reaction was carried out for 2 h at 37°C and was terminated by adding 500 µl of a 1 M bicarbonate solution, pH 9.5. The resulting absorbance was measured at 420 nm using the DU-65 UV-VIS spectrophotometer from Beckman GmbH (München, Germany).

4.7.9 DNA fragmentation assay

In some of the experiments, the DNA fragmentation assay was performed to examine the degree of cellular apoptosis. Following treatment with cam, thiol-free medium and rMIF or control buffer (see above), 1 x 10^6 cells were lysed in 1 ml extraction buffer (SDS 1 %, NaCl 0.4 M, EDTA 10 mM, Tris-hydrochloride pH 8.0, 10 mM). After 1 h incubation at 37°C, 10 µg/ml proteinase K was added and the samples were incubated for 3 h at 50°C. Samples were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) followed by a chloroform extraction, and precipitation of the DNA by a routine protocol. The DNA was resuspended in 40 µl ddH_2O, 0.5 µl RNase A was added, and the solution was incubated for 45 min at 37°C. The DNA was then directly applied to a 1 % agarose gel for electrophoretic separation and bands were visualized by ethidium bromide staining.

4.7.10 Cell death ELISA

The degree of cellular apoptosis was mainly scored by a commercial apoptosis-specific cell death ELISA from Roche Diagnostics (Mannheim, Germany). The ELISA provides a quantitative determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of a cell lysate. The presence of mono-and oligonucleosomes is a typical feature of cells undergoing apoptosis.
Briefly, the assay represents a sandwich ELISA applying monoclonal antibodies directed against DNA and histones. First, the anti-histone antibody (clone H11-4) was adsorbed to the microtiter plate. Following blocking, the cell lysates containing the free nucleosomes were added. During the incubation step, the nucleosomes, via their histone components (histone H2B), bound to the immobilized anti-histone antibody. Next, the formed complexes were incubated with an anti-DNA antibody conjugated with peroxidase (clone MCA-33). Immune complexes were then determined photometrically using ABTS® (2, 2- azino-di-[3-ethylbenzthiazoline sulfonate]), as a substrate. Quantification was performed by spectrophotometric measurement at 405 nm.

4.8 Biochemical and immunological characterization of MIF and MIF peptide fragment MIF(50-65)

4.8.1 Insulin reduction assay

The enzymatic redox activity of wtMIF, mutants, and MIF(50-65) was measured by their ability to reduce insulin. MIF-catalyzed insulin reduction activity was performed following the procedure of Chandler (Chandler & Varandani, 1975) and Holmgren (Holmgren, 1979). Briefly, the insulin assay is based on the reduction of insulin and subsequent insolubilization of the insulin β-chain. The time-dependent increase in turbidity is then measured spectrophotometrically at 650 nm.
The reaction was started by adding X µl of renatured MIF, mutant, or a control solution (X adjusted to a final concentration of 20 µM MIF dissolved in 20 mM sodium phosphate buffer, pH 7.2), and 22.5 µl 200 mM reduced glutathione (GSH), 470.4 µl of the ice-cold reaction mixture containing 1 mg/ml insulin, Y µl sodium phosphate buffer, pH 7.2 (X+Y= 407.1 µl, final volum of reaction was 900 µl). MIF or mutant-catalyzed insulin reduction was measured against the control solution (containing GSH) in the same experiment. MIF(50-65) was added from a 1 mM stock solution and tested at a concentration of 40 µM. Scores were taken by analyzing both the time needed for precipitation to occur (time of onset) and the rate of precipitation (Holmgren, 1979).

4.8.2 Dopachrome tautomerization assay

Tautomerase activity was measured using the D-dopachrome tautomerase test as described previously (Rosengren *et al*., 1996). Briefly, a fresh solution of D-dopachrome methyl ester was prepared by mixing 4 mM L-3,4-dihydroxyphenylalanine methyl ester with 8 mM sodium peroxidate for 5 min at room temperature and then placed directly on ice before use. Activity was then determined at room temperature by adding 100 µl dopachrome methyl ester to a cuvette containing 800 µl 25 mM potassium phosphate buffer, pH 6.0, and 0.5 mM EDTA. After 30 sec, 100 nM MIF or 3.3 µM MIF peptide was added to the cuvette and the resulting decrease in absorbance at 475 nm was followed for 4 min using a Speckol spectrophotometer (Jena Analytics, Jena, Germany).

4.8.3 Glucocorticoid overriding assay

Glucocorticoid counter-regulating activity of MIF and MIF peptide MIF(50-65) was assayed in the glucocorticoid overriding test as described previously (Calandra *et al.,* 1995). Briefly, overriding by MIF and MIF peptide MIF(50-65) of the anti-inflammatory effect of dexamethasone was analyzed using LPS-stimulated tumor necrosis factor (TNF) production by peripheral blood monocytes (PBM) and THP-1 cells.

PBM were isolated from an unstressed donor by Ficoll gradient and adherence, washed, and plated at a concentration of 1 x 10^6 cells/ml. Cells were incubated for 1 h with 10^{-9} M dexamethasone and 0.16 nM wtMIF or 3.3 µM MIF peptide (MIF50-65). LPS was added at a concentration of 1 µg/ml together with another 0.16 nM of test protein or 3.3 µM MIF peptide MIF(50-65) and cells incubated further for 14 h. Culture supernatants were prepared and analysed for TNF content using a commercial TNF ELISA (R & D Systems GmBH, Wiesbaden, Germany)(data not shown).

THP-1 cells plated at a concentration of 1 x 10^6 cells/ml were incubated for 1 h with 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M dexamethasone and differently concentration of rMIF or 3.3 µM MIF peptide fragment MIF(50-65). LPS was added at a concentration of 100 ng/ml, 500 ng/ml, 1 µg/ml and 10 µg/ml together with another concentration of test protein or 3.3 µM MIF peptide fragment MIF(50-65) and cells incubated further for 4 h, 8 h and 16 h. Culture supernatants were prepared and analyzed for TNF content using a commercial TNF ELISA (R&D Systems GmbH, Wiesbaden, Germany).

5 Results

5.1 Investigation of the redox potential of MIF and peptide MIF(50-65)

Previous work from different groups has shown that the redox potential of protein thiol: disulfide oxidoreductases is influenced by the amino acid residues located between the two active-site cysteines (Krause *et al.,* 1991). MIF exhibits functional similarities with the thioredoxin (Trx) family of oxidoreductase enzymes, i.e. the thiol-protein oxidoreductase activity of MIF is dependent on the presence of a redox-active Cys-Xaa-Xaa-Cys motif (CXXC*)* (Kleemann *et al*., 1998a; Kleemann *et al*., 1998b; Kleemann *et al*., 1999; Nguyen, T. M., 1998; Kleemann *et al*., 2000b). Studies in our lab (Kleemann *et al*., 1998a; Kleemann *et al*., 1998b; Kleemann *et al*., 1999) had shown that MIF exhibited enzymatic oxidoreductase activity in the insulin and HED reducing test. In the insulin test, disulfides are cleaved by the reducing agent glutathione (GSH) and the resulting insolubility of the free β-chain of insulin is monitored over time by turbidity measurements at 650 nm.

MIF displays a pro-inflammatory spectrum of action that is most strikingly represented by its capacity to override the anti-inflammatory and immunosuppressive actions of glucocorticoids (Bernhagen *et al.*, 1994; Bernhagen *et al.,* 1998; Calandra *et al*., 1995; Donnelly *et al.,* 1997b). The glucocorticoid assay is an independent immunological assay that is specific for MIF (Calandra *et al*., 1995), and was used to further investigate and possibly confirm the physiological relevance of the oxidoreductase activity of MIF. Kleemann and colleagues showed that mutant C60SMIF had no activity in the HED transhydrogenase assay. Similarly, in the overriding test, mutant C60SMIF had significantly reduced activity when compared with wtMIF, exhibiting only 37 % activity of wtMIF (data not shown). They also showed that mutant C57SMIF exhibited 67 % activity when compared with wtMIF (data not shown). Testing the Pro2 mutants in the glucocorticoid overriding assay, they also demonstrated that these mutants were essentially as active as wtMIF, confirming that the cysteine residues of CALC are required for the cytokine functions of MIF (Kleemann *et al.,* 1999).

However, whereas for the Trx proteins a decade of research has elucidated the precise biochemical properties of this activity including the redox potentials involved (Bartoluci *et al*., 1997; Dyson *et al*., 1990; Mulrooney *et al*., 1997; Siedler *et al*., 1994) these parameters have not yet been investigated for MIF. Thus, determination of the redox potentials of the CXXC motif of MIF was one major task of this thesis.

5.1.1 Establishing a HPLC-based detection system for distinguishing reduced versus oxidized MIF

One approach to investigate the redox potentials of protein thiol oxidoreductases is to incubate the CXXC-containing protein with equilibrium mixtures of reduced and oxidized glutathione (GSH and GSSG) and analyze the relative portions of the oxidized and reduced species generated. For example, analysis may be performed by HPLC separation of the protein species. By this and related methods, Trx has been determined to have a more reducing potential of -270 mV, whereas CXXC proteins like the bacterial analogy DsbA (-124 mV) have more oxidizing potentials (Aslund *et al*., 1997; Hennecke *et al*., 1997; Lundström and Holmgren, 1995).

A redox potential for MIF has not yet been determined, because no assay systems have been available to distinguish quantitatively the oxidized and reduced species of MIF. I elected to try to establish a HPLC-based system that was able to distinguish between the oxidized and reduced species of MIF (i.e. oxMIF and redMIF) as a prerequisite to perform GSH/GSSG equilibrium studies with the ultimate goal to determine the redox potential of the CXXC site of MIF.

As a first step towards this aim, I tried to separate oxMIF and redMIF by various HPLC protocols. 20 μg of human MIF were incubated for 90 min under either reducing (in the presence of an excess of DTT or GSH or GSH/GSSG) or oxidizing (air oxidation) conditions. The following protocol was found to be best for reducing MIF (Table 6).

Table 6: Optimized protocol for the reduction of MIF. GSH, reduced glutathione; DTT, dithiothreitol; GSH/GSSG, mixture of reduced and oxidized glutathione; HCl, hydrochloric acid.

Components		Volume (µl)
HuMIF	20 µg	100
GSH	200 mM	2.5
DTT	1 M	1.5
GSH/GSSG	4/1 mM	2.5
HCl	0.1 M	100

The reaction mixtures were stopped by „freezing-in“, i.e. 0.1 M HCl was added to the reactions, the mixtures centrifuged, and subjected to a semi-preparative C18 reverse phase HPLC column. The investigated HPLC elution conditions are summarized in Table 7.

Table 7: Investigation of the HPLC elution conditions for the separation of reduced and oxidized MIF; AN, acetonitrile; TFA, trifluoroacetic acid.

Time (min)	rate (ml)	Pump A (H_20+0.05 % TFA)	Pump B (90 % AN+0.05 % TFA)	Duration
5	1	90	10	30
35	1	10	90	2
37	1	0	100	8
45	1	90	10	10
55	1	90	10	

Time (min)	rate (ml)	Pump A (H_20+0.05 % TFA)	Pump B(90 % AN+0.05 % TFA)	Duration
7	1	30	70	30
37	1	0	100	2
39	1	0	100	5
44	1	70	30	2
46	1	70	30	

Representative HPLC chromatograms of the reduced and oxidized MIF preparations are shown in Figure 3.

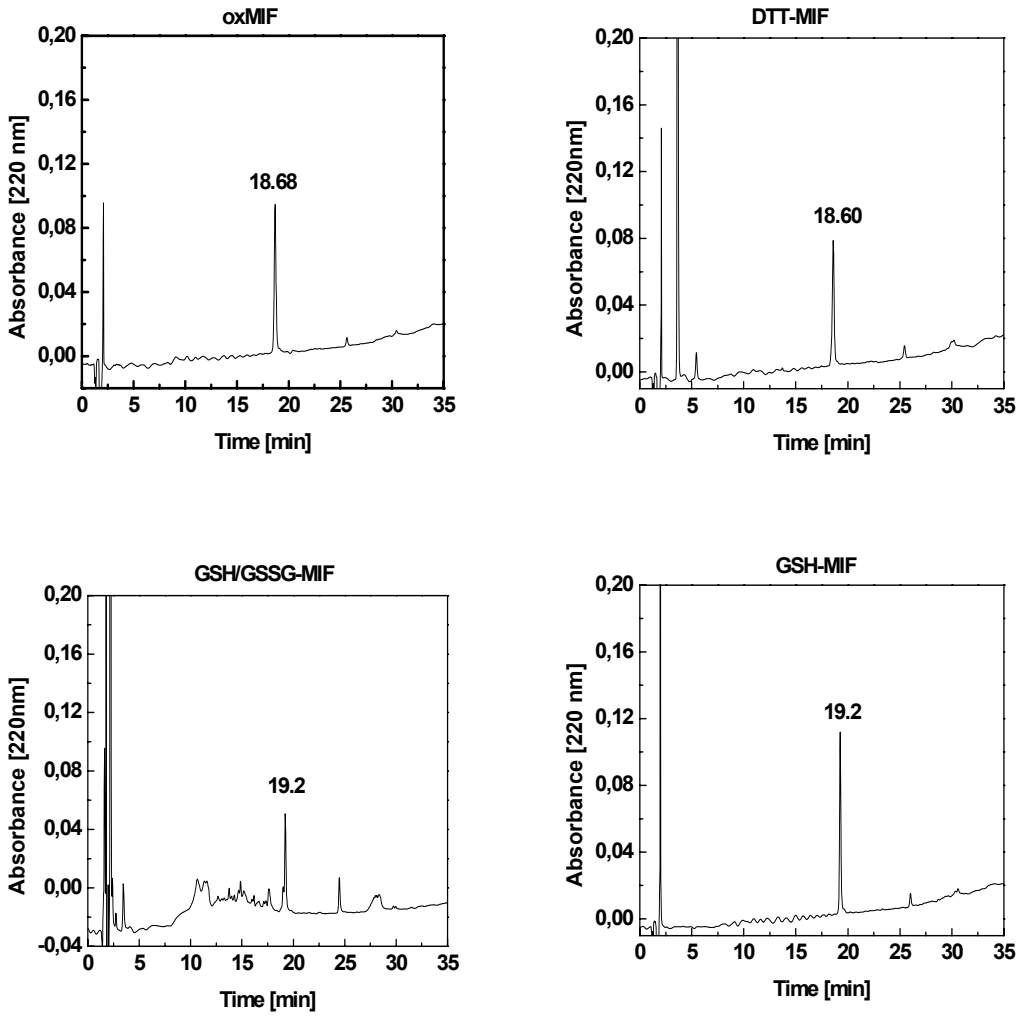

Figure 3: HPLC chromatograms of redMIF and oxMIF under various incubation and elution conditions. oxMIF, oxidized MIF; DTT-MIF, MIF with dithiothreitol; GSH-MIF; MIF with reduced glutathione; GSH/GSSG-MIF; MIF with mixture of reduced and oxidized glutathione.

The chromatograms show that no significant separation of oxMIF and redMIF was achieved. The elution times of red and oxMIF were too close to one another. A larger sample of separations then showed that oxMIF eluted ~ 0.3 min later than redMIF, but differences were not statistically significant (Table 8).

Table 8: Statistics of the retention time of oxidized and DTT-reduced human MIF on a C18 reverse phase HPLC column. wtMIF, human MIF; redMIF, reduced MIF; oxMIF, oxidized MIF.

wtMIF	Time (min)					Mean
redMIF	17.0	17.66	17.24	18.04	17.88	17.56 ± 0.44
oxMIF	18.1	17.41	17.56	18.05	18.14	17.85 ± 0.34

Together, these data showed that the investigated method was not suitable for the separation of the reduced and oxidized MIF species and was thus not a good basis to establish an assay for the measurement of the redox potentials of the MIF´s.
Attempts in my thesis to distinguish between the oxidized and reduced species of full length MIF (i.e. oxMIF vs. redMIF) by a HPLC-based system in conjunction with GSH/GSSG equilibrium studies were not successful, as no sufficient HPLC separation of the full length species was obtained.

5.1.2 Characterization of MIF peptides

As an alternative way to determine the redox potential of the CXXC site of MIF, I began to investigate the properties of elected MIF peptide fragments that covered the CXXC site. Initial conformational studies and studies on the redox enzyme activity had been promising and had suggested that such peptides could be excellent models to study the redox properties of MIF. Moreover, work by others in the field on Trx and Grx peptides had led to the successful determination of redox potentials (Siedler *et al.,* 1993; Siedler *et al*., 1994). The measurement of the redox potentials of MIF peptides were therefore investigated as part of this thesis (see below); these peptides show MIF-like redox activity although they are only 10-16 amino acids long; due to the short length much better separation profiles of the reduced and oxidized species were to be expected; similar work has been performed by Siedler and co-workers using peptides of the Trx proteins and they were successful in determining redox potentials with these peptides (Siedler *et al.,* 1993; Siedler *et al*., 1994). I concentrated on establishing a MIF peptide fragment-based system as a prerequisite to study the redox properties of MIF in more detail. The short peptides MIF(56-65) and MIF(50-

65) were designed to span the CXXC region of human MIF. Both peptides were synthesized by the collaborating laboratory of PD Dr. A. Kapurniotu at the University of Tübingen.
In this study, I sought to investigate these peptides fully as they were likely to represent mini-molecules with MIF-like redox activities and as they were expected to serve as experimental tools to estimate the redox potential of wtMIF (see above).
To better control for the effects and activities exerted by these peptides, control peptides were designed and synthesized that had either one or both of the CXXC Cys residues substituted by Ser residues. The following list summarizes the peptides investigated.

MIF-like peptides

MIF(56-65)	Pro-**Cys-**Ala-Leu-**Cys**-Ser-Leu-His-Ser-Ile
MIF(50-65)	Phe-Gly-Gly-Ser-Ser-Glu-Pro-**Cys-**Ala-Leu-**Cys**-Ser-Leu-His-Ser-Ile

Control peptides

Ser^{57}MIF(56-65)	Pro-Ser-Ala-Leu-**Cys**-Ser-Leu-His-Ser-Ile
$Ser^{57}Ser^{60}$MIF(56-65)	Pro-Ser-Ala-Leu-Ser-Ser-Leu-His-Ser-Ile
Ser^{57}MIF(50-65)	Phe-Gly-Gly-Ser-Ser-Glu-Pro-Ser-Ala-Leu-**Cys**-Ser-Leu-His-Ser-Ile
$Ser^{57}Ser^{60}$MIF(50-65)	Phe-Gly-Gly-Ser-Ser-Glu-Pro-Ser-Ala-Leu-Ser-Ser-Leu-His-Ser-Ile

MIF(56-65) had been shown prior to this thesis to have enzymatic redox activity (Kleemann *et al.,* 1998a).
I elected MIF(50-65) as the first choice model peptide in this thesis because the conformational and activity data were most clear with this peptide (see below):

MIF(50-65)	Phe-Gly-Gly-Ser-Ser-Glu-Pro-**Cys-**Ala-Leu-**Cys**-Ser-Leu-His-Ser-Ile

Before HPLC separation of the peptides was attempted, I sought to characterize the biophysical and enzymatic properties of the peptides more carefully. Figure 4 shows near- and far-UV circular dichroism spectra of oxidized versus reduced MIF(50-65) in comparison to $Ser^{57}Ser^{60}$MIF(50-65).

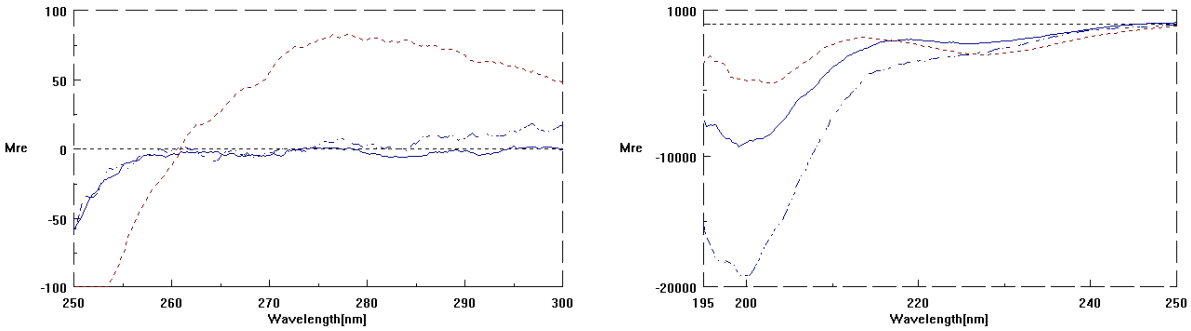

Figure 4: Near-UV-CD spectropolarimetry (left) of oxidized versus reduced MIF(50-65) versus $Ser^{57}Ser^{60}$MIF(50-65). Hatched line, oxidized MIF(50-65); dotted line, reduced MIF(50-65); filled line, $Ser^{57}Ser^{60}$MIF(50-65); Mre, mean residue ellipticity. (Right) Far-UV-CD spectropolarimetry of oxidized versus reduced MIF(50-65) versus $Ser^{57}Ser^{60}$MIF(50-65).

The near-UV CD analysis showed that the CXXC peptide MIF(50-65) contained a CD-active disulfide (signal in the 280 nm region) when present in it's oxidized form, whereas reduced MIF(50-65) and $Ser^{57}Ser^{60}$MIF(50-65) did not have a CD signal at 280 nm, indicating that they did not form a disulfide bond. In addition, the far-UV CD analysis demonstrated that disulfide bond formation went along with β-turn formation, a notion that is established for disulfide-active CXXC peptides (Siedler *et al.,* 1993; Siedler *et al.,* 1994), further confirming that the oxidized wildtype peptides should be able to serve as redox shuttles.

Comprehensive analysis of the peptides in the insulin reduction assay, which is one of the established enzymatic assays for the testing of oxidoreductase-active proteins or peptides, was begun next (Figure 5). The disulfide reductase activity of MIF(50-65) was compared to that of the control peptide $Ser^{57}Ser^{60}$MIF(50-65) with the insulin reduction assay. The three disulfide bridges of insulin are reduced by a slight excess of glutathione in this assay, and the aggregation of reduced insulin is recorded by the increase of the optical density at 650 nm. As shown in Figure 5, MIF(50-65) reduced insulin with an approximately 2 fold higher maximal rate (ΔOD_{650}/min) than the control peptide.

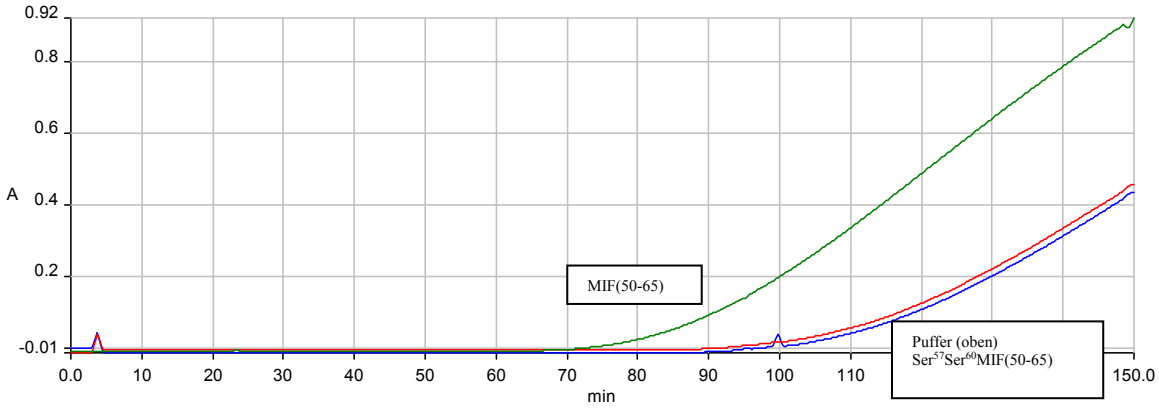

Figure 5: Catalytic redox activity of the CXXC-spanning MIF peptides in the insulin reduction assay. The peptides were tested at a concentration of 40 µM. MIF(50-65) and the control peptide $Ser^{57}Ser^{60}$MIF(50-65) are shown and compared to the buffer control. Turbidometric measurements of the insulin reduction/(-precipitation) assay after Holmgren (Holmgren, 1979b) are shown. A, absorption at 650 nm.

Testing of peptide MIF(50-65) versus the double serine control peptide showed that the MIF peptide exhibited significant enzymatic activity, whereas the control peptide showed only background activity, which was no higher than that exhibited by the reaction buffer.

Analysis of peptide MIF(50-65) in the dopachrome tautomerase assay, which is one of the established enzymatic assays for the testing of the tautomerase/isomerase activity of MIF, was also investigated. The result showed that peptide MIF(50-65) was inactive in the tautomerization of D-dopachrome (Figure 6). This finding confirmed that Pro2 is the catalytic center of the tautomerase activity. The redox and the tautomerase activity of MIF are not directly related to each other through their respective active site residues.

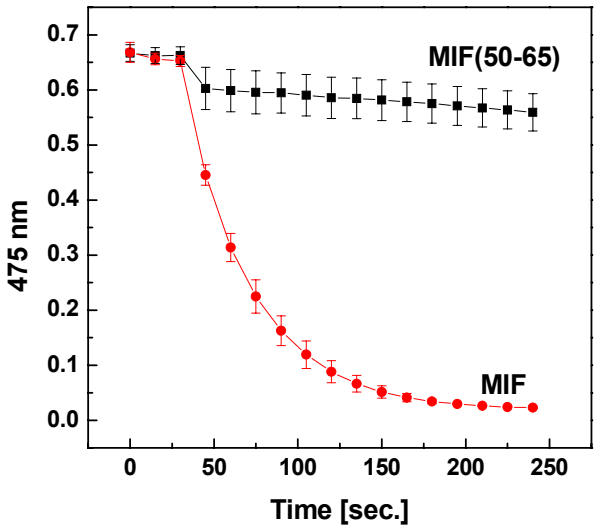

Figure 6: Tautomerase activity of MIF(50-65) using D-dopachrome as a substrate. The peptide was assayed at a final concentration of 3.3 µM. Recombinant full length MIF was used as a positive comparative control. Data represent the mean ± SD of three independent determinations.

5.1.3 Establishing a HPLC-based detection system for distinguishing the reduced versus oxidized peptides

As the initial results with the peptides were promising, I began to establish a HPLC separation system for the peptides as prerequisite to measure the redox potentials of the peptides, which could then serve as models for the redox potential of MIF. Figure 7 shows that oxidized MIF(50-65) and reduced MIF(50-65) were separated nicely and could be quantified on a C18 reverse phase HPLC column.

The result showed that oxidized MIF(50-65) could be nicely separated from the reduced peptide species. These peaks were analysed by MALDI-TOF mass spectrometry and the results revealed that redMIF(50-65) had a M+1 mass of 1648.6 Da (Fig. 7C) and oxMIF(50-65) had a M+1 mass of 1646.9 Da (Fig. 7B). The theoretical masses were 1648.7 Da and 1646.7 Da, respectively. Confirming that HPLC-based quantification of the two species could be a suitable read-out system for glutathione and cysteine equilibrium measurements. It was thus applied to the glutathione and cysteine reactions in this thesis.

Mass peaks in Fig. 7B and C represent the $M+Na^+$ peaks of the peptides. Potassium adducts were in some occasions observed with MALDI-MS technique applied. By mass, the peak

could possibly represent a small occuring fraction of a potassium adduct of a mono mixed disulfide with MIF(50-65) and DTT.

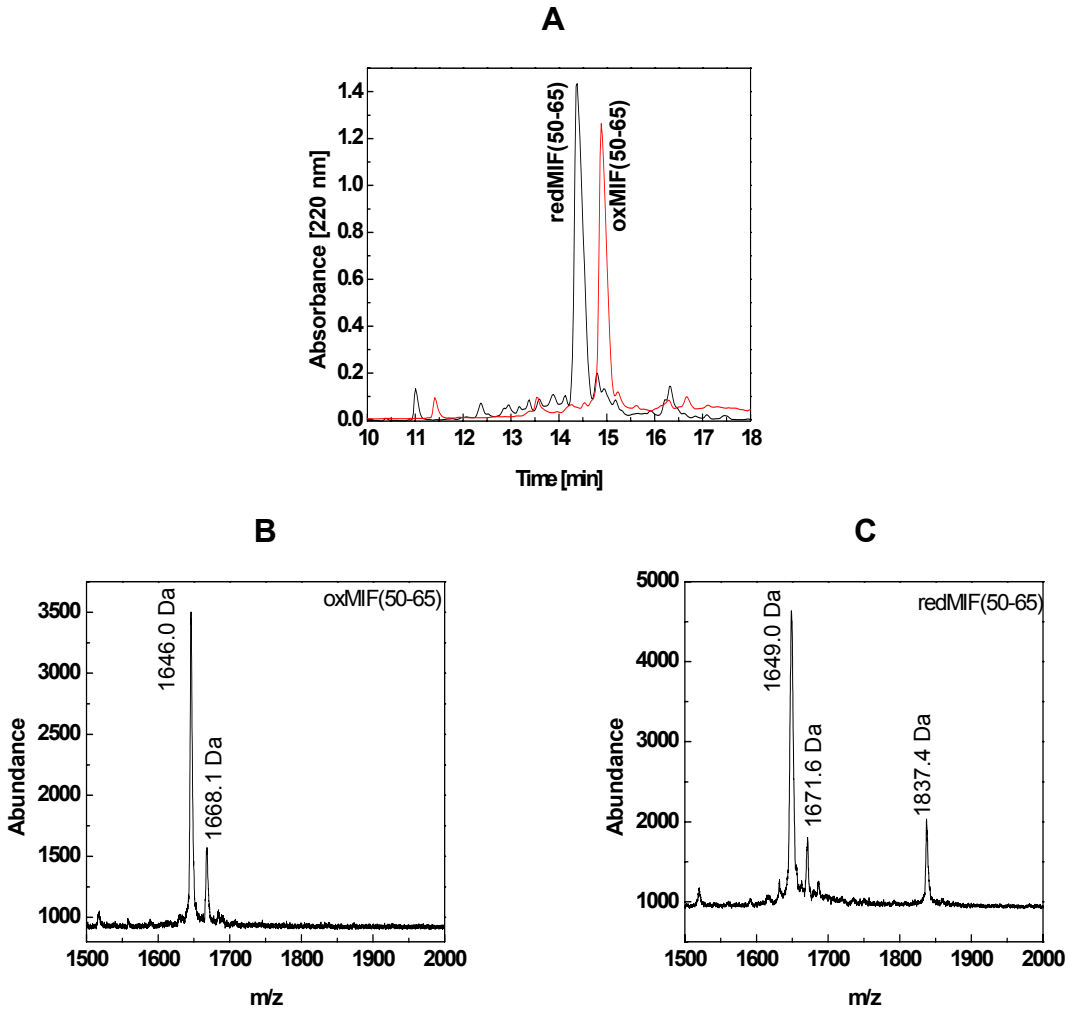

Figure 7: HPLC separation and mass spectrometric measurements of oxidized and reduced MIF(50-65). **A**: HPLC separation of oxdizedMIF(50-65) versus reducedMIF(50-65). Linear gradient over 30 min from 10 % to 90 % acetonitrile in 0.05 % trifluoroacetic acid. Flow rate = 1ml/min; wavelength = 220 nm; C18 reverse phase HPLC column = 125 x 4.6 mm. **B** and **C**: Mass spectrometric measurement of oxidized and reduced MIF(50-65), respectively. Reduced MIF(50-65) [redMIF(50-65)], oxidized MIF [oxMIF(50-65)].

5.1.4 Investigation of kinetic and equilibrium parameters of peptide MIF(50-65)

From the results described, it is apparent that it would be useful to investigate the kinetics and equilibrium parameters of the reduction of MIF(50-65) by mixtures of GSH/GSSG.

Reduction of MIF(50-65) as achieved in Figure 7 was obtained by maximal reducing conditions, i.e. by reducing the peptide with a large excess of the strong reductant DTT for an extended period of time. To characterize the redox behaviour of the peptide more precisely, GSH/GSSG redox mixtures were applied and various kinetic and equilibrium parameters determined.

The experiments began by mixing oxidized MIF(50-65) with GSH/GSSG at a ratio of 50/1 in 100 mM sodium phosphate, pH 7.0, 1 mM EDTA at 30°C for different time periods. Aliquots were removed and 1M H_3PO_4 added to lower the pH and to stop the reaction. Quenched aliquots were frozen and loaded on a C18 reverse phase HPLC column. Figure 8 shows that a GSH/GSSG ratio of 50/1 led to a rapid and almost complete reduction of oxidized MIF(50-65) after 2 h of incubation.

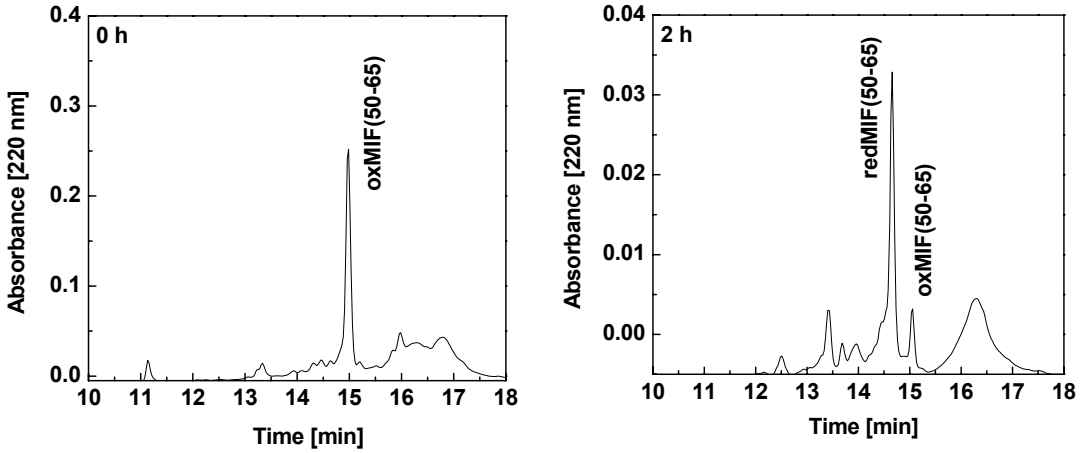

Figure 8: HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by GSH/GSSG (50/1) at time 0 min (left); and at time 2 h (right).

I also tried mixing the oxidized MIF(50-65) peptide with GSH/GSSG at a ratio of 50/0.001 in the same buffer employed above. The results showed that reduction at a GSH/GSSG ratio of 50/0.001 was even more complete (Figure 9).

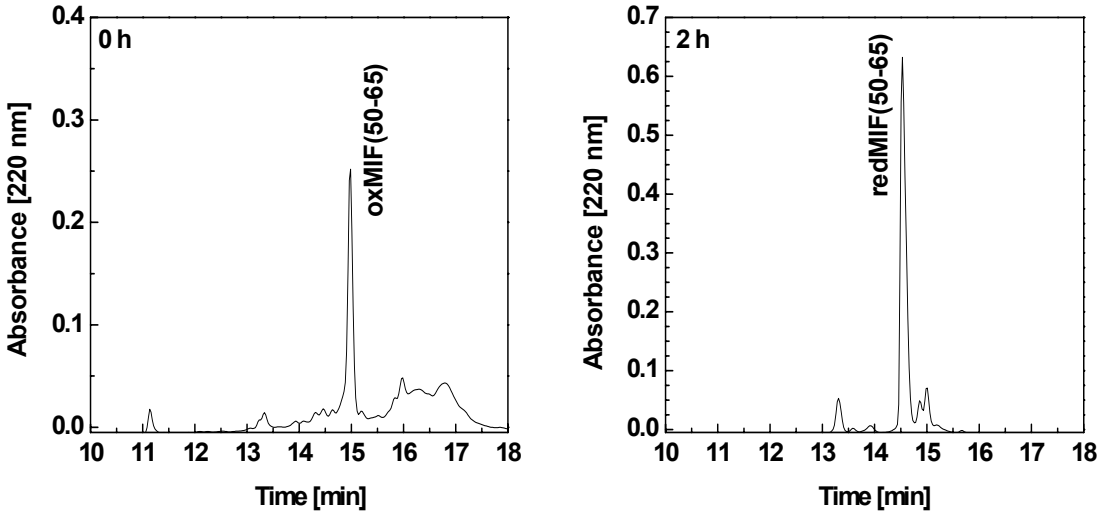

Figure 9: HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by GSH/GSSG (50/0.001) at time 0 min (left), and at time 2 h (right).

By contrast, Figure 10 shows an experiment involving a more a oxidizing GSH/GSSG ratio (50/10). Under such conditions, complete reduction was not reached even after 42 h of incubation. This experiment also showed that the equilibrium between oxidized and reduced peptide was reached at around 20 h of incubation. Mixtures expected to also contain a significant amount of the two possible glutathione mixed disulfide forms [GS-S-MIF(50-65); GS-S-MIF(50-65)-S-SG] showed 2 additional peaks at around 12.4 min and 13.4 min retention time, respectively.

Under these more oxidizing incubation conditions, it was also noted, that additional peaks appeared and then disappeared, a phenomenon that also had been seen for the early time points in the HPLC chromatograms using GSH/GSSG ratios of 50/1.

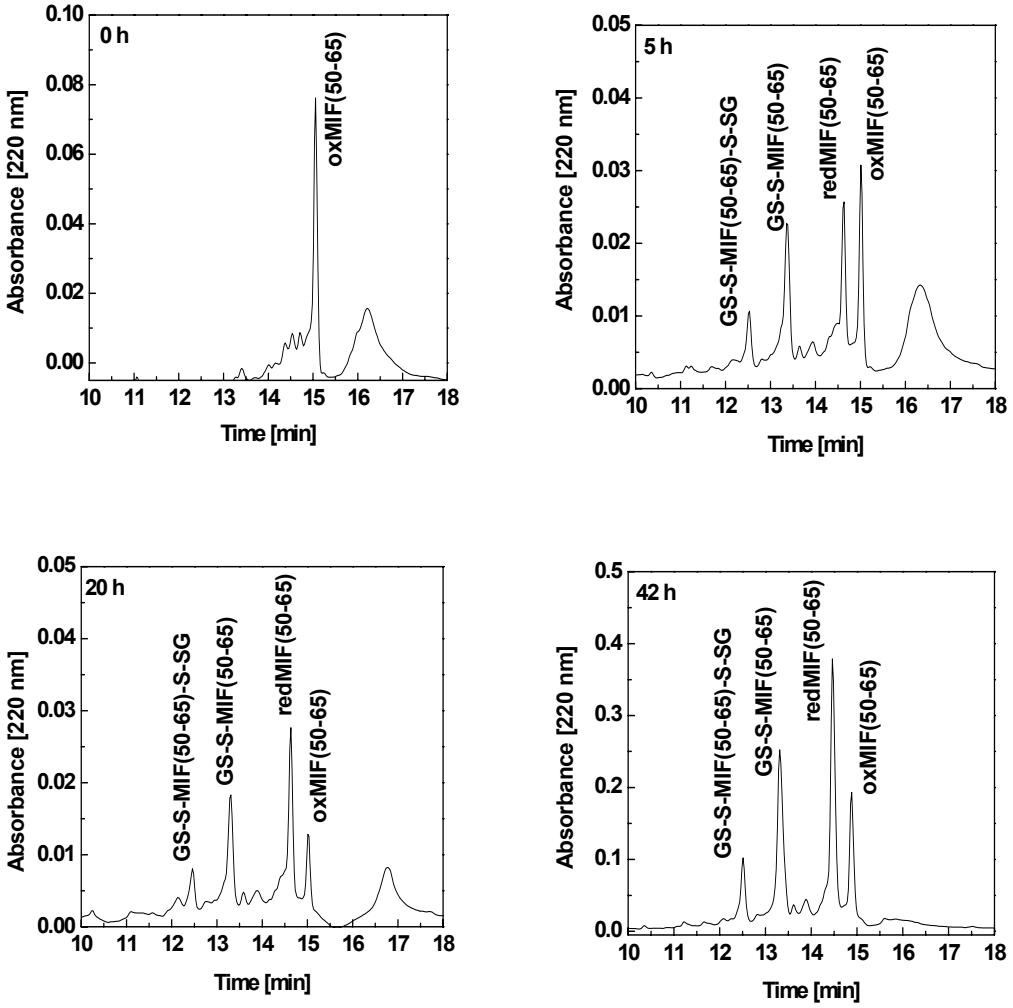

Figure 10: HPLC separation of MIF(50-65) following reduction of oxidized MIF(50-65) by GSH/GSSG (50/10) at time 0 min (top left), at time 5 h (top right), at time 20 h (bottom left) and at time 42 h (bottom right). Reduced MIF(50-65) [redMIF(50-65)], oxidized MIF(50-65) [oxMIF(50-65)], mono-mixed disulfide [GS-S-MIF(50-65)], di-mixed disulfide [GS-S-MIF(50-65)-S-SG].

Equilibrium measurements were then performed for peptide MIF(50-65) using a fixed total amount of the glutathione cofactor. Oxidized MIF(50-65) was equilibrated with a glutathione redox buffer containing varying ratios of [GSH/GSSG] at total concentrations of glutathione ([GSH] + [GSSG]) of 50 mM at 30°C in the same buffer as above. After 20 h of incubation, acid was added and the quenched aliquots were frozen and loaded on a C18 reverse phase HPLC column as before. The results showed that after 20 h a reducing ratio of GSH/GSSG

ratio of 48/2 led to almost complete as expected from the experiment using the 50/1 ratio (Figure 11). The result also confirmed that the equilibrium between oxidized and reduced peptide was reached at around 20 h of incubation.

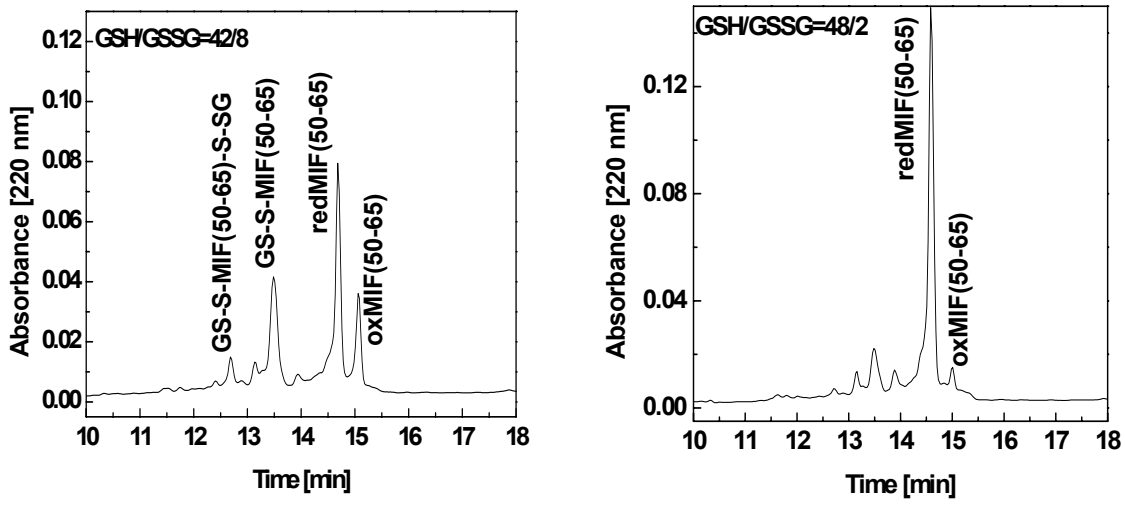

Figure 11: HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by GSH/GSSG at a ratio of 42/8 mM (left), and at a ratio of 48/2 mM (right) at 20 h.

For these conditions, all peaks were collected, lyophilized and analyzed by MALDI-TOF mass spectrometry to test whether GSH-MIF peptide mixed disulfide species may have formed as transition states (Figure 12).

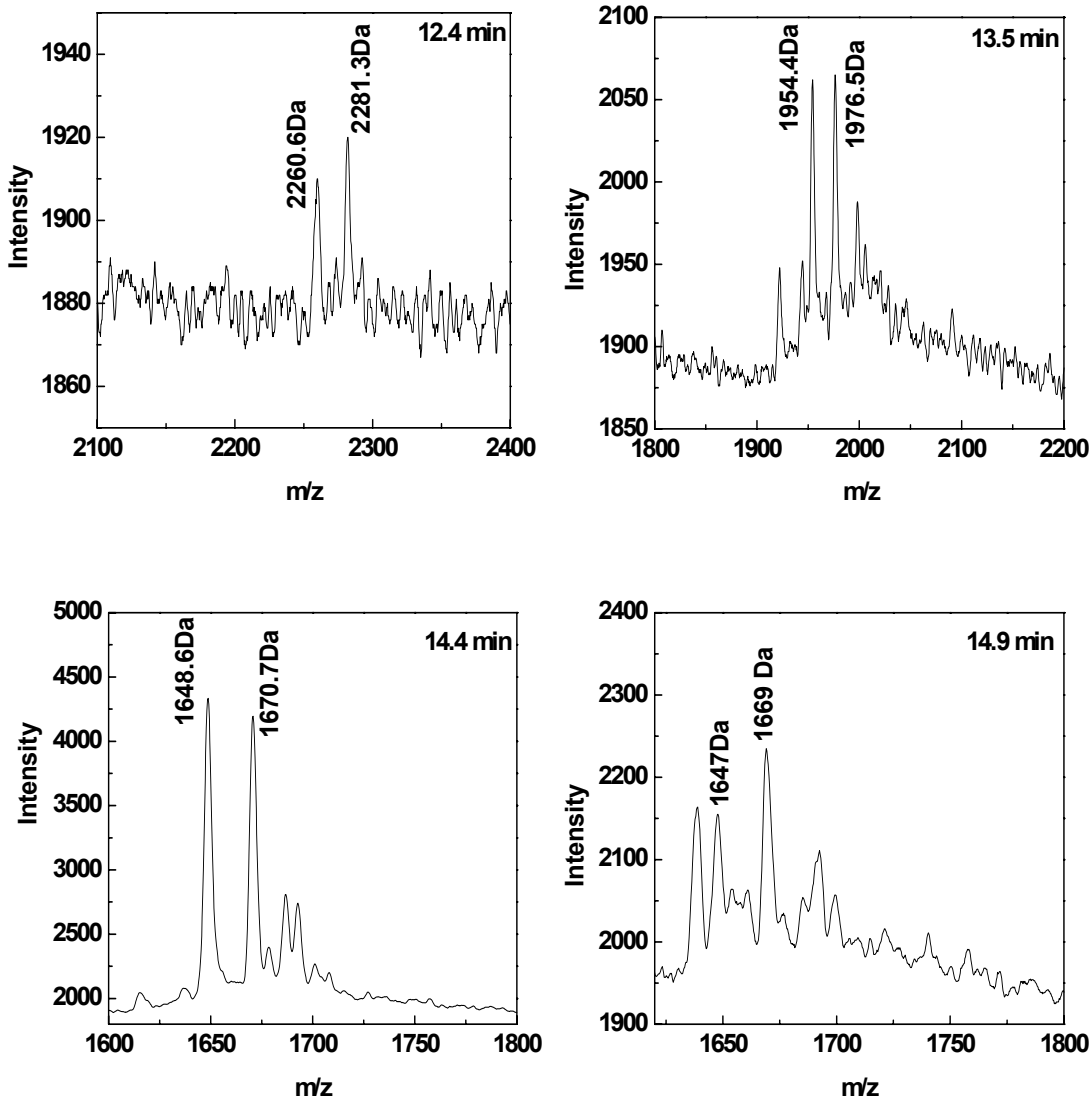

Figure 12: Mass spectrometric measurements of oxidized and reduced MIF(50-65) and the isolated mixed glutathione-MIF peptide species.

Table 9 summarizes the obtained mass spectrometric data and shows that both di- and mono-mixed disulfides can form during the reduction process of oxMIF(50-65). The data implicate such species as transition state species that may be critical to the redox process catalyzed by MIF.

Table 9: Summary of the mass spectrometric measurements of oxidized and reduced MIF(50-65) and the isolated mixed glutathione-MIF peptide species.

Species	Relative abundance at time 20 h (estimated)	Retention time (min)	Expected $M+Na^+$ mass for species (Da)	Experimentally determined $M+Na^+$ mass (Da)
oxMIF(50-65)	18%	14.9	1668	1669.0
redMIF(50-65)	40%	14.4	1670	1670.7
mono-mixed disulfide [GS-S-MIF(50-65)]	30%	13.5	1976	1976.5
di-mixed disulfide [GS-S-MIF(50-65)-S-SG]	10%	12.4	2281.6	2281.3
Dimer	< 2%	~ 16	3292 Da*	3295.1*

* $M+H^+$ mass

A typical kinetics experiment was performed by mixing oxidized MIF(50-65) with GSH/GSSG at a ratio of 42/8 mM at 30°C and pH 7.0 in the same buffers employed above. At the different time points, aliquots were removed and 1 M H_3PO_4 was added to lower the pH and to stop the reaction. Quenched aliquots were frozen and loaded on a C18 reverse phase HPLC column. oxMIF(50-65) and redMIF(50-65) were observed through the time course of the reaction.

Figure 13 A and B shows the kinetic data for such an experiment. The starting reagent was oxidized MIF(50-65). This was reduced by the excess redox buffer to produce reduced MIF(50-65), mono-mixed disulfide [GS-S-MIF(50-65)] and di-mixed disulfide [GS-S-MIF(50-65)-S-SG] in addition to oxMIF(50-65). Over time, reduced MIF(50-65) increased and oxidized MIF(50-65) decreased.

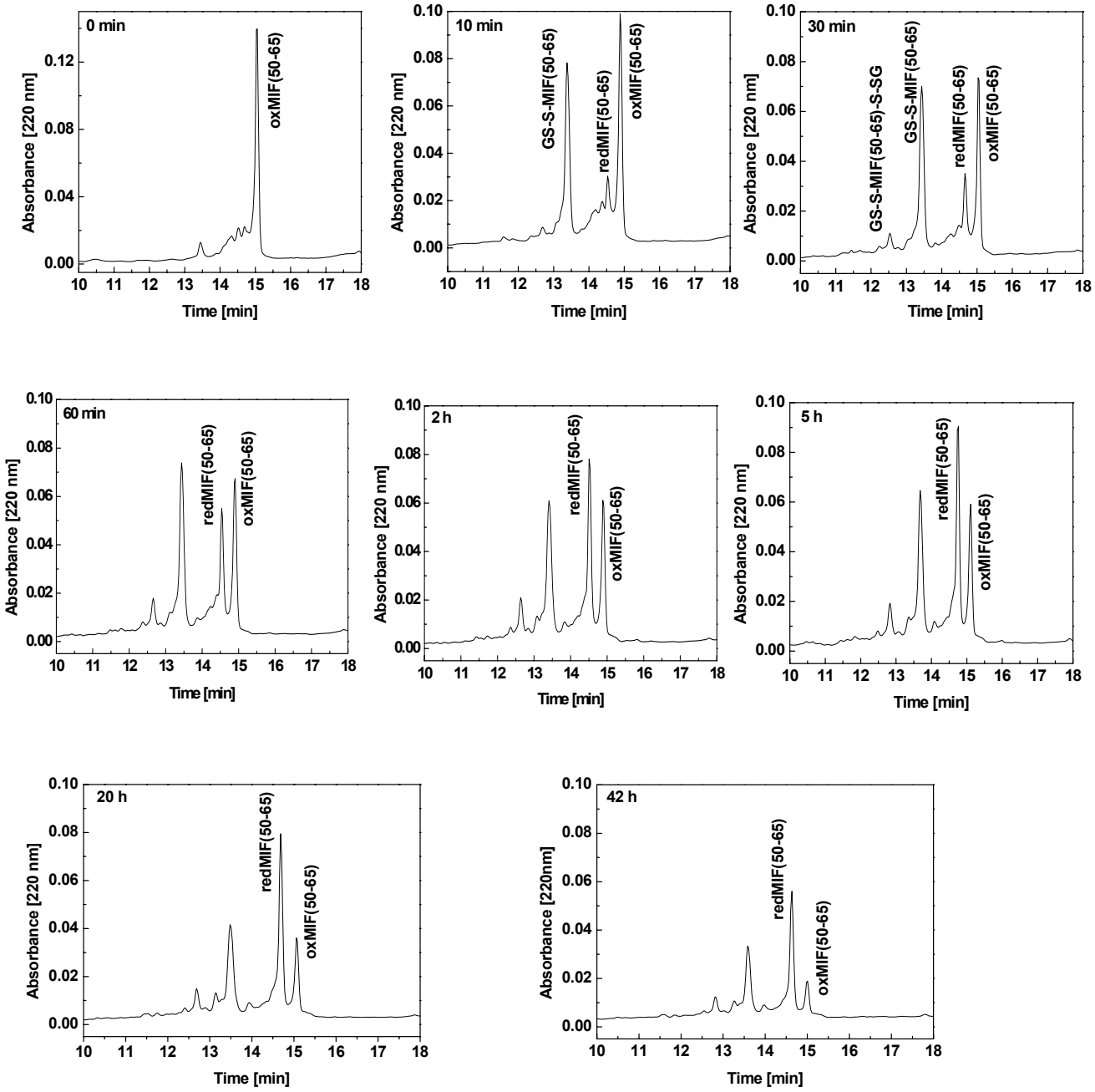

Figure 13A: HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by GSH/GSSG (42/8) as measured at various time intervals. Top left, 0 min; top middle, 10 min; top right, 30 min. Middle left, 60 min; middle middle, 2 h; middle right, 6 h. Bottom left, 20 h; and bottom right, 42 h.

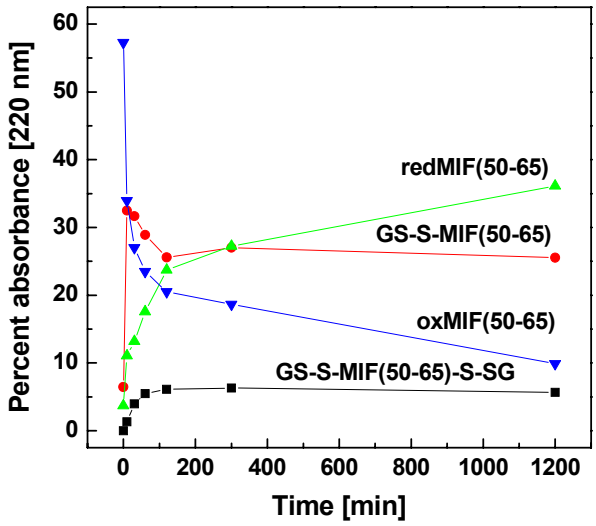

Figure 13B: Percent absorbance of the isolated glutathione-MIF peptide species from HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by GSH/GSSG (42/8) at various time intervals.

To study the redox behaviour of the peptide more closely and investigate whether MIF(50-65) could form disulfides with other small molecule thiols as well, cysteine/cystine redox mixtures were applied and various kinetic and equilibrium parameters determined.

The experiment was optimized and adjusted to a total cysteine content of 50 mM. This experiment showed that the equilibrium between the oxidized and reduced peptide was also reached at around 20 h of incubation. The kinetic parameters were analyzed by mixing oxidized MIF(50-65) with cysteine/cystine at a ratio of 42/8 mM under the same conditions as for the glutathione redox buffer. Figure 14 shows that reduction at a cysteine/cystine ratio of 42/8 is seen already for an early time point (10 min) compared to the corresponding glutathione experiment.

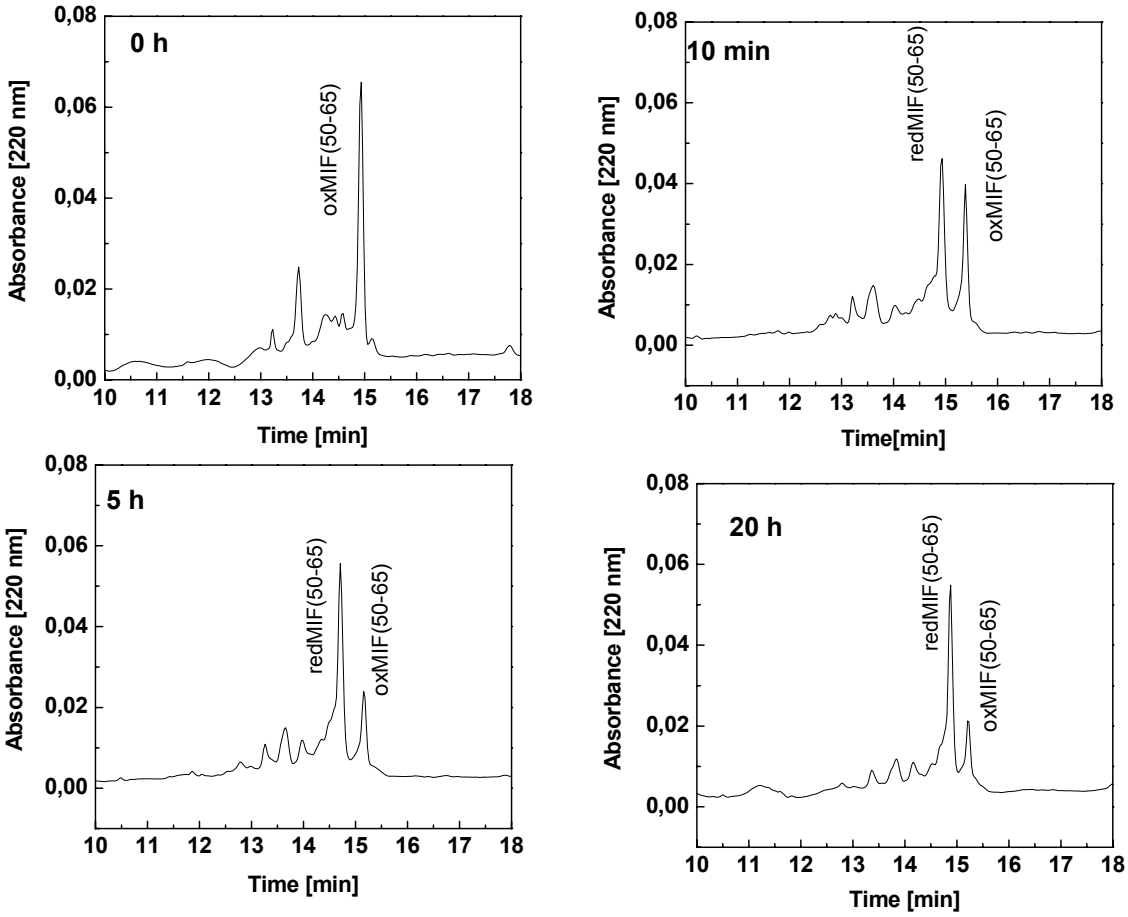

Figure 14: HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by cysteine/cystine (42/8) as analyzed at various time intervals. Top left, 0 min; top right, 5 h; bottom left, 2 h; and bottom right, 20 h.

This observations led us to investigate the kinetics of peptide MIF(50-65) under conditions as above but at early time points. After 30 seconds of incubation oxidized MIF(50-65) was already significantly reduced by the excess cysteine/cystine redox mixture (Fig. 15).

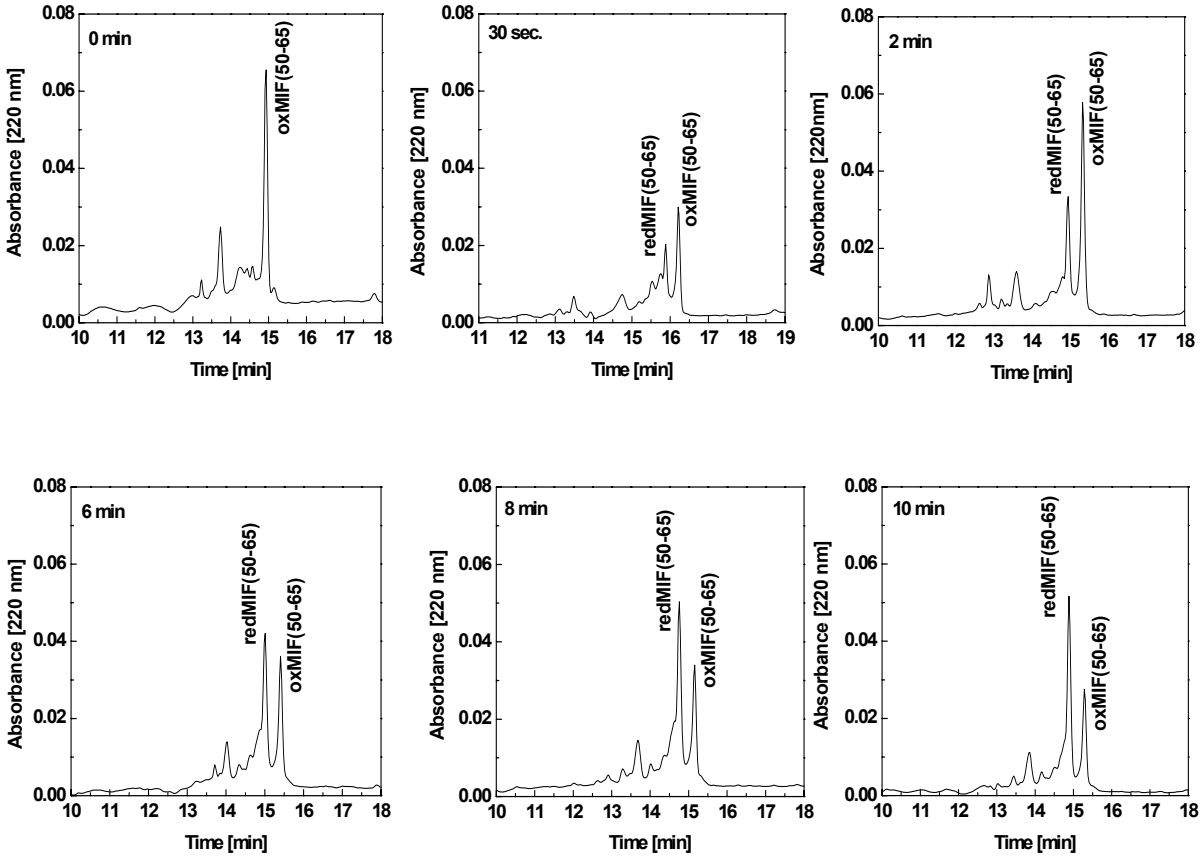

Figure 15: HPLC separation of MIF(50-65) following reduction of oxMIF(50-65) by cysteine/cystine (42/8) as analyzed at various time intervals. Top left, 0 min; top middle, 30 sec; top right, 2 min. Bottom left, 6 min; bottom middle, 8 min; and bottom right, 10 min.

The redox potential was determined at pH 7.0 and 30°C by equilibration of the MIF(50-65) with reference redox pair GSH/GSSG and cysteine/cystine. The experiments were performed at different GSH/GSSG ratios. All the expected redox species present at equilibrium i.e., redMIF(50-65), oxMIF(50-65), the mono-mixed disulfide and the di-mixed disulfide were identified by mass spectroscopy and the percentages are shown in Table 10.

Table 10: Relative abundance (%) of the isolated mixed glutathione-MIF peptide species by different GSH/GSSG ratios at time 20 h.

Species	Relative abundance at time 20 h (% estimated)						
	[42/8] (mM)	[43/7] (mM)	[44/6] (mM)	[45/5] (mM)	[46/4] (mM)	[47/3] (mM)	[48/2] (mM)
redMIF(50-65)	36.2	34.79	38.43	43.44	50.62	55.38	55.16
oxMIF(50-65)	17.2	14.0	12.89	11.47	8.3	6.59	5.4
mono-mixed disulfide [GS-S-MIF(50-65)]	27.5	25.2	21.76	25.47	21.25	20.76	17.61
di-mixed disulfide [GS-S-MIF(50-65)-S-SG]	6.8	6.75	5.77	4.84	2.99	1.2	2.06

Using these data, the redox potential of the peptide was calculated. The $[GSH]^2/[GSSG]$ or $[cysteine]^2/cystine]$ value used for each experiment and the corresponding [ox]/[red] ratio estimated from the HPLC were introduced into Equation:

$$Kox = \frac{[ox][GSH][GSH]}{[red][GSSG]}$$

The Kox values that had been derived from the experiments were then averaged and transformed into the equation: $Y = m X$

Finally, the E'o values were obtained from the Nernst Equation:

$$E'_0 = E'_0 (GSH/GSSG) – 0.03 \log Kox$$

A redox potential of - 0.258 V for peptide MIF(50-65) at 30°C and pH 7.0 was calculated from the Nernst equation using a value of - 0.205 V (Szajewski *et al*., 1980) for the glutathione standard potential . Using a value of - 0.223 mV for the cysteine standard potential (Millis *et al*., 1993), a redox potential of - 0.255 V for peptide MIF(50-65) also was calculated from the Nernst equation.

MIF is important for the regulation of the host immune and inflammatory response. The CALC motif was shown to form an intramolecular disulfide bond and the oxidoreductase activity of MIF was found to be dependent on the presence of the cysteines in this region (Kleemann *et al*., 1998a; Kleemann *et al*., 1998b; Mischke *et al*., 1997). A link between the oxidoreductase activity of MIF and its physiological function was found (Kleemann *et al*., 1999). In this thesis, I was not able to distinguish quantitatively the oxidized and reduced species of MIF. Accordingly, I was not able to determine a redox potential for full length MIF. However, I was able to determine a redox potential of - 0.258 V for peptide MIF(50-65). The data showed that the redox potential of MIF(50-65) was more reducing rather than oxidizing. The result agrees that the potential for MIF would also be a strongly reducing one (see Discussion). Interestingly, the redox potential of the peptide MIF(50-65) was stronger than the redox potentials of the Trx and Grx peptides that were analyzed by Siedler and colleagues (Siedler *et al*., 1993). Knowledge about the redox potential of MIF(50-65) will be helpful to elucidate the molecular mechanism of the immunological function of MIF.

5.2 Biological activities of MIF(50-65)

5.2.1 Investigation of the immunological activity of MIF(50-65)

MIF is induced by glucocorticoids and has the unique ability to override the anti-inflammatory and immunosuppressive effects of glucocorticoids on macrophages and T cells (Calandra *et al.,* 1995; Bacher *et al.,* 1996; Mitchell *et al*., 1999). Previous studies (Kleemann *et al.,* 1999) have indicated that the cysteine residues of the CALC motif are important for the immunologic effects of MIF. They showed that substitution of one or both cysteines of the CALC motif led to a striking alteration of the solubilities of these proteins, and suggested that both the cysteine residues of the catalytic CALC region influence the conformational and/or stability properties of MIF. Of note, in the overriding test, mutant C60SMIF had only 37 % activity of wtMIF.

The observed full activity in the glucocorticoid overriding test of both the PDI- and Trx-like mutants indicated that in addition to a potential conformational effect, the oxidoreductase and cytokine effect of MIF are governed by different redox requirements. Analysis of the PDI/Trx-like variants in the GC-OR, indicated that the redox potential is important. Examination of the Trx- and PDI-like MIF variants provided the first evidence concerning the redox properties of the CALC site. The redox properties of the catalytic centers of some thiol-protein oxidoreductases had been determined (Siedler *et al*., 1993). The redox properties were dependent on the X-X dipeptide sequences within the CXXC region of thiol-protein

oxidoreductases. Siedler and colleagues demonstrated that short peptide fragments retained some of the biochemical activity of full length Trx/Grx (Siedler *et al*., 1993; Siedler *et al*., 1994). I showed that MIF(50-65) has MIF-like redox properties. The reducing redox potential for MIF(50-65) as found in this thesis, led to the hypothesis that the short MIF peptide, although covering only 1/10 of the MIF molecule, could have MIF-like immunologic function.

To investigate the potential immunologic effects of peptide MIF(50-65), I first needed to establish a routine immunology assay, representing the biologic activity of MIF. Because the previously established GC-OR assay in PBMC was found to be unsuitable, I sought to establish a reliable cell line-based assay. Preliminary studies (data not shown) indicated that the THP-1 monocytic cell line could be suitable.

5.2.1.1 Establishing the THP-1 glucocorticoid overriding assay

I first tested the LPS responsiveness of these cells. LPS-stimulated tumor necrosis factor (TNF) production in THP-1 was evaluated. Figure 16 shows that a final concentration of 1 µg/ml of LPS was optimal for the experiment. Thus, TNF induction in this system was obtained by the same LPS concentration as that previously used by Calandra in PBMC.

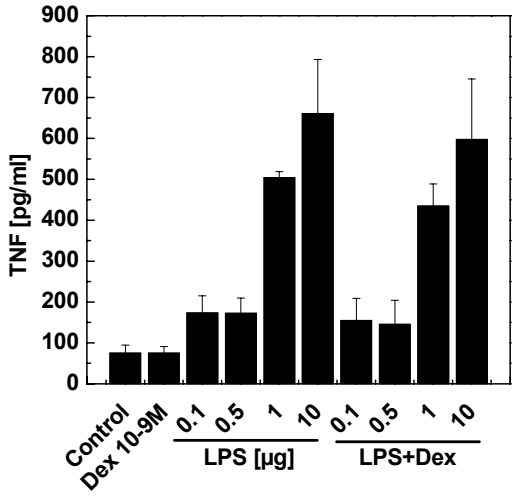

Figure 16: Effect of various concentrations of endotoxin (LPS) on the TNF production of THP-1 cells and inhibition by a concentration of 10^{-9} M dexamethasone (Dex). Data represent the mean ± SD of three independent assays.

On this basis, I then tested the dexamethasone (Dex) responsiveness of these cells. Figures 17 and 18 show that a reduction of at least 30 % was obtained with 10^{-6} M Dex, and thus shows that a final concentration of 10^{-6} M Dex was optimal for the experiment in connection with a maximal incubation period of 4 h for Dex and LPS (data not show). Similar results were reported by Gatanaga and Condino (Gatanaga *et al.,* 1991; Condino *et al.,* 1998). For the same assay time, the data showed that a concentration of 10^{-9} M Dex was not sufficient to suppress the effect of 1 µg/ml LPS on THP-1 cells TNF product (Figure 17).

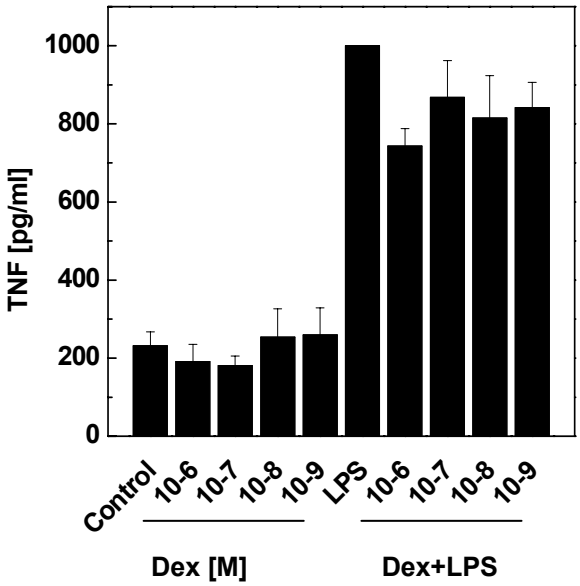

Figure 17*:* Basic studies – effect of endotoxin (LPS) on the TNF production of THP-1 cells and inhibition by various concentrations of dexamethasone (Dex). Data represent the mean ± SD of three independent assays.

Thus together, a typical glucocorticoid overriding experiment with THP-1 cells was performed as follows. THP-1 cells were incubated for 1 h with 10^{-6} M dexamethasone and rMIF at different concentrations (see Methods). LPS was added at a concentration of 1 µg/ml together with another rMIF dose and cells were incubated for 4 h. Culture supernatants were prepared and analyzed for TNF content using a commercial human TNF ELISA. Figure 18 demonstrates that MIF and MIF(50-65) had strong significant GC-OR activity at a concentration of 0.16 nM and 3300 nM, respectively. This finding confirmed that the concentration of 0.16 nM was the optimal dose for MIF in the glucocorticoid overriding assay

when compared to the results of the previous studies (Calandra *et al*., 1995, Kleemann *et al*., 1999).

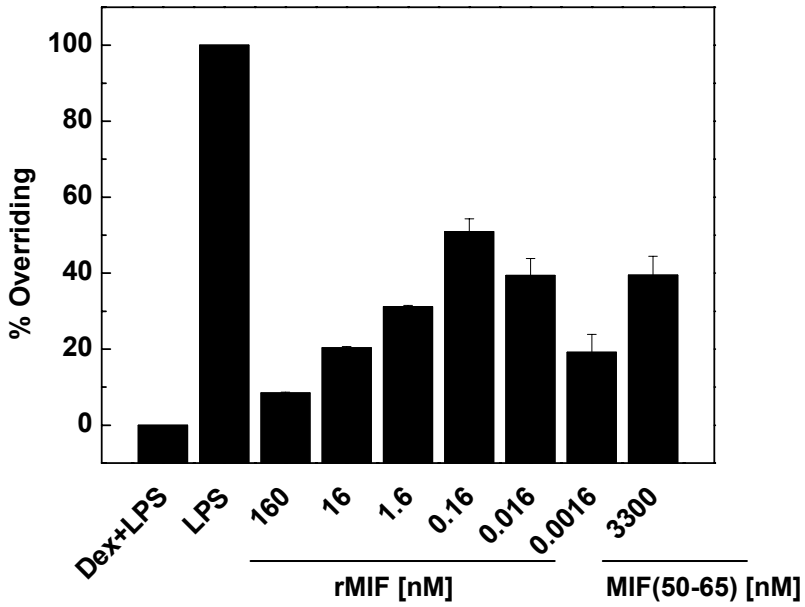

Figure 18: Immunological glucocorticoid overriding capacity of peptide MIF(50-65) in comparison to wtMIF. Data represent the mean ± SD of five independent assays.

5.2.1.2 Establishing a reporter gene-based glucocorticoid overriding assay for MIF

Redox regulation of transcription factors is an interesting and important issue. Previous studies reported that cellular glucocorticoid responsiveness is co-ordinately modulated by the redox state of the cell and the Trx level (Makino *et al*., 1996). In addition to analyzing the GC-OR assay in the above optimised form through a TNF ELISA read-out, I also devised an assay format, where the GC-OR assay was revealed through a NFκB-dependent transcription activity. NFκB activity was measured via a NFκB-Luc reporter construct. pNFκB-Luc is designed to monitor the activation of the NFκB signal transduction pathway and to measure the binding of transcription factor to the κ enhancer, providing a direct measurement of activation for this pathway.

The NFκB reporter assay includes the information obtained through the TNF ELISA, as TNF transcriptional activation, which precedes TNF protein production, is driven among other stimuli, through a NFκB site in the TNF promoter. In addition, the reporter assay was of interest as it naturally also covered earlier upstream signalling pathway.

In this study, I identified transcriptional suppression as the main mechanism for glucocorticoid suppression of TNF expression in THP-1 monocytic cells. THP-1 cells were transfected with pNFκB-Luc, rested overnight, and were preincubated with MIF along with Dex before LPS stimulation (see Methods). As shown in Figure 19, doses of 1.6 and 0.16 nM of MIF induced the transcription from the pNFκB-luciferase reporter construct at 6 h after exposure with LPS while Dex alone suppressed it. Thus, MIF counteracted the glucocorticoid-mediated inhibition of NFκB.

In principal, the data obtained were comparable to the data from the TNF ELISA except that the optimal MIF dose was discovered to be 1.6 nM rather than 0.16 nM.

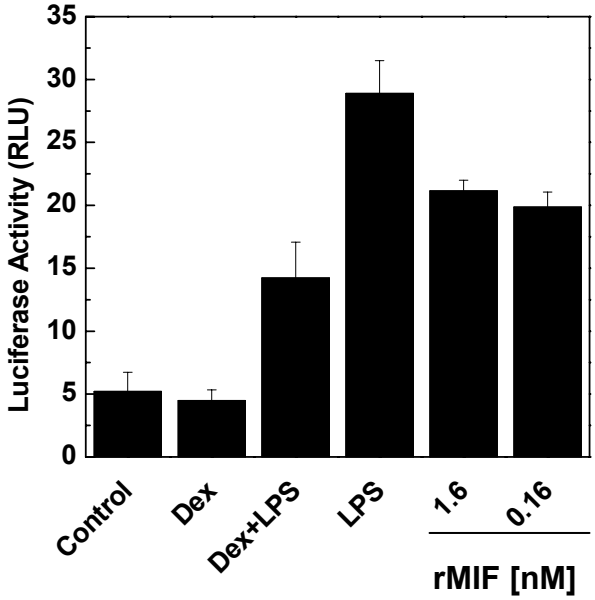

Figure 19: MIF enhances NFκB-driven luciferase activity. pNFκB-transfected cells were compared to the cells transfected in the presence of dexamethasone 10^{-6} M (Dex), lipopolysaccharide 1 µg (LPS), lipopolysaccharide and dexamethasone (Dex+LPS), and recombinant MIF (rMIF). This allowed monitoring of luciferase activity directly driven by activation of the pSV-β Gal transcriptional activator. Data represent the mean ± SEM of three independent assays.

5.2.1.3 Investigation of MIF(50-65) activity in the THP-1 reporter gene-based glucocorticoid overriding assay

To further confirm the influence of MIF(50-65) on the immunological activity of MIF, I tested the peptide in the established glucocorticoid overriding reporter assay. MIF(50-65), at a concentration of 3300 nM, was found to exhibit GC-OR reporter gene activity comparable to that of wtMIF and exhibited an overriding effect of about 40 % (Fig. 20). This further confirmed that the CALC region and the redox activity of MIF is important for the cytokine functions of MIF.

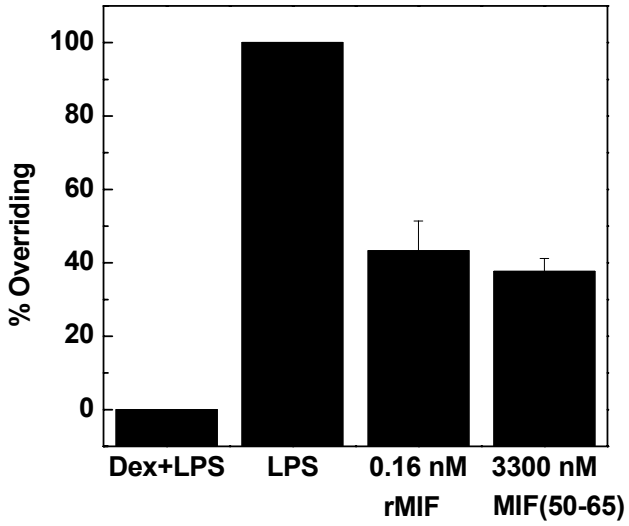

Figure 20: Immunological glucocorticoid overriding capacity of peptide MIF(50-65) in comparison to wtMIF, The established THP-1 reporter gene GC-OR assay was applied. Data represent the mean ± SD of five independent assays.

5.2.2 Intracellular MIF and MIF(50-65) activity: modulation of the cellular activity by interaction with JAB1

Another clue towards the elucidation of the molecular pathways of MIF action has come from the identification of the coactivator JAB1 as an intracellular binding protein of MIF (Kleemann *et al.,* 2000a). Following non-receptor-mediated endocytosis, MIF specifically interacts with JAB1 in both immune and non-immune cells (Kleemann *et al.,* 2000a). Intracellular interaction of MIF and JAB1 also occurs in MIF-producing cells (Kleemann *et al.,* 2000a). MIF can modulate activator protein-1 (AP-1) transcriptional and c-Jun N-terminal kinase (JNK) pathways and the cell cycle through JAB1. MIF specifically interacts with JAB1 (Claret *et al*., 1996), a coactivator of AP-1 transcription that promotes degradation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ (Tomoda *et al*., 1999). MIF also counter-regulates JAB1-dependent cell cycle processes. MIF increases $p27^{Kip1}$ expression and strongly inhibits JAB1 mediated rescue of fibroblasts from starvation-induced growth arrest. Depending on the

physiological context and the cell type, MIF can stimulate or inhibit cell proliferation (Mitchell and Bucala, 2000; Kleemann *et al.,* 2000a; Abe *et al.,* 2001).

The CALC redox motif of MIF is important for at least some of the JAB1-mediated activities of MIF, and MIF-induced $p27^{Kip1}$ levels in a dose-dependent manner (Kleemann *et al.,* 2000a). To investigate whether MIF peptide fragment MIF(50-65) could also negatively regulate JAB1 action with regard to non-AP-1-related activities as MIF, I also studied the effect of MIF(50-65) on the levels of the cell-cycle inhibitor $p27^{Kip1}$. MIF(50-65) also induced $p27^{Kip1}$ levels as indicated by immunoblots prepared from lysates of proliferating NIH 3T3 (Figure 21A and B). These data showed that MIF(50-65), at a concentration of 3.3 µM, had a similar JAB1-antagonizing activity as full-length MIF at a concentration of 1 µM.

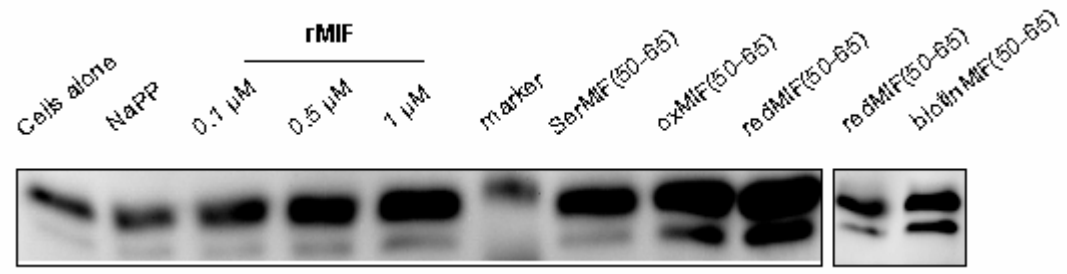

Figure 21A: Western blotting analysis: MIF and MIF(50-65) induce $p27^{Kip1}$ expression in NIH 3T3 fibroblasts in a dose-dependent fashion. Immunoblots were stained with anti-$p27^{Kip1}$ antibody. NaPP; control buffer.

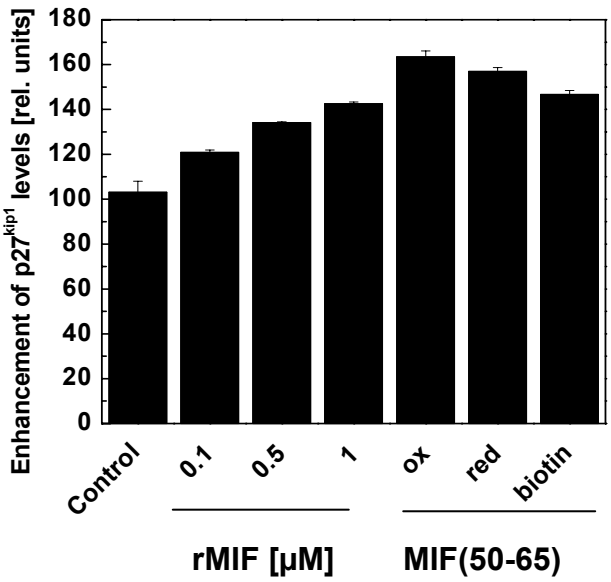

Figure 21B: Quantitation of the Western blot analyses as shown in Figure 21A. Quantification was performed by densitometric analysis of three independent assay (mean ± SEM). Pixel densities of % activity bands were standardized by calculating the ratios of the band intensities of $p27^{Kip1}$.

MIF is a cytokine that has anti-inflammatory growth-inhibitory activities. MIF(50-65) was designed to span the CXXC region of MIF; it exhibited numerous immunological and cellular activities comparable to the effects observed for MIF. Like MIF, MIF(50-65) exhibited GC-OR activity and also induced the cellular levels of $p27^{Kip1}$. These data suggested that the CXXC sequence region of MIF is important for intracellular functions of MIF and its immunological activities.

5.3 Investigation of the role of the redox activity of MIF for the molecular mechanism of action of MIF

The studies on the possible role of the redox activity of MIF on its molecular mechanism was further investigated. In particular, I was interested in the question whether redox regulation by MIF may be involved in cellular stress, oxidative stress, apoptosis, and other signalling pathways. Previous work showed that Trx, by a enzymatic redox mechanism, plays an important role in the regulation of oxidative stress, cell proliferation, and apoptosis (Hayashi *et al*., 1993).

MIF had been demonstrated to suppress p53-mediated apoptosis (Hudson *et al*., 1999). I first wished to establish whether MIF also had apoptosis-modulatory properties that were not directly related to p53-dependent apoptotic processes. As the investigation of some of the effects of MIF on cellular activity had been difficult experimentally due to the high endogenous MIF levels in the cells studied, I first devised a suitable experimental system exhibiting reasonable differences between the concentrations of MIF obtained following overexpression or ectopic addition and the baseline expression levels of MIF.

5.3.1 General tools

5.3.1.1 Cloning of the C60SMIF mutant in the eukaryotic Tet expression system

The construction of a human MIF cDNA-bearing tetracycline-responsive plasmid, pMIFRP, for the transient and stable transfection experiments, was performed by PCR cloning (see methods). pC60SMIFRP was constructed in an identical manner except that a pET11b plasmid bearing the human C60SMIF cDNA sequence (Kleemann *et al*., 1998a) was used as template in the amplification step. Briefly, I generated C60SMIF cDNA with the suitable restriction enzyme overhangs by PCR. Then pC60SMIFRP was generated by ligation of the C60SMIF insert in the response vector pUHD 10-3 and transformation in to competent *E. coli* DH5α. Positive colonies of the mutants were picked and the plasmid DNA isolated. The DNA of the transformed positive clones was purified by the maxiprep procedure (see Methods). All generated cDNA sequences were confirmed by bidirectional DNA sequencing. A gel of the cloned plasmid pC60SMIFRP is shown in Figure 22.

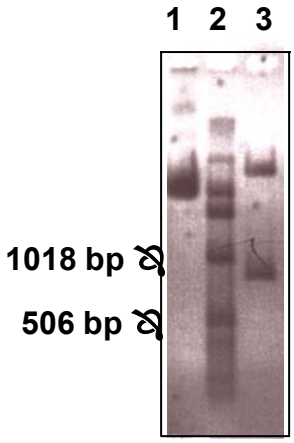

Figure 22: Analysis of the cloned plasmid pC60SMIFRP digested with *Sac* II/*Bam* HI by agarose gel electrophoresis. 1: pUHD 10-3 negative control; 2: Marker; 3: Plasmid isolated from colony and cut with *Sac* II/*Bam* HI.

5.3.1.2 Measurement of glutathione levels

Glutathione (GSH), a ubiquitous thiol, plays a major role in maintaining intracellular redox balance and regulating pathways augmented by oxidative stress (Meister, 1988; Haddad *et al.,* 2000). Thus, intracellular glutathione levels were measured in lysates from cells overexpressing human MIF. For overexpression, MIF was induced using the Tet-off system in cells which were cultured in thiol-free medium (TFM) to induce oxidative stress.
HtTA cells represent HeLa cells stably transfected with a Tet-off regulator gene cassette. HtTA cells stably expressing the human MIF gene (HtTAM) were generated by transfecting HtTA cells with phuMIFRP using the SuperFect transfection reagent (see Methods). For glutathione determinations from HtTAM cell lysates, the cells were cultured and assayed for intracellular GSH by a commercial glutathione assay (BIOXYTECH GSH-400 colorimetric assay) according to the manufacturers intructions (see Methods).

Exceptions to the general procedure were made in that the cells were either homogenized with a Dounce homogenization by vortexing for 1-5 min or by a ultrasonification procedure for 1-10 min.

Comparison of the three methods showed that direct addition of MPA to the cells followed by dounce homogenization was the best method to measure the glutathione levels of these cells, while various other homogenization procedures including ultrasonification in combination with mechanical methods and a ultraturrax treatment did not yield satisfactory results (data not shown).

5.3.2 Apoptosis assays

5.3.2.1 MIF inhibits apoptosis induced by a variety of stimuli

The Tet-off plasmid coding for the C60S variant of human MIF as well bacterial recombinant mutant C60SMIF (rC60SMIF), the later being available from former work in our lab (Kleemann *et al*., 1999), then served as the key molecular tools to investigate the role of the CXXC region of MIF and the redox activity of MIF for the various cellular and molecular pathways, in which MIF was assumed to be involved.

One such system was obtained by stably transfecting the Tet-off-regulated HeLa cell line HtTA with the cDNA for human MIF. HtTA cells contain significant baseline concentrations of MIF (123 ± 19 fg/cell; mean ± SD, n = 3), but preliminary experiments with transiently transfected Tet-off-regulated MIF plasmids had suggested that good specific induction levels could be obtained with this system. Out of a total number of 36 stable clones obtained, 3 were tested further and screened for their dox-dependent MIF expression levels. Clones 13 and 14 showed the most favorable combination of low baseline MIF expression values and high induction levels and had switch-on factors for the expression of MIF of 5.2 ± 0.2 and 2.6 ± 1.1, respectively (mean ± SD, n = 3) (Figure 23).

Clone 13 exhibited a baseline MIF concentration of only 96 ± 26 fg/cell (mean ± SD, n = 3) in the presence of 1 µg/ml dox (switch-off status), which is less than 50 % of the concentration of MIF measured in most other cell types (Bacher *et al.,* 1996). Clone 13 was thus used for the apoptosis experiments in the study. HeLa cells stably transfected with both the Tet-off gene regulation cassette and the human MIF gene were designated HtTAM cells. I used the anti-tumor drug and topoisomerase I inhibitor cam as a strong stimulus for the induction of apoptosis (Kaufmann, 1998; Darzynkiewicz *et al*., 1996).

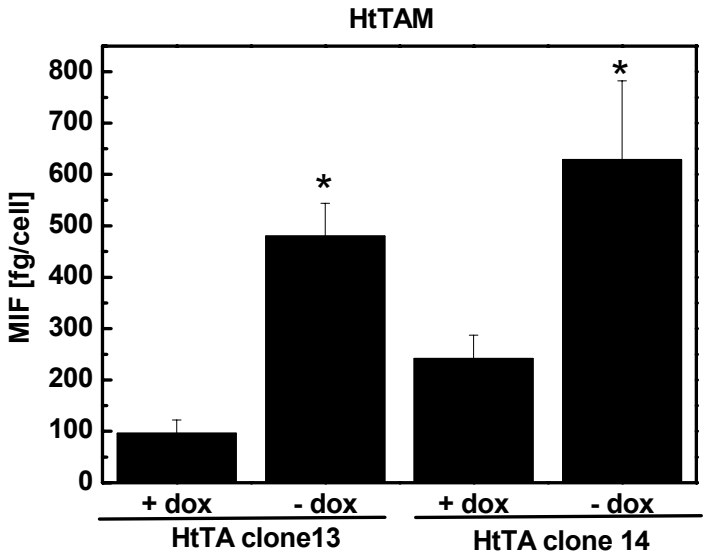

Figure 23: Stably transfected human MIF is efficiently and specifically overexpressed in Tet-off-regulated HeLa cells. G418-resistant HeLa cell clones stably carrying the Tet-off gene cassette and the human MIF gene (HtTAM cells) were induced by removal of dox from the culture medium and expressed MIF measured from the cell lysates by ELISA. MIF expression of the two most suitable clones (13 and 14) is shown. Data are means ± SD from three experiments. -dox represents MIF expression following induction; the +dox values show the non-specific "leak" expression of MIF in the switched-off state. *, Statistically significant differences with $p < 0.05$, the p value of -dox bars refers to the corresponding +dox bars.

Overexpression of MIF in HtTAM cells initiated by the removal of dox from the culture medium protected cells from cam-induced apoptosis by up to 40 %, depending on the concentration of cam applied (Figure 24).

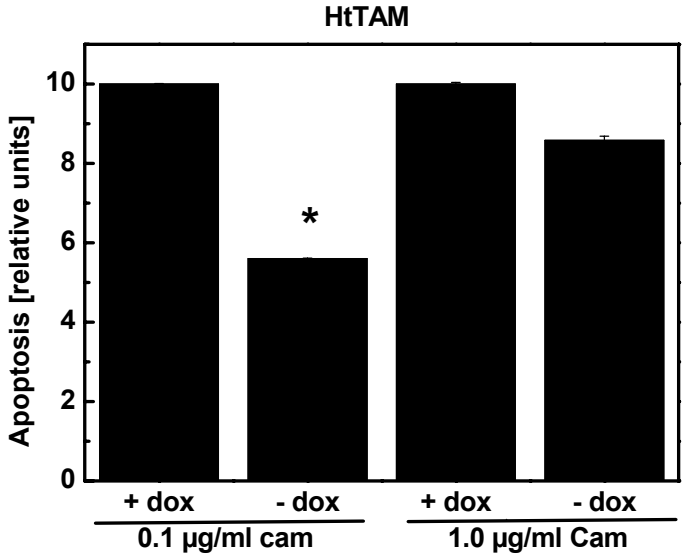

Figure 24: MIF specifically overexpressed by Tet-off gene regulation inhibits cam-induced apoptosis in HtTAM cells. Comparison of cellular apoptosis in the switched-on (-dox) versus switched-off (+dox) at two different cam concentrations. Apoptosis was measured by a nucleosome-specific cell death ELISA from the cell lysates and is expressed as relative units. Data represent mean values ± SD from three experiments. *, Statistically significant differences with $p < 0.05$, the p value of -dox bars refers to the corresponding +dox bars.

For comparison, when apoptosis was induced by growing the HtTAM cells to high density (cell density-induced apoptosis (Saeki *et al*., 1997)), the degree of suppression of apoptosis yielded by the overexpression of MIF was approximately 20 % (n = 3; data not shown). Overall I observed that, while MIF significantly inhibited apoptosis induced by these stimuli, the degree of protection provided, while significant, was only partial, ranging from 20 to 50 % (see also below).

Successful generation and expression of pC60SMIF was demonstrated by the β-galactosidase transfection efficiency control method in both COS-1 cells with a specific switch-on factor of > 300-fold (data not shown). The rhabdomyosarcoma cell line Kym-1 was chosen as another experimental system. Kym-1 cells only contain minute concentrations of

MIF protein if any (none by Western blotting and 7 ± 5 fg/cell (mean ± SD, n = 3) by MIF ELISA; see (Kleemann *et al.,* 1998a)), thus exhibiting a very favorable background expression level. I was not able to generate Kym-1 clones stably expressing both the Tet-off regulator and human MIF genes (data not shown). However, transient overexpression of the Tet-off and MIF plasmids in these cells led to excellent specific induction levels of MIF in Kym-1 cells (specific switch-on factor of 2-7-fold) (Figure 25). pC60SMIF behaved essentially like wtMIF, showing that the mutant plasmid was a good tool for comparing effects with those of wtMIF.

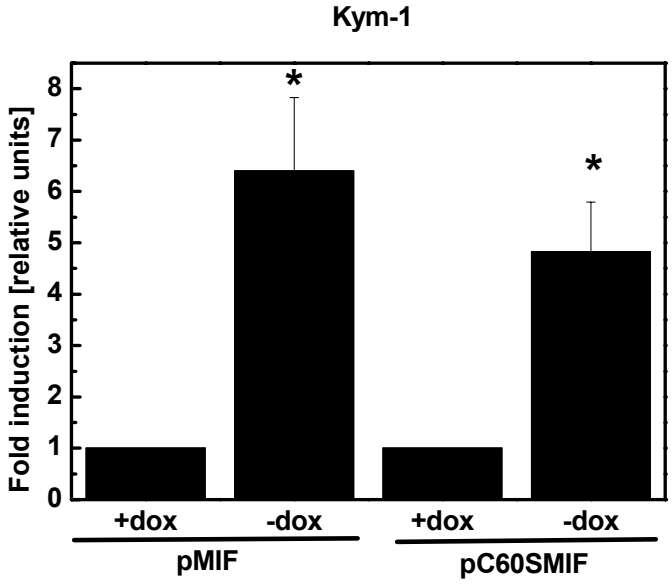

Figure 25: The degree of overexpression of the enzyme-dead MIF mutant C60SMIF was analysed in comparison. Data are the mean ± SD from three experiments. -dox represents MIF expression following induction; the +dox values show the non-specific "leak" expression of MIF in switched-off state. *, Statistically significant differences with $p < 0.05$, the p value of -dox bars refers to the corresponding +dox bars.

To further investigate a possible involvement of the MIF redox activity in the anti-apoptotic effect, first attempts were also made to utilize the pMIF expression system. Evidence obtained from transfecting Kym-1 cells with pMIF indicated, that expressed wtMIF inhibited apoptosis as expected. When apoptosis in these cells was induced by growing the cells to high density, MIF led to a reduction of apoptosis by 30 % (Figure 26).

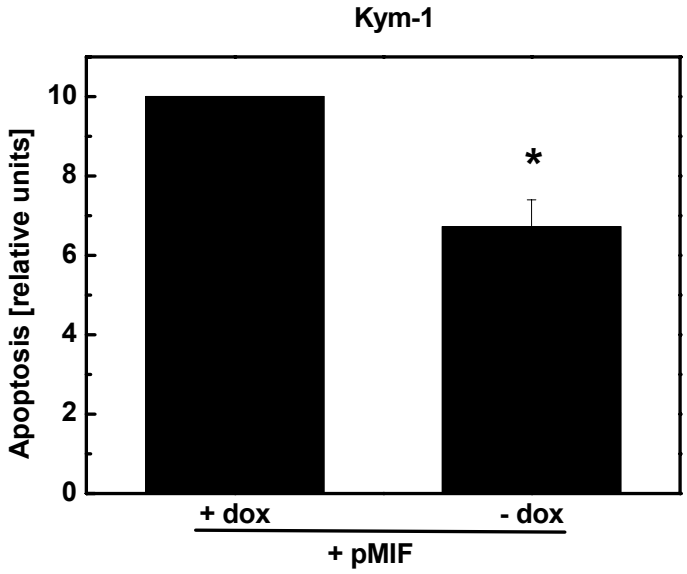

Figure 26: Overexpression of transiently transfected human MIF was analyzed in Kym-1 cells transiently expressing the TA regulator and induced to undergo apoptosis by cell density stress. Data are mean ± SD from three experiments. –dox represents MIF expression following induction; the +dox values show the non-specific "leak" expression of MIF in the switched -off state. *, Statistically significant differences with $p < 0.05$, the p value of -dox bars refers to the corresponding +dox bars.

Together, these data showed that intracellular MIF, following ectopic overexpression, was able to inhibit apoptotic processes induced by different stimuli. As these effects seemed to strictly depend on the enhancement of the concentration of intracellular MIF protein (Figure 23 and Figure 25), they may represent a MIF activity that may not be related to its repertoire of activities of a transcellularly acting cytokine. Thus, I next tested whether biologically active recombinant human MIF (rMIF) added exogenously to cells could also inhibit apoptosis. The human promyeloblast cell line HL-60 was applied for these experiments, as the monocyte/macrophage had been demonstrated to be the most important cellular target of the immunological and inflammatory cytokine activities of MIF (Calandra *et al*., 1995; Calandra *et al.,* 1994). Camptothecin led to a strong apoptotic response in these cells (Figure 27). Recombinant MIF, when added to HL-60 cells for 24 h before cam treatment, markedly

reduced the degree of apoptosis in a concentration-dependent manner. The protection from apoptosis provided by MIF ranged from a few percent (for 1 nM MIF) up to 50 % (for 3 µM MIF) (Figure 27).

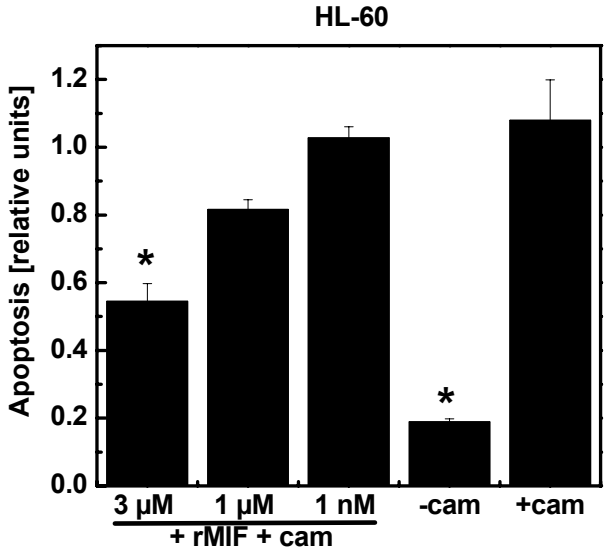

Figure 27: Exogenously added recombinant human MIF (rMIF) inhibits cam-induced apoptosis in HL-60 leukocytes in a dose-dependent manner. Apoptosis was measured as in Fig. 24 and Fig. 26. Data represent mean values ± SD from three independent experiments. *, Statistically significant differences with $p < 0.05$, the p value refer to +cam.

The potential anti-apoptotic activity of rMIF on immune cells was also probed in a cellular model of UV stress-induced apoptosis. I found that, when Jurkat T cells were exposed to UV irradiation for 1 or 6 h, pre-incubation with rMIF (2 µM) led to an inhibition of apoptosis of 20-30 % (Figure 28).

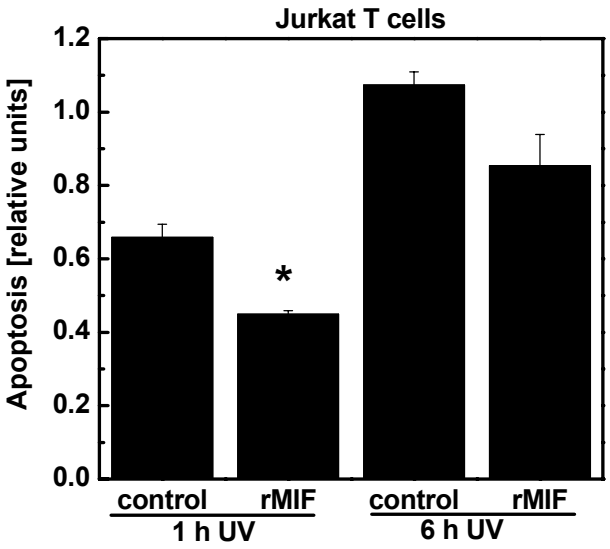

Figure 28: MIF inhibits UV stress-induced apoptosis in T cells. Jurkat T cells were treated with UV irradiation for 1 or 6 h and relative apoptosis compared for cells previously teated with rMIF versus cells treated with buffer control. Data represent mean values ± SD from three experiments. *, Statistically significant differences with $p < 0.05$, the p value refer to control.

5.3.2.2 Dependence of the apoptosis-suppressing activity of MIF on the presence of residue Cys-60

Together, these experiments demonstrated that MIF could inhibit apoptosis induced by several stimuli in a variety of cell types. It appeared that both intracellular MIF, possibly representing its role as an abundant enzyme and cell regulator, and exogenously added rMIF, representing the activities of MIF as a typical cytokine, can protect cells from apoptosis. MIF has been shown to share several functional homologies with proteins of the Trx family of oxidoreductases (Kleemann *et al.,* 1998a; Holmgren, 1985a). Like Trx, MIF exhibits an enzymatic thiol-protein oxidoreductase activity that is dependent on the presence of a CXXC redox motif (Kleemann *et al.*, 1998a; Kleemann *et al*., 1999). Ample evidence is now available to show that Trx exhibits cytokine-like properties (Bertini *et al.,* 1999; Pekkari *et al*., 2000; Arner and Holmgren, 2000) and participates in the regulation of cellular redox homeostasis (Holmgren, 1985a; Arner and Holmgren, 2000; Nishinaka *et al.*, 2001; Tanaka *et al.*, 2000; Powis *et al*., 2000). Trx acts as a potent inhibitor of redox stress-induced

apoptosis and this activity was found to be dependent on the presence of cysteine residues of Trx (Powis *et al*., 2000; Iwata *et al.,* 1997; Sato *et al*., 1995). These similarities between MIF and Trx as well as my previous finding on the redox properties of MIF(50-65) prompted me to investigate whether MIF may also play an important role in redox stress-induced apoptotic events. As the oxidoreductase enzymatic activity of MIF had been demonstrated to denpend on the presence of the CALC sequence motif of MIF, I thus compared the apoptosis-regulating properties of wtMIF with those of the enzyme-dead mutant C60SMIF. Induction of C60SMIF was comparable to that of wtMIF (Figure 25). However, when the apoptosis-regulating effect of overexpressed wtMIF in Kym-1 cells was compared to that of the cysteine mutant, I observed that overexpression of C60SMIF did not lead to any suppression of apoptosis. This would be in agreement with an involvement of the CXXC/redox mechanism. Enhanced levels of the mutant protein even appeared to slightly promote apoptosis (Figure 29).

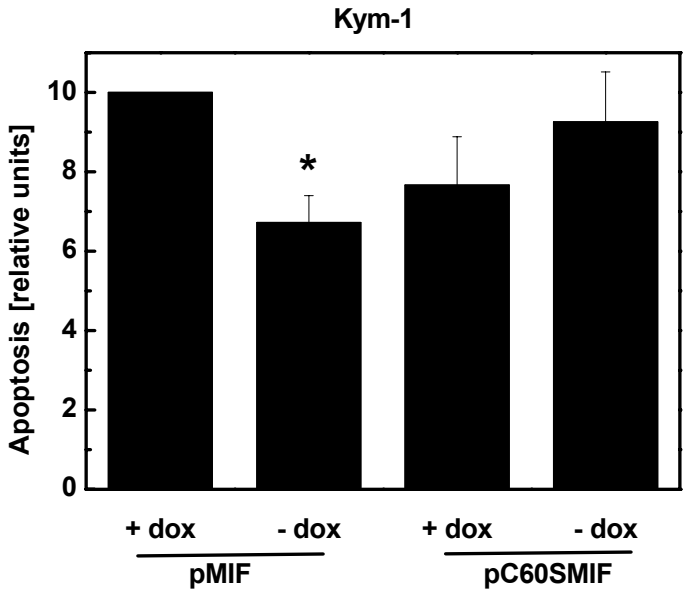

Figure 29: Endogenously overexpressed mutant C60SMIF does not exhibit apoptosis-inhibiting activity. Kym-1 cells transiently transfected with the Tet-off gene cassette and the human MIF gene and induced to undergo apoptosis by cell density stress as in Fig. 26 were analyzed for apoptosis and compared to cells transfected with the plasmid encoding for C60SMIF. The relative induction factor for wtMIF and C60SMIF were comparable as shown in Fig. 25. Data represent mean values ± SD from three experiments. *, Statistically significant differences with $p < 0.05$, the p value of -dox bars refers to the corresponding +dox bars.

Surprisingly, a different outcome was observed, when the recombinant proteins exogenously added to the Kym-1 cells were studied. Recombinant C60SMIF behaved similarly to rMIF, causing a marked and concentration-dependent apoptosis-suppressing effect (Figure 30).

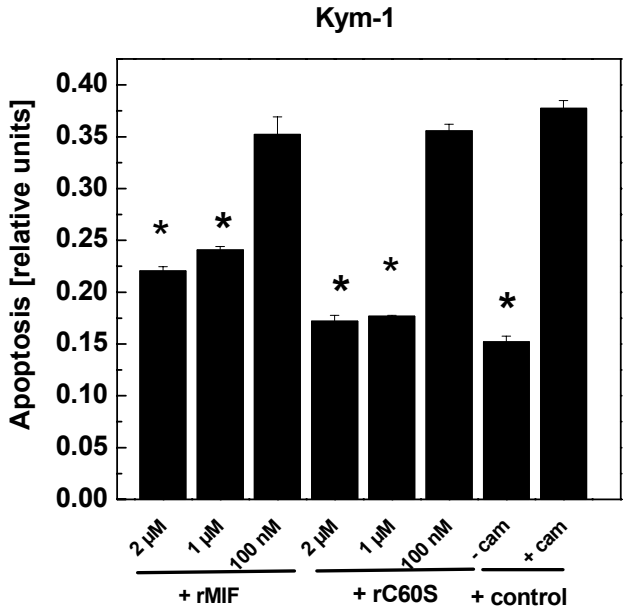

Figure 30: Exogenously applied recombinant C60S (rC60SMIF) exhibits comparable apoptosis-suppressing effects as rMIF in Kym-1 cells. Cells were treated with different concentrations of rMIF and rC60SMIF as indicated and apoptosis was induced by cam treatment. Data represent mean values ± SD from three experiments. *, Statistically significant differences with $p < 0.05$, the p value refer to + cam.

A concentration-dependent anti-apoptotic effect of rC60SMIF was also measured, when apoptosis by cam was induced in the HL-60 promyeloblasts (Figure 31). Again, the anti-apoptotic activity of rC60SMIF was even more pronounced than that of rMIF. The differential activities observed for ectopically overexpressed C60SMIF and rC60SMIF could indicate that different pathways of enzymatic action may exist for intra- and extracellular MIF, respectively. The investigation of these pathways and the role of a Cys60-based enzymatic activity in these mechanisms is beyond the scope of this thesis and will be pursued in detailed future studies that will also include the role of potential MIF target proteins such as JAB1

(Kleemann *et al.,* 2000a), PAG (Jung *et al.*, 2001), or the putative membrane receptor (Bucala, 2000) (see Discussion).

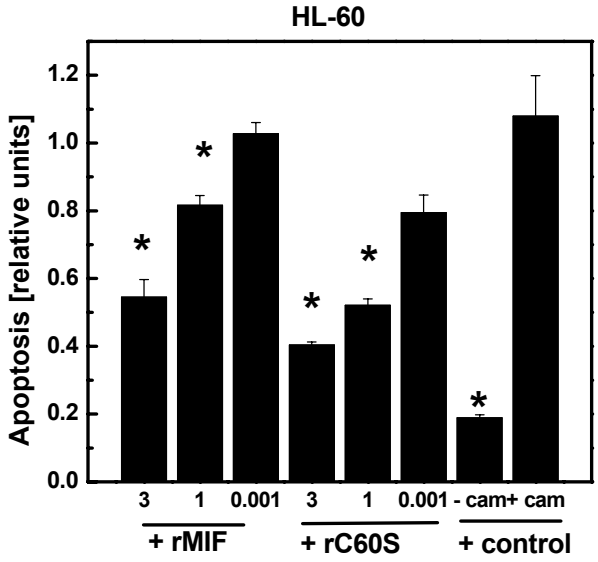

Figure 31: Exogenously applied recombinant C60S (rC60SMIF) exhibits comparable apoptosis-suppressing effects as rMIF in HL-60 leukocytes cells. Cells were treated with different concentrations of rMIF and rC60SMIF as indicated and apoptosis was induced by cam treatment. Data represent mean values ± SD from three experiments. *, Statistically significant differences with $p < 0.05$, the p value refer to +cam.

So far, the obtained data indicated that the central CXXC motif of MIF is essential for its oxidoreductase activity; in particular the presence of cysteine residue 60 (Cys60) is critical, when MIF is expressed intracellularly. Prior studies showed that mutant C60SMIF exhibited a lower enzymatic activity when compared with wtMIF, with respect to the MIF peptides, my data showed that SerMIF(50-65) did not have the oxidoreductase activity, and MIF(50-65) induced $p27^{kip1}$ levels to a slightes stronger degree than SerMIF(50-65). Thus, the CXXC region is essential for MIF´s activity.

5.3.2.3 MIF inhibits pro-oxidative stress-induced apoptosis

To further establish a potential role for MIF as an inhibitor of redox stress-induced apoptosis as suggested by my findings in Fig. 29, I directly studied the role of MIF in pro-oxidative stress-induced apoptosis in HL-60 and HtTAM cells.

Apoptosis of HL-60 cells was induced by controlled mild thiol starvation and a potential protective effect of MIF was compared to the well-known protective effects of small molecule thiol compounds such as β-mercaptoethanol and L-cystine. I found that rMIF added at a concentration of 2 µM led to an inhibition of thiol starvation-induced apoptosis of about 30 %. Interestingly, rC60S, at the same concentration, inhibited thiol starvation-induced apoptosis even stronger than rMIF. For comparison, 2 µM β-ME reduced apoptosis by approximately 50 % and 200 µM L-cystine rescued about 15 % of the starved HL-60 cells (Figure 32).

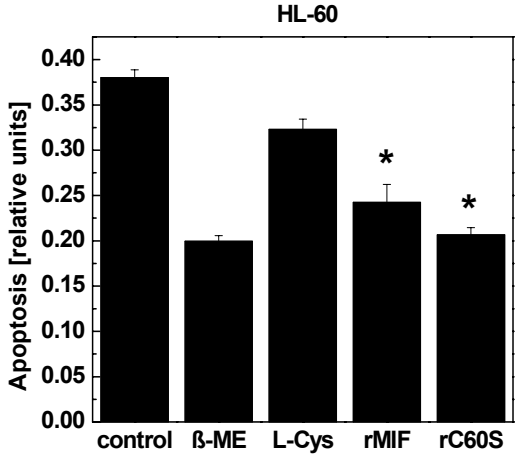

Figure 32: MIF inhibits pro-oxidative stress-induced apoptosis in leukocytes. Mild pro-oxidative stress was induced in HL-60 by thiol starvation through culturing cells in thiol-free medium. Comparison of apoptosis in cells cultured in presence of rMIF, rC60S or buffer control with the protective effects of the small molecular weight disulfides β-mercaptoethanol and L-cystine. Data represent mean values ± SD from three experiments. *, Statistically significant differences with $p < 0.05$, the p value refer to control.

The result therefore resembled that found for apoptosis inhibition by exogeneously added rMIF and rC60S as observed previously in this thesis (see also Discussion).

The biochemical and morphological changes caused by apoptosis affect all aspects of the cell from the plasma membrane to the nucleus. Plasma membrane alterations at the cell surface are observed in the initial stages of apoptosis. One of these alterations is the

translocation of phosphatidylserine (PS). The Ca^{2+}-dependent phospholipid-binding protein annexin-V has a high affinity for PS. In the early phase of apoptosis, the cells maintain an intact plasma membrane that enables them to exclude propidium iodide but expose phosphatidylserine to the outer membrane. Then annexin-V interacts with PS, which is translocated from the cytoplasmic surface of the cell to the cell surface early in the apoptotic response (Fadok et al., 1992).

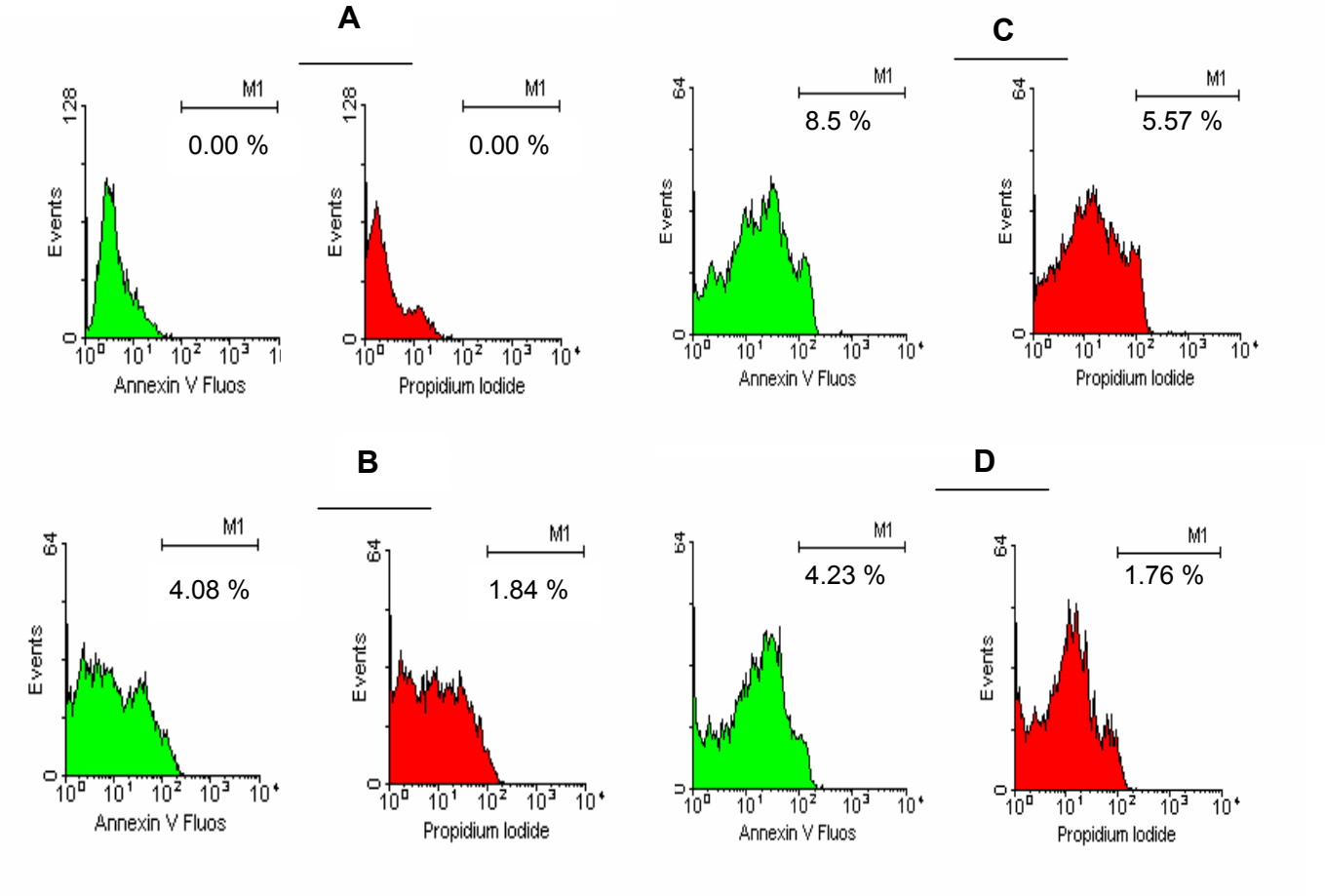

Figure 33: Flow cytometric assay of HtTAM apoptotic changes. HtTAM cells were incubated in full medium without and with dox (control), and then cells were cultured in TFM medium for 18 h with and without dox. Cells were incubated with annexin-V-fluos and propidium iodide, and 10,000 cells were analyzed by flow cytometry. The percentages of apoptotic cells by fluorescence staining were 0.00 % (A), 4.08 % (B), 8.5 % (C), and 4.23 % (D) for the background staining (A), normal cells with dox (B), TFM cells with dox (C), and TFM cells without dox (D). M1: Apoptotic cells or necrotic cells.

To assay for apoptosis, a biochemical approach using annexin-V to detect an early apoptotic marker was used. As Fig. 33 shows, HtTAM cells treated in the TFM medium with dox (Figure 33C) showed an increase of apoptotic cells when compared with HtTAM cells in the

TFM medium without dox (Figure 33 D). Normal cells did not induce annexin-V staining (Figure 33B). This finding indicated that MIF inhibits thiol starvation induced-apoptosis.
Induction of apoptosis according to this procedure was at a small rate, but this was due to the mild oxidative stress conditions chosen.
To further confirm this observation, HtTAM cells potentially undergoing apoptosis (cells cultured in normal medium (FM) were compared with cells in thiol-free medium (TFM) inducing pro-oxidative stress with dox) were studied for apoptotic nuclei by staining with PI post-fixation, HtTAM cell nuclei exhibited a change in chromatin structure in their nuclei; nuclei in TFM-treated HtTAM cells showed micronucleation and smaller diameters than those of untreated controls. Figure 34B shows that under TFM-culturing conditions a greater number of cells underwent apoptosis as compared to the cells treated in FM (Fig. 34A). In the TFM cultures, approximately 40 % apoptotic cell nuclei were detected (see also Fig. 35C), whereas in FM, only a few apoptotic cell nuclei were found, probably due to cell density stress.

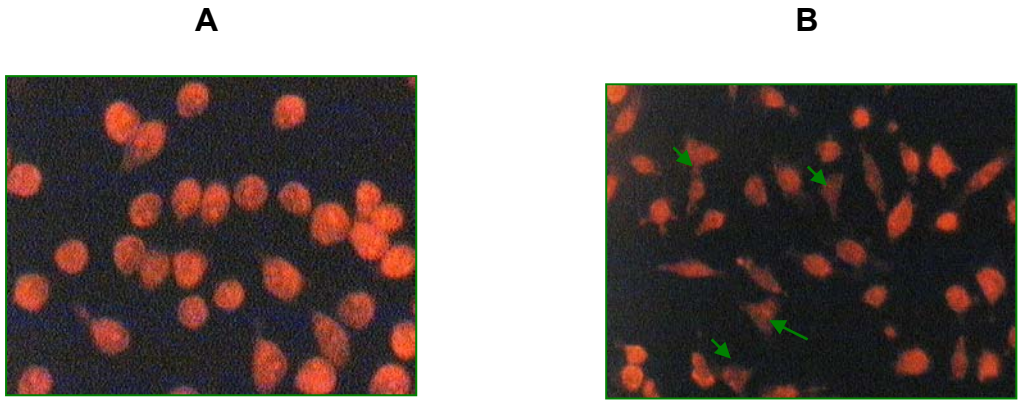

Figure 34: Morphology of HtTAM cell nuclei, stained with propidium iodide following induction of apoptosis by oxidative stress. Cells cultured in normal medium (A) were compared with cells in thiol-free medium inducing pro-oxidative stress (B). Both cultures were treated with dox to suppress the overexpression of MIF. Apoptotic cell nuclei versus normal cell nuclei appearance was determined by fluorescence microscopy.

Figure 35 A and B show that the morphology of a significant number of HtTAM cell nuclei was different in the presence of dox compared to cells that were treated without dox. In the absence of dox, i.e. with MIF overexpressed and pro-oxidative stress-induced apoptosis potentially inhibited, less cells with morphologic changes in PI-stained nuclei were seen. By this analysis, MIF was found to reduce pro-oxidative stress-induced apoptosis by approximately 50 % in thiol-starved HtTAM cells (Figure 35C) i.e. reducing the number of

apoptotic cells from 40 to 20 %. Thus, MIF was able to significantly protect cells from pro-oxidative stress-induced apoptosis.

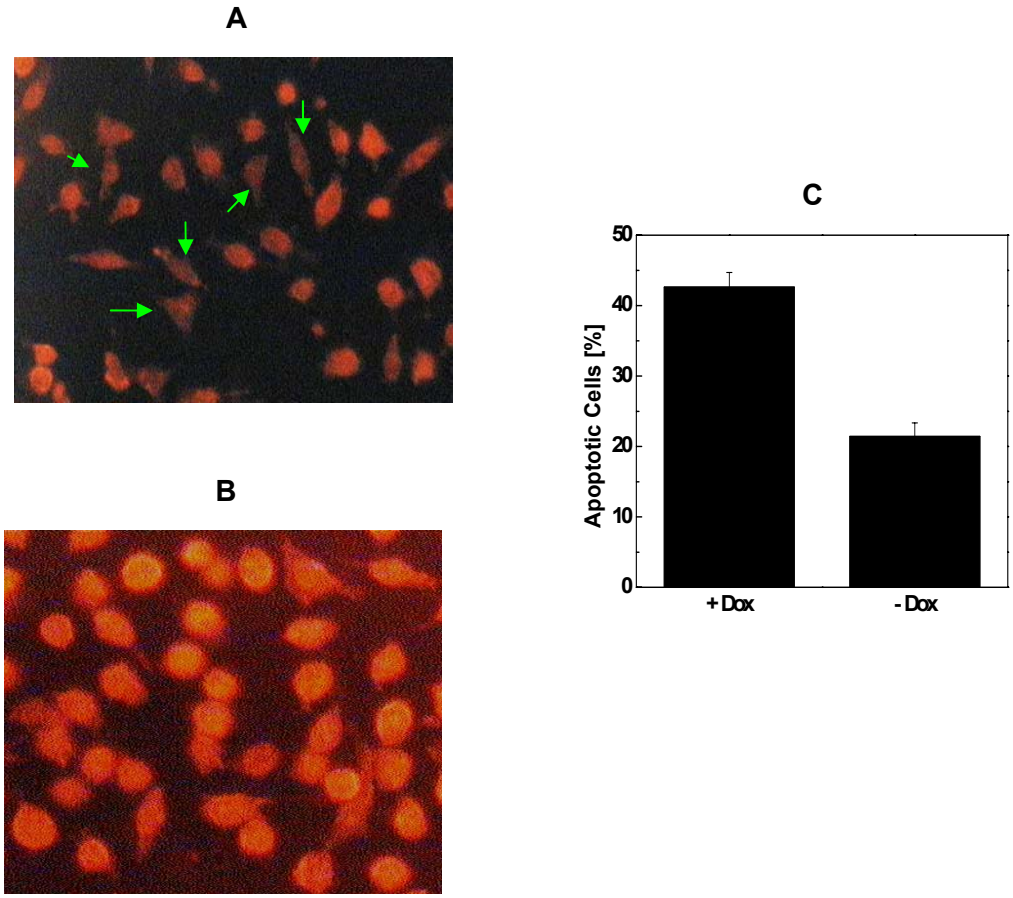

Figure 35: MIF inhibits pro-oxidative stress-induced apoptosis in HtTAM cells as analyzed by cell staining. Apoptosis in HtTAM cell nuclei thiol-starved through culturing the cells in TFM medium for 18 h in the presence (+ dox, - MIF) versus absence (- dox, + MIF) of dox. Cells were stained with propidium iodide and apoptotic versus normal cell nuclei appearance was determined by fluorescence microscopy. The morphology of the HtTAM cell nuclei in TFM medium with dox (A) and TFM medium without dox (B) is compared. The graph shows the percentage (mean ± SD of three independent assays) of apoptotic cell (C) out of the total number of cells counted.

Together, these data demonstrated that MIF was able to significantly protect cells from pro-oxidative stress-induced apoptosis. As concentrations of MIF below 1 µM did not exhibit a significant anti-apoptotic effect in this assay setting (data not shown), these data also

suggested that a substantial amount of MIF-derived thiol equivalents was necessary for this effect to occur. A similar observation has been made for Trx (Iwata *et al.,* 1997).

5.3.2.4 MIF modulates the cellular levels of glutathione

Haddad and colleagues showed that modulating the cellular redox equilibrium by pharmacological thiols can lead to a differential regulation of pro-inflammatory cytokines (Haddad *et al.*, 2000). A role for glutathione as an immunopharmacological regulatory thiol driving cytokine pathways has been derived. On the other hand, the concentration of intracellular reduced glutathione (GSH) also has been shown to play a role in rescuing the cell from redox stress (Cotgreave and Gerdes, 1998). Immune mediators such as Trx have been shown to participate in the modulation of the cellular GSH concentration. Therefore, I tested whether MIF was able to modulate the levels of intracellular GSH under conditions of mild oxidative stress. Inhibition by MIF of pro-oxidative stress-induced apoptosis correlated with MIF-induced enhancement of cellular glutathione (GSH). In fact, when MIF was overexpressed in thiol-starved HtTAM cells by turning on tTA-dependent target protein expression, cytosolic GSH levels were found to be raised by approximately one fifth (Figure 36).

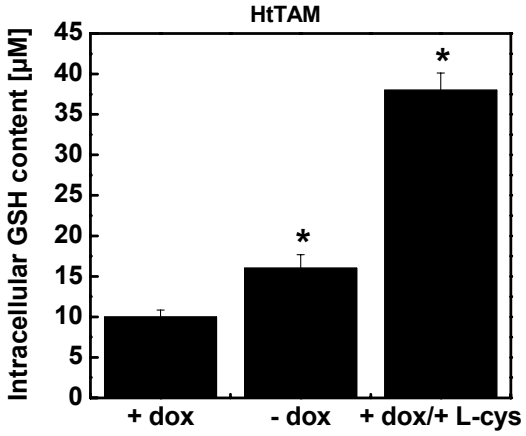

Figure 36: MIF enhances cellular GSH levels in thiol-starved HtTAM cells. Cells thiol-starved for 18 h were analyzed in the presence (+dox, -MIF) versus absence (-dox, +MIF) of dox and compared to cells incubated with L-cystine. Intracellular GSH was measured by a colorimetric GSH assay. Concentrations reder to 4 x 10^6 cells and standard dilution factor of the assay. *, Statistically significant differences between the +dox and –dox/L-cys states with $p < 0.05$.

Enhancement of GSH levels by MIF corresponded to MIF-mediated suppression of thiol starvation-induced apoptosis in these cells (Figure 37).

Figure 37: Induction of MIF in Tet-off-regulated HtTAM cells reduces the degree of pro-oxidative stress-mediated apoptosis. Cells were treated as in Figure 36 and apoptosis was analyzed by a DNA fragmentation assay.

Similarly, GSH levels were enhanced by approximately 20 % in thiol-starved Jurkat T cells, when MIF was transiently expressed in these cells by Tet-off gene expression regulation (data not shown). When apoptosis was induced by cell density stress or cam, no significant effect of MIF on the cytosolic levels of GSH could be observed (data not shown).

5.3.2.5 MIF-induced reduction in phosphorylation of endogenous c-Jun

The c-Jun N-terminal kinase family (JNKs) of mitogen-activated protein kinases (MAPKs) have emerged as important players in cellular stress responses. Prior studies showed that

MIF activated ERK1/2 in NIH fibroblats and that MIF increased phosphorylation of ERK1/2. (Mitchell *et al*., 1999). The effect of MIF on JNK activity had previously been observed when JNK activity was stimulated by overexpression of the coactivator JAB1 (Kleemann *et al.,* 2000a). I tested whether the apoptosis-suppressing properties of MIF were accompanied by corresponding changes in cellular JNK activity. To study this potential correlation, thiol-starved HtTAM cells were checked for their concentrations of endogenous phosphorylated c-Jun in the presence versus absence of dox treatment. Thiol starvation led to an enhancement of the endogenous phospho-c-Jun levels. Significant induction of phospho-c-Jun was first observed 4 h after treatment with thiol-free medium (TFM) and slightly declined at 6 h (Figure 38).

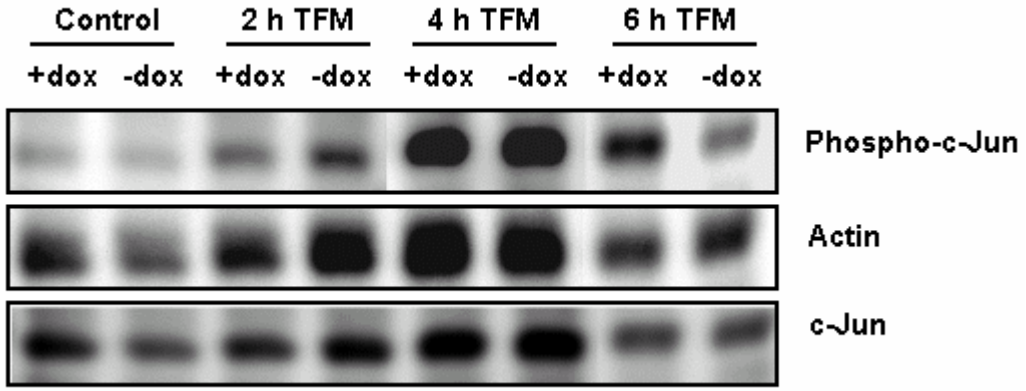

Figure 38: MIF inhibits pro-oxidative stress-stimulated JNK activity in HtTAM cells. Same as Figure 36 and 37 except that thiol starvation treatment (culturing in thiol-free medium, TFM) was performed for 2, 4, and 6 h instead of 18 h. JNK activity was assessed by analysis of the phosphorylation of endogenous c-Jun using phospho-c-Jun-specific SDS-PAGE/Western blotting. For standardization, Western blot analysis for c-Jun and β-actin was performed. A representative blot is shown.

Specific overexpression of MIF by omitting dox from the culture medium led to a reduction of induced phospho-c-Jun, which was most pronounced at the 6 h time interval (Figure 38). Densitometric analysis of the blot showed that the reduction of the level of phospho c-Jun mediated by MIF was approximately 40 %. These data indicated that protection by MIF from pro-oxidative stress-induced apoptosis could be mediated, at least in part, by a MIF-

mediated inhibition of induced JNK activity. Of note, an inhibitory effect of MIF on JNK activity had previously been observed when JNK activity was stimulated by overexpression of the coactivator JAB1 (Kleemann *et al.,* 2000a).

Thus together, it appeared that suppression by MIF of pro-oxidative stress-induced apoptosis was accompanied by concomitant effects of MIF on the cellular GSH content and on the activity of JNK/SAPK, both components of the cellular homeostasis-regulating system that are typically modulated during cellular redox stress.

6 Discussion

6.1 Investigation of the biochemical redox properties of MIF(50-65)

MIF is a unique cytokine that reportly overrides the anti-inflammatory effects of endogenous glucocorticoids. MIF has been suggested to be an enzyme. MIF exhibits a tautomerase/isomerase activity and a thiol-protein oxidoreductase activity. Interestingly, the thiol-protein oxidoreductase (TPOR) activity of MIF was found to be dependent on a CALC sequence motif as catalytic center and linked directly to MIF-mediated macrophage activation (Mischke *et al*., 1997; Kleemann *et al*., 1998a; Kleemann *et al*., 1998b). The catalytic center of thiol-protein oxidoreductases (TPORs) is contained within thiol/disulfide segments which exhibit the characteristic sequence motif CXXC (Holmgren, 1985b; Puig *et al.,* 1994). However, numerous proteins containing CXXC motives do not function as redox catalysts (Ellis *et al.,* 1992). Thus, both sequence-dependent information provided by the residues within the CXXC motif and closely neighboured ones, and the conformational constraint imposed by the overall structural architecture of the protein determine the functional potential of a CXXC motif.

The reported X-ray crystallographic data of human and rat MIF did not reveal a disulfide bridge between Cys residues 57 and 60 (Sun *et al.,* 1996a; Suzuki *et al.,* 1996b). However these analyses were performed using *E. coli*-derived recombinant MIF that was expressed and isolated under reducing conditions. Crystallization of oxidized MIF has not yet been reported. It has been shown in our lab and by Tomura (Tomura *et al*., 1999) that trimeric MIF is not the biologically active species and that MIF forms monomers, dimers and trimers under physiological conditions, but the biologically active MIF unit could not yet be determined.

It has also been reported that a disulfide-stabilized loop structure may form when MIF is oxidized at physiological concentrations. Renatured MIF contained both oxidized and reduced MIF species, this finding would suggest that there is an equilibrium between these two species at physiological conditions. The presence of an intramolecular disulfide bond was confirmed by near-UV CD measurement that showed a significant spectral difference between oxidized and reduced wtMIF in the region between 275 and 285 nm. Moreover, near-UV CD analysis of oxidized versus reduced mutants C57SMIF and C60SMIF was found to be identical to wtMIF. However, MIF might function as a catalyst for the reduction of disulfide structures that are close to equilibrium ΔG values (Kleemann, Ph.D thesis 2000).

In addition to the overall tertiary structure features that are specific to the individual protein, the X-X dipeptide sequences within the CXXC region of thiol-protein oxidoreductases have been shown to determine redox properties of these enzymes (Chivers *et al.,* 1996; Holst *et al.,* 1997; Krause *et al.*, 1991; Grauschopf *et al.,* 1995; Kortemme *et al.,* 1996). It was one aim of my thesis to determine the redox potential of MIF.

An important prerequisite to determine the E'_0 was first to be able to separate the oxidized and reduced species. Therefore, I tried to distinguish between the oxidized and reduced species of full length MIF (i.e. oxMIF vs. redMIF) by a HPLC-based system in conjunction with GSH/GSSG equilibrium reactions. Although previous work using near-UV CD had shown a spectral difference between oxidized and reduced wtMIF in the region between 275 and 285 nm, under the experimental conditions and for HPLC, I was not successful, to separate these species by HPLC separation of the full length species. Consequently, I was unable to determine the redox potential of full length MIF.

It has been shown for the oxidoreductase Trx, that a short peptide fragment retained some of the biochemical activity of full length Trx (Siedler *et al*., 1993; Siedler *et al*., 1994). As MIF also was found to exhibit TPOR activity and because there are several similarities between MIF and Trx, such as dependence of the redox activity of MIF on the presence of a redox-active Cys-Xaa-Xaa-Cys motif (CXXC), I investigated the redox potential of certain designed MIF peptides. The short peptide MIF(50-65) covering a 16 amino acids long stretch was designed to span the CXXC region of human MIF was studied in this thesis.

Systematic studies of the air oxidation of model peptides further demonstrated that dicysteine containing peptides of the type Cys-Gly_m-Cys (m= 0 – 6) did not form intramolecular disulfide bonds for m = 0 or m = 1. When m was 2 or 3, 15 and 40 % monomeric loops were found, respectively, and 90 % or more monomeric loops were detected for m = 4 – 6 (Zhang and Snyder, 1989). The latter study indicates that average peptides containing a CXXC sequence (m = 2) have low tendency (15 %) to form intramolecular disulfide bonds. Air oxidation studies of MIF peptides showed that intramolecular disulfide formation was strongly favored and occurred at $>$ 90 %, strongly arguing that the MIF-specific CXXC sequence and the sequence surrounding CXXC favor disulfide bond formation (Bernhagen, 1999). MIF (50-65) was fully reduced and then subjected to air oxidation. Analysis of the oxidized peptide solution showed a strong propensity of the peptide for the formation of an intramolecular disulfide bridge, whereas almost no peptide dimers, that would have been formed by intermolecular disulfide bridging were detected.

When analysed by near-UV CD and far-UV CD measurements, MIF(50-65) showed that the CXXC peptide contained a CD-active disulfide in the 280 nm region when present in it´s oxidized form, whereas reduced MIF(50-65) did not have a CD signal at 280 nm. These results support the data obtained by HPLC analysis that the CXXC spanning sequence indicated the tendency of the high intramolecular disulfide. The tendency of excised active site fragments of TPORs to form intramolecular disulfides, confirmed the product distribution in air oxidation experiments. This spectral region had shown a β-turn structure, it can be concluded that disulfide formation depended on the formation of a β-turn structure. Conditions of the proportions of random coil structure behave in a reverse proportion. Thus,

the oxidized peptide is more structured than the reduced one or the control peptide. The far-UV CD analysis demonstrated that disulfide bond formation went along with β-turn formation, the β-turn structures in short peptides has also been observed by Siedler (Siedler *et al*., 1994). Further confirming that the oxidized peptide should be able to serve as redox shuttles.

To further analyze that MIF(50-65) not only shows structural similarities with full length MIF, but also exhibited enzymatic activity, peptide MIF(50-65) was tested in the insulin oxidoreductase assay. In this test, the MIF peptide exhibited significant enzymatic activity. These results confirmed the role of the CALC sequence for the oxidoreductase activity of MIF.

MIF has been shown to have structural similarities with the two bacterial enzymes 5-carboxymethyl-2-hydroxymuconate isomerase (CHMI) and 4-oxalocrotonate tautomerase (OT), and to catalyze the isomerization of the D-isomer of dopachrome (Rosengren *et al*., 1996). In this study, MIF(50-65) was tested in a tautomerase assay with D-dopachrome as a substrate. MIF(50-65) did not catalyze the tautomerization of the non-naturally occurring D-isomer of dopachrome. This finding confirmed that the role of the Pro2 residue is essential for MIF in tautomerase reactions. Thus, peptide MIF(50-65) only shows one aspect of the enzymatic properties of MIF.

Prior studies showed that oxidized MIF is not soluble at high concentrations, and when alkylation was performed under oxidizing conditions, non-alkylated and cysteines had been protected from alkylation by a disulfide bond (Kleemann, Ph.D thesis 2000). However, a short synthetic peptide of MIF(56-65) spanning the putative redox center were found to almost exclusively forum intramolecular, rather than intermolecular disulfides (Kleemann *et al*., 1998a), confirming that the CXXC motif of MIF might function as catalytic center.

Initial conformational studies and studies on the redox enzyme activity of MIF(50-65) had been promising and had suggested that such MIF-derived peptides could be excellent models to study the redox properties of MIF. For this reason, I hoped that it could be possible to distinguish between oxidized MIF(50-65) and the reduced species. The data in my thesis show that oxidized MIF(50-65) and reduced MIF(50-65) can be well separated and quantified on a C18 reverse phase HPLC column. Thus, HPLC analysis was suitable as an assay system to determine the redox potential of MIF-derived CXXC-spanning peptides, with implications to be drawn for the redox potential of full-length MIF.

Siedler and co-workers confirmed that the redox potentials of the thiol-protein oxidoreductases are largely dictated by the juxtaposition of the cysteine residues resulting from interactions in the folded state of the protein. MIF is not found in the endoplasmic reticulum (ER) fractions and, moreover, secretion of MIF does probably not follow conventional ER-mediated secretion pathways (Flieger, Ph.D thesis 2002). Thus, unlike PDI, MIF does not appear to be exposed to highly oxidizing conditions of the ER compartment

(Freedman *et al.,* 1994). In this work, I characterized the kinetics and equilibria for reduction and formation of the disulfide bonds of MIF(50-65) by thiol/disulfide exchange with reduced glutathione and oxidized glutathione. Determination of the redox potential of MIF was one aim of my thesis. The redox potential for the peptide MIF(50-65) was determined to lie at E'_0 = – 0.258 V, which is a realtively electronegative E'_0 value. Although all the thioredoxin-like enzymes have similar active site sequences, they can fulfil very different redox functions. The active site cysteine residues can be highly oxidizing in the case of PDI (Lundström & Holmgren, 1993), DsbA (Zapun *et al*., 1993), or much more reducing in the case of thioredoxin (Holmgren, 1985b). Thus, the redox potential of MIF-derived peptide is close to that of thioredoxin (- 0.23 to - 0.27 V, Krause *et al*., 1991, Holmgren, 1989) and is therefore significantly more reducing than eukaryotic PDI (-0.11V, Hawkins *et al*., 1991) and DsbA, whose redox potential -0.089V was also determined by the equilibrium with glutathione (Zapun *et al*., 1993; Wunder and Glockshuber, 1993). Siedler and colleagues showed that the redox potentials of TPOR peptides differed in the order Grx-[10-17] > Trr-[134-141] > PDI-[34-41] > Trx-His^{37}-[31-38], and they reported that the redox potentials of the peptides differed significantly from that of the native proteins. Although, I was not able to determine an E'_0 value for full-length MIF and although the redox properties of a smaller peptide are not necessarily identical to that of the corresponding parent-molecule (see above), I would conclude that MIF itself would also have a fairly electro-negative E'_0 value. In addition, previous indirect studies by Kleemann and colleagues connected with the variations of the X-X residues with the CXXC motif of MIF have also indicated that the redox potential of MIF is rather a negative one (Kleemann *et al*., 1999). The redox potential of MIF(50-65) was found to be a reducing one, when compared with the redox potentials of TPOR peptides that were investigated by Siedler. On the other hand, this study showed that the equilibrium between reduced and oxidized peptide was reached not before 20 h of incubation. The expected redox species present at equilibrium, i.e. reduced MIF(50-65), oxidized MIF(50-65), two mono mixed-disulfide derivatives with glutathione and the mixed di-disulfide MIF(50-65), I only detected one type of mono mixed disulfide derivative with glutathione by HPLC. This could mean that other mono mixed-disulfide did not form in this reaction. Alternatively, due to similar retention times, the two mono mixed-disulfide may not have been resolved in the HPLC system applied. Lastly, a very low concentration of one mixed mono-disulfide in comparison with the other one could be the reason for only detection one mono mixed-disulfide.

Moreover, the finding that the cysteines of the CXXC motif are involved in conjunction with the measured specificity for the substrate GSH suggested that MIF may play a role in the regulation of cellular redox homeostasis. Glutathione is a potent sulfhydryl reductant and plays a key role in maintaining the cellular redox balance (Williams, 1976). In this function,

GSH serves to protect cellular function against oxidative stress and apoptosis (Watson *et al*., 1996). While intracellular GSH levels were also investigated in my thesis. I concentrated on the determination of whether the redox potential of MIF and the related redox regulation through MIF is a general principle of MIF action and is part of its role in the immune system. This potential association was studied by investigating the connection between the redox activity of MIF and its apoptosis-inhibiting activity.

One of the most specific MIF-mediated activities, is its ability to override the effects of glucocorticoids, which is probably also the most important effect currently known to be exerted by MIF (Calandra *et al.,* 1995; Donnelly *et al,* 1997). Of note, only picomolar quantities of MIF are needed for this activity, whereas other proinflammatory activities of MIF require nano- to micromolar amounts (Bernhagen *et al.,* 1998). MIF, when secreted from immune cells in response to low concentrations of glucocorticoids and proinflammatory stimuli, was found to counter-regulate the immunosuppressive effects of glucocorticoids on the immune system (Bernhagen *et al.,* 1993; Calandra *et al.,* 1995; Nishino *et al.,* 1995). But unlike prior studies that used PMBC for the *in vitro* GC-OR assay, I established a cell line-based assay using THP-1 monocytes. Preliminary studies indicated that the THP-1 monocytic cell line could be suitable. When tested in vitro, wtMIF and MIF(50-65) showed similar overriding activity of 40 % though at different concentrations, because small peptide derivatives usually have much lower than the corresponding proteins. Other mutants, e.g. C-terminal mutants were found to be not active and mutant C60S had only 37 % activity when compared with wtMIF. Together, these findings confirmed that the CALC region is not only important for the enzymatic activity of MIF, but also important at least some of the cytokine functions of MIF.

MIF was found to interact with an intracellular protein, the transcriptional coactivator and cell cycle regulator JAB1 and to modulate several cellular signalling events through JAB1 (Kleemann *et al.,* 2000a). In this thesis, I found that MIF(50-65) and biotinMIF(50-65) induced $p27^{Kip1}$ levels to a similar extent as observed previously for MIF.

MIF can antagonize several JAB1/CSN5-based cellular effects. MIF inhibits JAB1/CSN5-mediated AP-1 activity and reduces JNK activity stimulated by JAB1/CSN5. While JAB1/CSN5 rescues fibroblasts from G1 growth arrest, MIF was found to counteract this effect, resulting in a net growth-inhibitory effect on fibroblast, and this effect is JAB1/CSN5-dependent and appears to occur through stabilization of $p27^{Kip1}$ by MIF (Kleemann *et al*., 2000a). It is known, that redox reactions are intimately involved in the control of biological processes including modulation of the function of transcription factors, e.g., AP-1 and NFκB (Bauskin *et al.,* 1990; Abate *et al.,* 1990). Redox regulation of transcription factors is an interesting and important issue. Debarbieux and Beckwith showed that the redox function of a thiol-disulfide oxidoreductase is necessary to determine its *in vivo* redox state (Debarbieux

and Beckwith, 1998). Oxidation/reduction-based regulation of gene expression appears to be a fundamental regulatory mechanism in cell biology. This context was confirmed by the finding that the sequence 50-65 is important for at least some of the JAB1-mediated activities of MIF.

The presence of the CXXC motif is essential for the formation of native disulfide bonds in the cell (Chivers *et al*., 1996). However, large variations in the redox potentials for individual protein disulfides/dithiols are apparent although yet largely unknown. My data confirm that the short MIF peptide, MIF(50-65), although covering only 1/10 of the MIF sequence exhibits MIF-like activity, suggesting that MIF(50-65) and MIF can have related immunological functions. Together with previous studies of our lab, this study adds to the increasing evidence that there may be an interplay between cellular redox effects and MIF action. Lastly, MIF(50-65) offers a novel molecular approach to design peptide-based MIF therapeutics.

6.2 The role of the redox activity of MIF for the molecular mechanism of action of MIF

I conclude that MIF can act to potently suppress apoptosis induced by a variety of stimuli. In particular, I have demonstrated that MIF suppresses pro-oxidative stress-induced apoptosis. My results are in line with observations describing the suppression of p53-mediated apoptosis by MIF (Hudson *et al*., 1999; Mitchell *et al*., 2002) and offer a previously unrecognized molecular link between the enzymatic redox function of MIF (Kleemann *et al*., 1998a) and its role in the regulation of cell survival.

Nevertheless, the observation that MIF inhibits redox stress-induced apoptosis needs to be reviewed in detail in the context of what is known about the effects of MIF on cell proliferation, cell differentiation and tumorigenesis.

Over the past several years, MIF has been recognized as a pluripotent immunomodulator with broad inflammation-regulatory properties including stimulatory effects on cell proliferation and cellular activation (i.e., summarized in: (Mitchell and Bucala, 2000; Lue *et al*., 2002)). Moreover, it appears that MIF could be one of the few target proteins identified which constitutes a connection between innate immunity and cancer (Hudson *et al*., 1999; Mitchell *et al*., 2002). However, the precise molecular changes induced by MIF in such situations have not yet been fully elucidated. Initial studies have indicated that activation of cell proliferation by MIF could be due to a bypassing of p53-mediated growth arrest. It has been proposed that this functional inactivation of p53 by MIF is based on the suppression of p53-dependent transcriptional activity by MIF (Hudson *et al*., 1999). Mitchell *et al.* have found that MIF-mediated inhibition of p53 requires cyclooxygenase activity (Mitchell *et al*., 2002).

Stimulation of Erk1/2 MAPK activity was identified as potential pathway by which MIF modulates cell proliferation (Mitchell *et al*., 1999). In contrast, other studies have indicated that MIF can potently inhibit cell proliferation in fibroblasts (Kleemann *et al.,* 2002a), that it may decrease $CD8^+$ T cell life span (Abe *et al*., 2001), and that it may inhibit the proliferation of microsvascular endothelial cells (R. Kleemann and J. Bernhagen, to be published in detail elsewhere). Furthermore, MIF acts to antagonize JAB1/CSN5-mediated rescue of fibroblasts from starvation-induced growth arrest (Kleemann *et al.,* 2000a).

It has been suggested that MIF could be a critical mediator of tumorigenesis and a MIF-based therapeutic strategy has been implicated as a promising clinical approach to treat cancer (Mitchell *et al*., 2000; Chesney *et al*., 1999). This notion has mainly been based on the observed correlation of enhanced MIF expression levels and tumor occurrence as well as on the potent anti-tumor activity of anti-MIF antibody treatment (Mitchell *et al*., 2000; Chesney *et al*., 1999). However, as the anti-tumor activity of anti-MIF antibodies appears to be due to an inhibition of angiogenic processes (Chesney *et al*., 1999) and because in some tumor models, MIF expression inversely correlates with tumor progression (del Vecchio *et al.,* 2000), it appears that MIF's role in tumorigenesis is a complex one and probably not simply due to a MIF-mediated enhancement of cell proliferation. Rather, the effects of MIF on cell proliferation, cell differentiation, growth arrest, the cell cycle, and apoptotic processes need to be carefully discerned.

As to a contribution of MIF to the regulation of cell survival, the results of the present study together with the previous data by Hudson and co-workers and Mitchell *et al*. demonstrate that MIF participates in modulating cellular apoptosis. The studies unanimously suggest that MIF acts to suppress apoptosis. Upon first sight, MIF's inhibitory effect on apoptosis correlates with its reported stimulatory effect on cell proliferation. However, cellular apoptosis and cell proliferation are processes that are regulated in a very complex fashion and that do not simply oppose each other. Cytokines are typical participants in this regulatory network. In fact, several cytokines have been shown to be able to both promote and inhibit apoptosis and cell proliferation, depending on the cell type, the specific cellular activation state and the physiological context (van den Berg *et al*., 2001; Thomson, 1998). The observed apoptosis-suppressing effects of MIF are not complete. I found that inhibition of apoptosis by MIF induced by most stimuli applied generally ranged from 20-60 % with a higher extent of inhibition seen, when apoptosis was induced by redox stress. Hudson *et al.* have obtained similar results; they have observed that in starvation and estradiol treatment-induced apoptosis of fibroblasts, MIF reduced cell death by a margin of approximately 20 %, while NO radical-induced apoptosis of macrophages is much more drastically suppressed by MIF (up to ~ 70 %) (Hudson *et al.,* 1999; Mitchell *et al*., 2002). Together with my results, the latter data suggest that suppression of apoptosis by MIF is most profound, when apoptosis is

caused by oxidative or nitrosative stress. Nevertheless, the notion that MIF is unable to completely suppress apoptosis, indicates that it only contributes to some of the pathways constituting the complex molecular network of apoptosis-regulating processes in the cell.

MIF's role in apoptosis is closely associated with its inhibitory effects on p53 function, with three downstream targets of p53-dependent transcriptional activation, namely $p21^{Cip1}$, cyclin G and MDM2, identified to be regulated by MIF and p53 (Hudson *et al.,* 1999). In part, this activity could be due to the observed MIF-mediated inhibition of p53 and phospho-p53 accumulation (Mitchell *et al*., 2002). However, the precise molecular mode of these activities has remained open. The finding that MIF suppresses pro-oxidative stress-induced apoptosis indicates that MIF could alter p53 activity by a redox mechanism. Such a scenario appears to be plausible because redox regulation of apoptosis has been shown to be one of the pathways to be critical for cell viability (Arner and Holmgren, 2000; Nishinaka *et al*., 2001; Powis *et al*., 2000). Moreover, the co-cytokine Trx has been demonstrated to directly suppress apoptosis by virtue of its redox-regulating activity (Arner and Holmgren, 2000; Iwata *et al*., 1997; Sato *et al.,* 1995). p53 function is based on the zinc-mediated sequence-specific DNA binding activity of p53 which is dependent on at least three reduced cysteine residues. Oxidation of these cysteines can lead to conformational changes in the p53 DNA binding domain, resulting in impaired DNA binding activity. Thus, p53 activity is potently and directly regulated by redox-based mechanisms (Rainwater *et al*., 1995; Hainaut and Mann, 2001). Trx, but also redox factor-1 (Ref-1), another cellular redox stress protein (Evans *et al*., 2000) that modulates transcription factor activity by keeping critical cysteine residues of these proteins in a reduced state, have been implicated to participate in sustaining p53 conformation and activity through their reducing properties (Hainaut and Mann, 2001). By analogy, MIF might therefore also be capable of reducing the cysteine thiol groups of p53, conferring protection from cysteine oxidation during redox stress. Two additional lines of evidence support the notion that p53 activity could be regulated by MIF-mediated redox processes. First, MIF was recently shown to interact with the transcriptional coactivator JAB1/CSN5 and to modulate the cell cycle through JAB1 (Kleemann *et al*., 2000a). At least some of the JAB1-modulating activities of MIF appear to be connected to the presence of the redox-active cysteine 60 residue of MIF. Secondly, JAB1 and p53 have been demonstrated to specifically interact with each other (Bech-Otschir *et al*., 2001). Together, these observations offer the intriguing possibility that a ternary MIF-JAB1-p53 complex could be the molecular basis for MIF-mediated, p53-dependent suppression of apoptosis. However, neither has complex formation between MIF and p53 been demonstrated, nor has an involvement of MIF in p53-JAB1 complexes yet been investigated. Alternatively, it may be hypothesized that MIF could cause a redox-mediated change in the activity or distribution of the JAB1 protein. For either possibility, the available evidence does not yet offer direct proof;

it is therefore also speculative at this time, whether redox regulation of apoptosis by MIF would occur by redox catalysis or whether the redox-competent cysteine thiol groups of MIF would serve to enhance the cell's pool of reducing thiol equivalents.

Yodoi and coworkers performed a detailed investigation of the role of the redox-active cysteine residues of Trx in the apoptosis of T cells (Iwata *et al*., 1997). Applying various cysteine mutants of Trx, they found that Trx-mediated suppression of redox stress-induced apoptosis is dependent on the presence of the cysteine thiols. However, as also mutant C31S/C34STrx, with both CXXC cysteines changed, as well as partially oxidized Trx suppressed pro-oxidative-stress-induced apoptosis to some extent, it appears that Trx-type oxidoreductases modulate cell viability by both CXXC-dependent catalysis, but also by direct thiol group donation that may also be mediated by the non-CXXC cysteine residues. The latter mode of action is also implied by the observation that nano- to micromolar concentrations of Trx were needed for inhibition of apoptosis to occur. In fact, increasing evidence suggests that cellular redox homeostasis is regulated not only by the ratios of oxidized versus reduced small molecule thiols such as GSH, but that oxidoreductases such as Trx directly participate in regulating the cellular redox state by "donation" of proteinaceous thiol equivalents (Powis *et al*., 2000; Iwata *et al*., 1997). My data that suppression of redox stress-induced apoptosis and enhancement of intracellular GSH levels by MIF was most pronounced, when micromolar concentrations of rMIF where added, indicate that MIF may also function through donation of proteinaceous thiol groups.

A membrane receptor for MIF has not yet been identified, but indirect evidence suggests that a receptor may exist (Mitchell *et al*., 1999; Bucala, 2000). It has been suggested that both receptor-mediated and intra-/autocrine pathways of MIF action may be distinguished and may occur in parallel (Bucala, 2000). My finding that the redox-dead mutant C60SMIF strongly suppresses apoptosis, when applied exogenously as recombinant protein, but is inactive, when overexpressed endogenously, could imply that the redox activity of MIF acts differently on the potential target proteins. Exogenously applied rC60SMIF inhibits apoptosis comparable to wtMIF. In fact, rC60SMIF even appears to exhibit stronger apoptosis-suppressing activity than wtMIF. A cysteine 60 variant of the MIF-like protein GIF exhibits enhanced cell-binding, possibly enhanced receptor-binding, activity (Sugie *et al.,* 1997), suggesting together with the above notions that MIF-mediated suppression of apoptosis could occur through a receptor-based pathway and that redox regulation by MIF may not interfere with such a pathway. As the glucocorticoid-overriding activity of MIF is at least partially dependent on the presence of the CXXC cysteines, this would imply that this activity occurred via a receptor-independent pathway. However, there has been no evidence yet, whatsoever, as to the nature of the molecular events leading to MIF-mediated overriding of glucocorticoid function. The observed lack of apoptosis-suppressing activity of endogenously

overexpressed mutant C60SMIF would indicate that the redox activity of MIF could be critical for its intracrine spectrum of actions. As discussed above, such activities may include the binding to PAG or redox regulation of JAB1 and p53.

An intracellular role for the redox activity of MIF is also suggested by the finding that the redox stress-treated HtTAM cells showed a lower degree of JNK activity, when MIF was induced. Because I previously demonstrated that MIF inhibition of JNK activity is connected to the interaction of MIF with JAB1, I conclude, that there might be a molecular link between apoptosis regulation, MIF function, and the cellular proteins JNK, JAB1, and p53. This notion is further supported by the recent demonstration that CSN signalosome function is linked to JNK and p53 activity (Chamovitz and Segal, 2001). The observed induction of phospho-c-Jun stimulated by TFM treatment occurred several hours before significant apoptosis could be measured under these conditions. These data are consistent with the suggested role for JNK as an upstream stimulatory event during the stress-induced apoptotic cascade (Leppa and Bohmann, 1999).

The similarities between MIF and Trx in the regulation of apoptosis add to the previously noted functional homologies between MIF and Trx-type proteins and their relation to redox processes (Kleemann *et al*., 1998a). For example, the redox activity of MIF has been implicated to be important for the interaction between MIF and the peroxiredoxin PAG (Jung *et al*., 2001) and with the transcriptional coactivator JAB1 (Kleemann *et al*., 2000a). Likewise, interaction of Trx and Ref-1 has been found to be redox-dependent (Hirota *et al*., 1997). Also, the various immunoregulatory activities of MIF and Trx have been shown to be at least partially dependent on the presence of the corresponding redox-active cysteine residues (Kleemann *et al*., 1998a; Bertini *et al*., 1999). In this respect, it is noteworthy of mentioning that Trx has recently been re-defined as a co-cytokine (Bertini *et al*., 1999; Pekkari *et al*., 2000; Arner and Holmgren, 2000), suggesting together that the roles of Trx as a redox regulator and as an immune mediator are closely linked.

For MIF, a correlation between its redox activities and its functions in immune regulation and physiology is also becoming increasingly evident. For example, MIF redox activity is at least in part critical for the glucocorticoid-overriding activity of MIF (Kleemann *et al*., 1999). Furthermore, MIF also functions as a redox-sensitive cytokine in myocardial ischemia (Takahashi *et al*., 2001). MIF is a redox enzyme with an oxidoreductase-type of activity that is based on a CXXC motif as catalytic, and the TPOR activity of MIF is directly connected to its immunological and cellular activities. In summary, modulation of oxidative stress-induced apoptosis by MIF could be an important novel mechanism, linking the redox function of MIF and its role in immune and stress regulation.

6.3 Concluding remarks

MIF is a small protein and contains the amino acid sequence motif Cys-Ala-Leu-Cys in its active site. Previous studies (Kleemann *et al*., 1999) have confirmed that the cysteine residues of the CALC motif are important for the cytokine functions of MIF. Unfortunately, I was not able to determine the redox potential for full length MIF. However, peptide MIF(50-65) was shown to have MIF-like redox properties, and I could demonstrate that MIF(50-65) and MIF are also related functionally. In this work I was successful in determining the redox potential for peptide MIF(50-65). This result also confirmed the role of the CXXC region of MIF and the redox activity of MIF for various cellular and the molecular mechanism of the immunological function of MIF.

MIF is released at the site of inflammation from T cells and macrophages, inflammatory loci are characterized by high rates of cell death. The ability of MIF to override the glucocorticoid effect, as well as acting together with TNF as a proinflammatory mediator may be tightly regulated by the redox properties of the MIF. Prior studies showed that thiol-disulfide oxidoreductases have active dithiol moieties and are known to play a central role in redox-sensitive signal transduction. MIF has been chacracterized as a thiol-disulfide oxidoreductase that is composed of two redox active cysteines. MIF was also shown to scavenge reaction oxygen species (ROS) such as H_2O_2 by its reducing activity to protect against H_2O_2 and TNF-induced cytotoxicity. The data in this thesis also show that MIF inhibits apoptosis induced by a variety of death stimuli in a variety of cell types. The anti-apoptotic activity of rC60SMIF was found to be even more pronounced than that of rMIF. This finding showed the complexity of the involvement of the redox activity of MIF in various cellular and molecular pathways. Moreover, MIF has been observed to be protective for cells under conditions of L-cystine and glutathione depletion. These findings suggested that intracellular redox regulation might be involved in thiol-stress induced apoptosis, and redox-based apoptotic regulation could be part of MIF´s mechanism of action *in vivo*. My results as well as these by others provide evidence that MIF is involved in the regulation of apoptosis and that the role of MIF is important.

The functions of MIF within the immune system are both unique and diverse, although a molecular mechanism of action remains to be elucidated. The data of my thesis confirm that it is becoming increasingly evident that MIF plays an important role in immune processes that appears to be in part dependent on MIF-based redox processes. Redox modulation by MIF of cellular apoptosis could be an important novel mechanism, linking the redox function of MIF and its role in immunity.

The determined strongly negative redox potential value of – 0.258 V for the MIF-like peptide MIF(50-65) makes it likely that MIF at a molecular basis is involved in this regulatory network by reducing receptor and/or target proteins.

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Publications

Parts of this thesis were published in:

1. Robert Kleemann, Hans Rorsman, Evald Rosengren, Ralf Mischke, Nguyen Tuyet Mai, and Jürgen Bernhagen (2000). Dissection of the enzymatic and immunologic functions of macrophage migration inhibitory factor: Full immunologic activity of N-terminally truncated mutants. *Eur. J. Biochem.* 267: 7183-7192.

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3. Nguyen Tuyet Mai, , Robert Kleemann, Hongqi Lue, Pieter Koolwijk, Jürgen Beck, Aphrodite Kapurniotu, Jürgen Bernhagen (2003). A sixteen residue peptide fragment of macrophage migration inhibitory factor, MIF(50-65), exhibits redox activity and has MIF-like biological functions. *J. Biol. Chem*. in revision 2003.

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Publications:

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Declaration

I, hereby, declare that this submission is my own work and, to the best of my knowledge and belief, has not been accepted for the award of any degree or diploma of a university or other institute of higher learning.

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Stuttgart, 28. November 2002