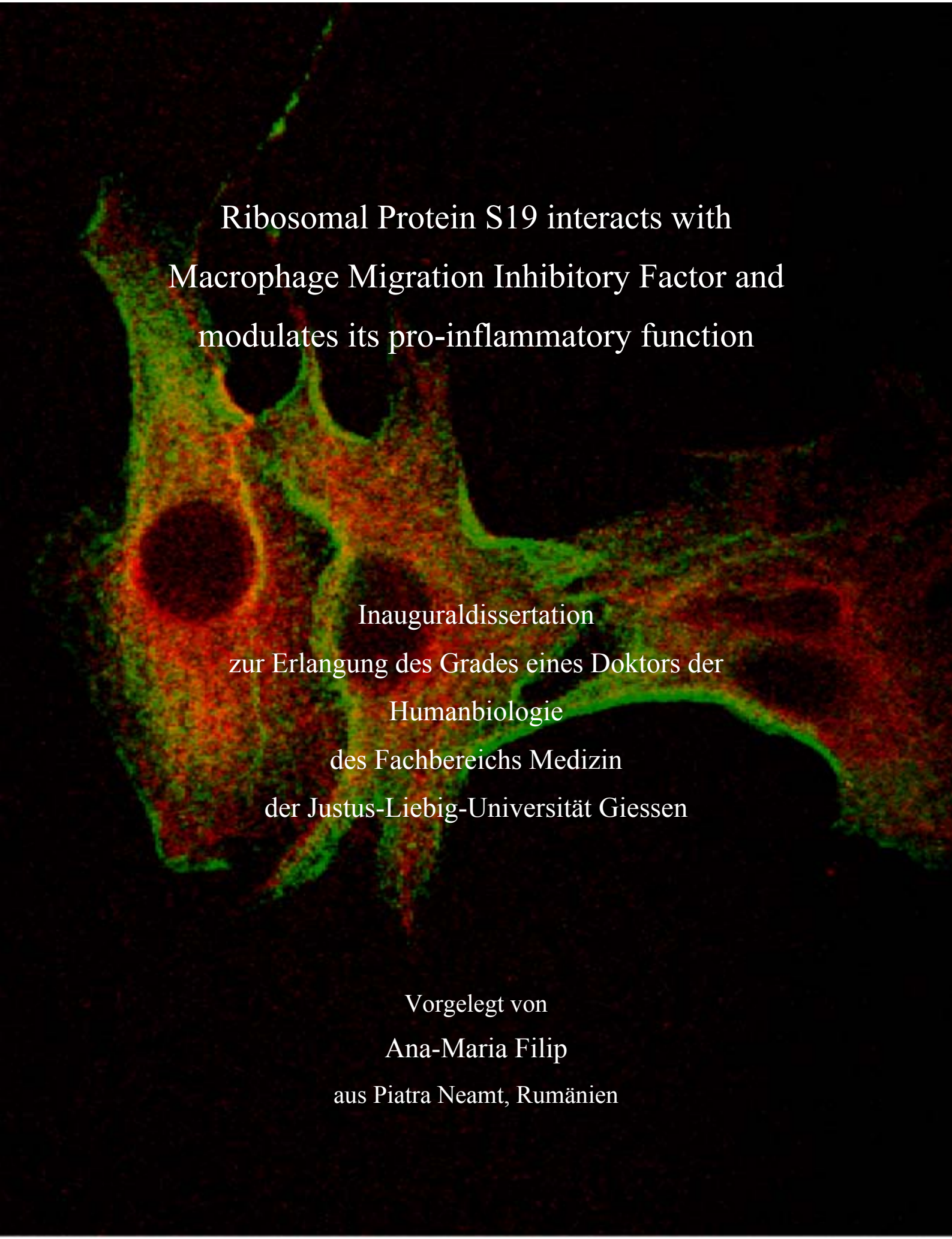

Ribosomal Protein S19 interacts with Macrophage Migration Inhibitory Factor and modulates its pro-inflammatory function

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

Vorgelegt von
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1. INRODUCTION

1.1. Discovery of MIF

Macrophage migration inhibitory factor (MIF) is one of the oldest known immunological mediators. The name macrophage migration inhibitory factor was coined in 1966 after the observation that a soluble material released by sensitized T-lymphocytes was able to inhibit the random migration of peritoneal exudate macrophages which was characterized (Bloom and Bennett 1966; David 1966). After almost two decades in 1989, the human protein was successfully cloned (David 1966; Weiser et al. 1989) and within a few years, both bio-active MIF protein and a neutralizing monoclonal antibody were produced, and a proinflammatory profile for MIF action was emerged (Bernhagen et al. 1994).

A separate line of investigation that aimed at identifying novel mediators which could regulate glucocorticoid action at the systemic level, led to the discovery of an apparently novel 12.5 kD protein released by cells of the anterior pituitary gland which was finally identified as MIF (Bernhagen et al. 1993). Intraperitoneal injection of lipopolysaccharide in mice resulted in a dramatic fall in the pituitary content of MIF and a concomitant increase in plasma level of this factor followed by a gradual elevation of MIF mRNA expression in pituitary tissue. MIF was thus rediscovered as a pituitary-derived mediator of systemic stress response (Bucala 1996).

1.2. MIF gene and protein structure

Only one MIF gene is found in the human genome located on chromosome 22. The human MIF gene contains three short exons and two introns. Its 5' regulatory region contains several consensus DNA-binding sequences for transcription factors, notably activator protein 1 (AP1) and nuclear factor-κB (NF-κB). However, little is known about the relevance of these putative DNA-binding sites in the regulation of expression of the human MIF gene. Searching of the human genome for homologues of MIF indicated that *D*-dopachrome tautomerase (DDT) is the only gene with marked homology to MIF

(Esumi et al. 1998). As both genes are located relatively close on chromosome 22, it was speculated that the MIF and DDT genes are duplications of a common ancestral gene that have evolved to have different biological functions (Calandra and Roger 2003). All mammalian MIFs (human, mouse, rat and cattle) have ~ 90% homology, and homologues of mammalian MIF have been found in chicken, fishes, parasites and plants. Conservation of the MIF gene across species indicates that MIF must have important biological functions. The cDNA for MIF encodes a 114-amino acid protein with an apparent molecular weight of 12.5 kD (Fig. 1.1.).

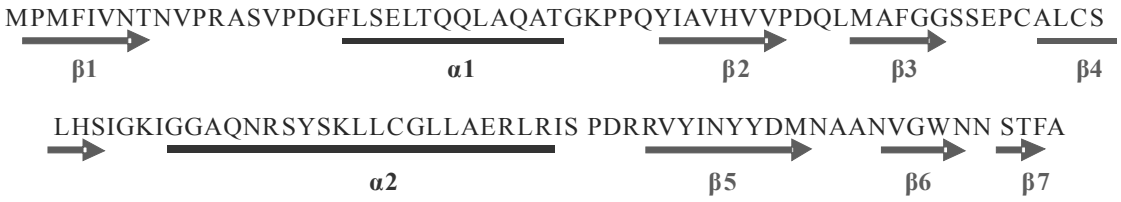


Fig. 1. Secondary structure of the human MIF monomer. The amino acid sequences forming the β sheets and the α helices are underlined.

The unique ribbon structure of rat and human MIF was defined using X-ray crystallography (Sugimoto et al. 1996; Sun et al. 1996a; Suzuki et al. 1996) (Fig. 1.2.). In addition, solution conformation data have been obtained by two-dimensional NMR (Muhlhahn et al. 1996). While the tertiary structure of the MIF monomer may resemble that of the IL-8 dimer and major histocompatibility complex (MHC) structures, the folding of MIF is unique.

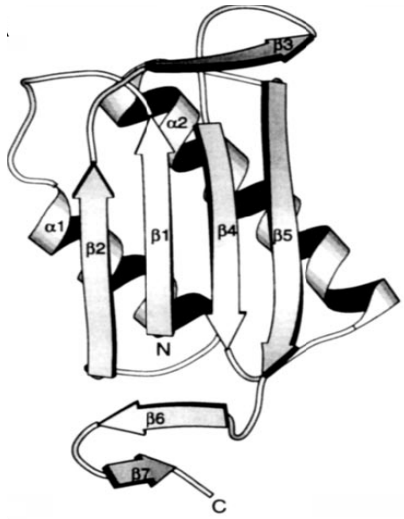


Fig. 2. Three-dimensional structure of MIF monomer (Kleemann et al. 2000b)**.**

Structural data show that this cytokine exists both as a trimer in the crystal form (Sun et al. 1996b) and as a dimer in solution (Muhlhahn et al. 1996). Recently, cross-linking experiments have provided evidence that under physiological conditions MIF exists as a mixture of monomers, dimers and trimers, the monomers being the major species (Mischke et al. 1998). MIF monomer consists of a core of four-stranded β-sheet flanked by two anti-parallel α-helices and a further three very short β-strands. The short β-strands extend the core four-stranded β-sheet of a neighboring monomer on either side, to create a seven stranded β-sheet, thus linking the monomers together into the trimer (Tan et al. 2001). Several hydrogen bonding sites between the monomers, and a hydrophobic core act to stabilize the MIF trimer. The C-terminal domain is believed to be important for stable trimer formation (Bendrat et al. 1997). A channel is formed in the centre of the trimer. This channel has a dimension varying from 4 Å to 15 Å in diameter and is predominantly lined with hydrophilic atoms which could possibly interact with negatively charged moieties (Baugh and Bucala 2002).

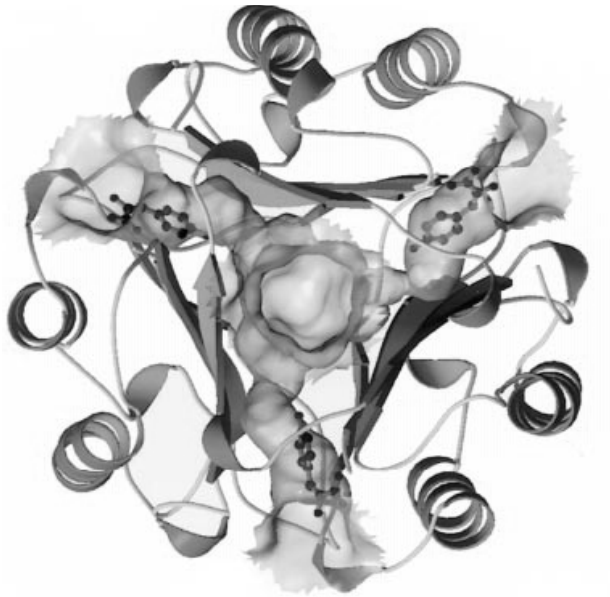


Fig. 3. Top view of the MIF trimer with the central channel (Tan et al. 2001)**.**

While its primary sequence is unrelated to that of other proteins, the three dimensional crystal form of human MIF is structurally homologous to the small bacterial enzyme 4-oxalocrotonat-tautomerase (4-OT), 5-carboxymethyl-2-hydroxymuconat-isomerase (CHMI) and chorismat-mutase (Chook et al. 1994; Subramanya et al. 1996). The structural similarity between MIF and 4-OT or CHMI also extends to the enzymatic active site. Each protein has an N-terminal proline with an unusually low pK_a that acts to facilitate proton transfer in the substrate (Stamps et al. 1998).

1.3. Enzymatic activity of MIF

The three dimensional structure and its resemblance to prokaryotic enzymes led to the observation that MIF possesses enzymatic activity. Thus, MIF has been reported to have two different catalytic activities: tautomerase (Rosengren et al. 1996); (Bendrat et al. 1997; Rosengren et al. 1997; Swope et al. 1998) and thiol-protein oxidoreductase(Kleemann et al. 1998a; Kleemann et al. 1999; Kleemann et al. 1998b). Therefore, MIF not only shares a three-dimensional architecture with several microbial enzymes, but also is itself an enzyme. To what extent these enzymatic functions have

physiological relevance is not known, because a natural substrate for MIF enzymatic activity was not yet found.

1.3.1. Tautomerase activity

MIF tautomerase activity was discovered during the investigation of melanin biosynthesis (Zhang et al. 1995), which involves the conversion of 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) into 5,6-dihydroxyindole-2-carboxylc acid (DHICA). Subsequent studies revealed that MIF catalyze tautomerisation of the non-physiologic substrates, D-dopachrome and L-dopachrome methyl ester (Rosengren et al. 1996). The first proline (Pro-1) appears to be a critical residue for enzymatic activity as replacement of Pro-1 with serine or glycine eliminates the tautomerase activity (Bendrat et al. 1997; Swope et al. 1998). Current data support the idea of a correlation between tautomerase activity and pro-inflammatory functions of MIF and a lot of efforts were employed in developing molecules that can inhibit the tautomerase activity (Dios et al. 2002; Lubetsky et al. 2002; Swope et al. 1998). In an attempt to identify natural ligands for MIF, the keto-enol isomerizations of p-hydroxyphenylpyruvate (HPP) and phenylpyruvate were discovered to be catalyzed by MIF (Rosengren et al. 1997). The separate localization of these substrates from MIF as well as the kinetic parameters for the tautomerization reaction suggests that these molecules are unlikely to be physiological substrates for MIF (Swope et al. 1998). Tautomerase activity is an evolutionarily ancient phenomenon, which early life forms presumably utilized for synthesis, but there is no evidence that modern species use this synthetic pathway.

1.3.2. Thiol-protein oxidoreductase activity

The catalytic thiol-protein oxidoreductase (TPOR) activity of MIF is mediated by a Cys57-Ala-Leu-Cys60 (CALC) motif that can undergo reversible intramolecular disulfide formation. These residues in the catalytic active site are among the most highly conserved residues and that is a characteristic feature of thiol-protein oxidoreductases, such as thioredoxin (Takahashi and Creighton 1996) and protein disulfide isomerase (Puig et al. 1994). Oxidoreductase activity is dependent on the formation and reduction of

disulfide bridges between the two conserved cysteine residues. Based on this observation, MIF was assessed for oxidoreductase activity and was found to promote the reduction of the disulfides in insulin and 2-hydroxyethyldisulfide (Kleemann et al. 1998a). Mutation of either of this cysteines abrogates the TPOR activity of MIF, while mutation of another cysteine, Cys81 is without effect (Kleemann et al. 1998a). Over the years, the biochemical and biological evidence for a role of TPOR activity for various MIF functions were investigated using the C60SMIF mutant, which has no TOPOR activity. Ectopically overexpressed wtMIF inhibits pro-oxidative stress induced apoptosis, while the redox-dead C60SMIF does not exhibit this capability (Nguyen et al. 2003b). This effect seems to be different in the case of exogenously added recombinant proteins when both wtMIF and C60SMIF protect cells from apoptosis at a similar degree. In another biological study, C60SMIF showed no activity in the HED transhydrogenase assay and in the glucocorticoid overriding assay this mutant had significantly reduced activity when compared to wtMIF (Kleemann et al. 1999). MIF's role in cellular redox regulation seems to be connected with the cell signaling. Evidence for this was suggested by the finding of an intracellular interaction between MIF and COP9 signalosome JAB1/CSN5 (Kleemann et al. 2000a). Binding of MIF to JAB1 is dependent on the sequence region 50-67 of MIF, but it is no requirement for the presence of an intact CXXC motif. The redox-dead mutant C60SMIF can bind to JAB1 (Kleemann et al. 2000a) but the JAB1-antagonistic effects of MIF appear to be CXXC-dependent (Kleemann et al. 2000a). MIF's oxidoreductase activity is likely to play a role in MIF-mediated immune cell functions. In contrast to wt MIF, the redox-dead mutant C60SMIF is unable to activate macrophages to kill leishmania parasites (Kleemann et al. 1998a). All this data reveals the TPOR activity of MIF is not limited to an *in vitro* function of an evolutionary conserved local sequence site, but also as an intracellular property of this factor that is involved in the regulation of a variety of cellular processes (Thiele and Bernhagen 2005).

1.4. Biological activities of MIF

1.4.1. MIF regulation of innate immunity via TLR4 expression

During the development of MIF knock-out mice, it has become apparent that these animals are relatively resistant to lipopolysaccharides (LPS) (Bozza et al. 1999). Similarly, LPS-induced nuclear factor-κB (NF- κB) activity and steady-state TNF-α mRNA levels are markedly reduced by antisense MIF treatment of macrophages. By contrast, antisense MIF macrophages generated by transduction of an antisense MIF adenovirus or by stable transfection with an antisense MIF plasmid or obtained from MIF-knockout, were hyporesponsive to stimulation with LPS and gram-negative bacteria (Roger et al. 2003) and exhibited normal responses to other inflammatory stimuli, including gram-positive bacteria (Froidevaux et al. 2001). It was then shown that the hyporesponsivness of MIF-deficient macrophages to LPS and gram-negative bacteria is due to down regulation of TLR4, the signal transduction molecule of the LPS receptor complex (Roger et al. 2001; Roger et al. 2003), and is associated with decreased activity of the transcription factor PU.1 that is required for optimal expression of the TLR4 gene (Roger et al. 2001). Toll-like receptor (TLR) plays an essential role in the innate immune response by detecting conserved molecular products of microorganisms (Medzhitov 2001; Medzhitov et al. 1997). TLR4, for example, is the receptor for LPS, the major component of the cell wall of the gram-negative bacteria (Takeda et al. 2003). MIF upregulates the expression of TLR4 by acting on the ETS family of transcription factors (including PU.1), which are crucial for transcription of TLR4. Therefore, MIF facilitates the detection of endotoxin-containing bacteria, enabling cells that are at the forefront of the host antimicrobial defense system, such as macrophages, to respond rapidly to invasive bacteria. Rapid production of pro-inflammatory cytokines is absolutely essential for mounting the host defensive response. Increased susceptibility of MIF-deficient mice to infection was associated with reduced plasma levels of the pro-inflammatory cytokines tumor-necrosis factor α (TNFα), interleukin 12 (IL-12) and interferon-γ (IFN-γ) , but not of nitric oxide (NO), and with higher bacterial counts compared to wildtype mice.

This indicates that MIF promotes a protective T helper (T_H1)-cell immune response against bacteria.

1.4.2. MIF effects on p53 activity

p53 is a tumour suppressor gene that encodes a nuclear protein involved in the control of cell growth, regulating the entry of the cell into S-phase of the cell-cycle and apoptosis. p53 is activated only when cells are stressed or their DNA is damaged. p53 blocks the multiplication of stressed cells, inhibiting progress through the cell cycle. In many cases it causes apoptosis of those cells in an attempt to contain the damage and protect the organism. The p53 protein therefore provides a critical brake on tumour development, explaining why it is so often mutated and thereby inactivated in cancers (Vogelstein et al. 2000).

Tumour cell lines were found to express high quantities of MIF. One important turning point in MIF biology was the finding that MIF negatively regulates the activity of the p53 tumor suppressor and hence, apoptosis (Hudson et al. 1999), providing a link between MIF, inflammation, cell growth and tumorigenesis. It was reported that the proinflammatory function i.e. the production of TNFα, IL-1β and PGE_2, and the viability of MIF-deficient macrophages were reduced compared to wild-type cells after challenge with LPS (Mitchell et al. 2002). Despite the equal level of NO production by MIF-deficient and wild-type macrophages, NO was thought to be a crucial mediator of increased apoptosis of MIF-deficient macrophages stimulated with LPS (Mitchell et al. 2002). MIF was found to inhibit NO-induced intracellular accumulation of p53 and phosphorylation of p53 and therefore, p53-mediated apoptosis (Mitchell et al. 2002). Inhibition of p53 by MIF requires serial activation of ERK1/2, PLA2, cyclooxygenase 2 (COX2) and PGE_2 (Mitchell et al. 2002). In agreement with these results, MIF was reported to interact with the E2F-p53 pathway to sustain normal and malignant cell growth (Petrenko et al. 2003). All these studies have established MIF as an important inhibitor of p53-mediated apoptotic processes in macrophages and other cell types and have supported the notion that MIF could be a key mediator linking inflammation and cancer. Although it was found that MIF inhibition of p53 results in an inhibition of p53 transcriptional activity, the underlying mechanism by which MIF inhibits p53 tumor

suppressor activity and apoptosis has not yet been resolved. It was suggested that the redox effects could play a role as MIF reduces oxidative stress-induced apoptosis in several cell types, including immune cells (Nguyen et al. 2003b).

1.4.3. Role of MIF in inflammation

Increases in hypothalamic-pituitary-adrenal axis activation resulting in the production of adrenal glucocorticoids in response to inflammatory stress is well documented, as are the suppressive effects of this response on inflammation (Stephanou et al. 1992; Yang et al. 1997). Stimulation of the hypothalamus and pituitary by circulating proinflammatory cytokines such as IL-6, therefore provoke production of glucocorticoids which in turn inhibit the production of IL-6. This comprises a classical feedback control loop (Morand et al. 1996).

MIF is directly proinflammatory by activating or promoting cytokine expression (TNF-α (Calandra et al. 1994; Calandra et al. 2000), IL-1β, IL-2 (Bacher et al. 1996), IL-6 (Calandra et al. 1994; Satoskar et al. 2001), IL-8 (Benigni et al. 2000), IFN-γ (Abe et al. 2001; Bacher et al. 1996), NO release (Bernhagen et al. 1994; Bozza et al. 1999; Onodera et al. 2002), matrixmetalloprotease 2 (MMP-2) (Onodera et al. 2002) expression and induction of COX-2 pathway. A surprising observation, which at first seemed to be incompatible with the pro-inflammatory features of this cytokine, was that MIF secretion was induced rather than inhibited by glucocorticoid hormones (Calandra et al. 1995) and MIF was found to override the immunosuppressive effect of glucocorticoids (Bacher et al. 1996; Calandra et al. 1995; Mitchell et al. 1999). The counter-regulatory effect of MIF was confirmed in mouse models of endotoxaemia (Calandra et al. 1995) and antigen-induced arthritis (Leech et al. 2000). Similar to glucocorticoids, the circulating concentration of MIF is increased during inflammation, infection and stress (Beishuizen et al. 2001; Calandra et al. 1995; Calandra et al. 2000). Studies concerning the molecular mechanism of MIF and glucocorticods actions showed that MIF antagonizes the effect of glucocorticoids via effects on activity of NF-κB. NF-κB is an important regulator of inflammatory cytokine gene expression (Barnes and Karin 1997), and several lines of evidence suggest that glucocorticoids may inhibit the production of proinflammatory mediators such as TNF-α via modulation of NF-κB activity. Glucocorticoids have been

proposed to inhibit binding of the p65 subunit of NF-κB to the transcriptional machinery of target genes (De Bosscher et al. 2000) and to induce IκB synthesis (Auphan et al. 1995; Scheinman et al. 1995). Elevation of cytoplasmic IκB inhibits the ability of NF-κB to translocate to the nucleus whereas inhibition of NF-κB p65 binding to DNA directly inhibits expression of target genes. Thus, by blocking glucocorticoid-induced IκB synthesis, MIF promotes the translocation of NF-κB into the nucleus where it activates proinflammatory cytokine and adhesion molecule expression (Daun and Cannon 2000).

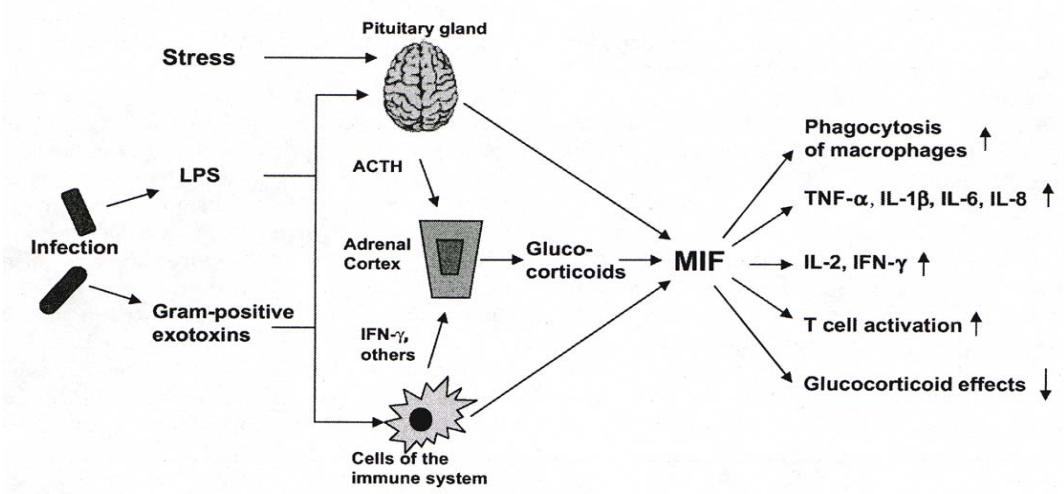


Fig. 4. Glucocorticoids and MIF. Glucocorticoids and MIF are in a tightly regulated balance. MIF is secreted upon glucocorticoid induction and then counter regulates glucocorticoid effects. This balance might be dysregulated in autoimmune diseases leading to an overexpression of MIF and of the proinflammatory cytokines (Denkinger et al. 2004).

1.5. MIF-modulated signalling pathways

1.5.1. MIF induces sustained ERK-1/2 activation

Studies of intracellular signaling events and proliferation of MIF-stimulated quiescent fibroblasts showed that MIF induces rapid (within 30 min) and sustained (up to 24 hours) phosphorylation and activation of the p44/p42 extracellular signal-regulated kinase-1/2 family of the mitogen-activated protein kinase (MAPK) pathway as well as cell proliferation (Mitchell et al. 1999). ERK1/2 are proline/serine/threonine kinases, components of the Ras-Raf-MEK-ERK MAP cascade. While ERK-1/2 has been characterized for its role in growth control, it also activates several downstream effector proteins that are involved in the inflammatory response such as transcription factors (c-myc, NF-κβ, Fos and Ets), cytoskeletal proteins mediating membrane activation and phagocytosis. Activation of ERK-1/2 by MIF is protein kinase A dependent and is associated with increased cytoplasmic phospholipase A_2 (PLA2) enzyme activity. PLA2 is and important intracellular link in the activation of the proinflammatory cascade, and its product, arachidonic acid, is the precursor of prostaglandins and leukotrienes (Hayakawa et al. 1993). PLA2 is also a key target of the anti-inflammatory effects of glucocorticoids, and ERK-1/2-mediated induction of PLA2 is one mechanism whereby MIF could override the immunosuppressive effects of steroids (Mitchell et al. 1999).

1.5.2. MIF inhibits Jab-1 activity

An alternative mechanism by which MIF may carry out its cellular actions was proposed by Kleeman et al. Yeast two-hybrid system showed that MIF interacts with a protein known as Jun-activation domain binding protein-1 (JAB1) or as COP9 signalosome subunit 5 (CSN5) (Kleemann et al. 2000a). The authors show that MIF is taken up into the cells where binds to JAB1 and then negatively affects the function of intracellular JAB1. JAB1 activates Jun N-terminal kinase (JNK) to phosphorylate JUN and functions as a co-activator of activator protein-1 (AP1), a transcription factor that is implicated in cell growth, transformation and cell death. JAB1 also binds and promotes

the degradation of p^{27Kip1}, a protein that halts the cell-division cycle. The binding of MIF to JAB1 results in a reduced degradation of p^{27Kip1}, and MIF overexpression inhibits the growth-promoting properties of JAB1 in fibroblasts (Kleemann et al. 2000a). Because JAB1 was shown to be an important regulator of several proinflammatory genes, the finding that MIF interacts with Jab-1 seemed to be contradictory to the proinflammatory action of MIF. The cell growth-promoting effects of MIF (Hudson et al. 1999; Mitchell et al. 1999) would conflict with the proposed role of MIF in the enhancement of p^{27Kip1}-regulated cell-cycle stasis. However, one characteristic feature of MIF action is its bell-shaped dose-response curve with respect to several biological phenomena. This implies that low versus high levels of MIF may have distinct regulatory effects on cellular processes.

1.6. Direct effects of MIF by means of protein-protein interaction

Although MIF was one of the first cytokines to be discovered, the understanding of its molecular mechanism of action is only fragmentary. Recent work has identified CD74, a MHC class II–associated invariant chain, as a cell surface binding protein/receptor for MIF (Leng et al. 2003). CD74 expression is required for MIF mediated ERK-1/2 phosphorylation, PGE2 production and cell proliferation (Leng et al. 2003). Because CD74 does not contain an intracellular domain for signal transduction, it has been suggested that CD74 could serve as an adaptor molecule which could present MIF to other transmembrane proteins in a process possibly involving CD44 (Meyer-Siegler et al. 2004).

However, a number of intracellular proteins have been shown to interact with MIF, supporting the earlier contention that MIF also possesses intracellular functions based on its uptake into numerous immune and non-immune cell-types by non-receptor mediated endocytosis (Kleemann et al. 2002), its enzymatic activity and constitutive expression profile. In a recent review (Thiele and Bernhagen 2005) it was pointed out that several of the MIF-interacting proteins identified to date are redox proteins or proteins which are directly connected to redox regulation. In this context, MIF was shown to interact with PAG, a thiol-specific antioxidant and low-efficiency peroxidase (Jung et al. 2001), with hepatopoietin (HPO), a flavin-linked sulfhydryl oxidase (Li et al. 2004) and

with insulin, which can be enzymatically reduced by MIF (Kleemann et al. 1998a). Insulin colocalizes with MIF in secretory granules of the pancreatic islets and MIF regulates glucose-induced insulin release (Waeber et al. 1997). Like HPO and thioredoxin, MIF also interacts with JAB1 (Kleemann et al. 2000a), subunit 5 of the COP9 signalosome (CSN/CSN5) that was originally identified as a coactivator of activator protein 1 (AP-1) transcription. The CSN modulates the ubiquitin-proteasome protein degradation pathway and enhances for example degradation of the tumor suppressor p53 (Bech-Otschir et al. 2001). More recently, a direct interaction of MIF with myosin light chain kinase isoform (MLCK) was identified (Wadgaonkar et al. 2005) which may have important implications for the regulation of both non-muscle cytoskeletal dynamics as well as pathobiologic vascular events that involve MLCK.

1.7. Pathophysiological effects of MIF and tissue distribution

Because of its broad regulatory properties, MIF is a critical mediator of a number of immune and inflammatory diseases. In septic shock MIF up-regulates TNF-α, NO, IL-1, IL-6, IL-8 expression levels and LPS signaling and inhibits the migration of monocytes (Bernhagen et al. 1998; Bernhagen et al. 1993; Bloom and Bennett 1966; David 1966; Muhlhahn et al. 1996; Tomura et al. 1999; Weiser et al. 1989). The best evidence for a role of MIF in chronic inflammation has been gathered for rheumatoid arthritis (RA). It was demonstrated that anti-MIF monoclonal antibodies markedly suppressed the inflammatory response in a mouse model of human RA (Mikulowska et al. 1997). Connective tissue degradation by matrix metalloproteases (MMPs) is a typical pathological feature of RA. MIF has been suggested to contribute toward this process via up-regulation of MMP-1 and MMP-3 mRNA levels in synovial fibroblasts (Onodera et al. 2000). Glucocorticoids repress transcription of the MMP-1 gene by interaction of the glucocorticoid receptor with the AP-1 complex (Vincenti et al. 1996). A connection between MIF and glucocorticoid/AP-1 interaction is implied by a recent study (Chauchereau et al. 2000) showing that JAB1/CSN5 can bind to the glucocorticoid receptor. These studies support the concept that MIF is a potent counter-regulator of glucocorticoid control of inflammation in general and synovial inflammation in particular. Evidence has also been obtained for an involvement of MIF in lung inflammation. Significant MIF quantities were found in the alveolar airspaces of patients with acute respiratory distress syndrome (Donnelly et al. 1997). MIF augmented

proinflammatory cytokine secretion (TNF-α and IL-8) and anti-MIF mAbs significantly attenuated TNF-α and IL-8 secretion (Abe et al. 2001). Accumulating data imply that MIF could be centrally involved in processes regulating cell proliferation and tumor angiogenesis. In murine colon carcinoma cells, cytosolic MIF levels are increased in response to growth factors and this was correlated with enhanced proliferation of these cells (Takahashi et al. 1998), a notion that was confirmed by the finding that overexpression of antisense MIF constructs led to an inhibition of cell proliferation. The mechanistic pathway of how MIF may regulate tumor progression and cell proliferation is unknown. However, a number of recent observations offer potential molecular explanations for the activities of MIF. A direct proliferation-enhancing effect of recombinant MIF in quiescent fibroblasts seems to be mediated through ERK1/2 (Mitchell et al. 1999), while a growth-inhibiting effect seems to be JAB1/CSN5/$p27^{Kip1}$-dependent (Kleemann et al. 2000a). Other recent studies suggest that the modulation of cell proliferation by MIF could involve a complex regulatory system in which the proteins p53, AP1/CSN5 and possibly other signalosome proteins may be involved.

The potential use of MIF-based therapeutic strategies has recently been underscored by successful application of anti-MIF monoclonal antibodies in pre-clinical models of sepsis, rheumatoid arthritis and tumorigenesis (Bernhagen et al. 1993; Calandra et al. 2000; Mikulowska et al. 1997; Sakai et al. 2003).

Disease/pathophysiologic condition	Corresponding MIF activity	Proposed involved mechanism(s)
Septic shock	↑ TNF, NO, IL-1, IL-6 and IL-8 ↑ LPS signaling (↓ Monocyte migration and chemotaxis)	Unknown (possibly counter-regulation of glucocorticoid action; desensitization of chemokine-induced chemotaxis; modulation of iNOS expression; up-regulation of component of LPS signaling pathway)
Stress and glucocorticoid function	Counter-regulation of glucocorticoid action	Overriding of glucocorticoid-mediated suppression of arachidonate release (Cys-60-based catalytic MIF oxidoreductase activity) (Glucocorticoid receptor- JAB1/CSN5-MIF-interaction-based mechanism)
Inflammatory lung disorders	↑ TNF, IL-8 ↑ Arachidonic acid release (Overrides suppressive effect of glucocorticoids on TNF-induced arachidonic acid)	Unknown (possibly counter-regulation of glucocorticoid action; ↑ arachidonic acid release by MIF)
Rheumatoid arthritis	↑ MMP-1 / MMP-3 in synovial fibroblasts ↑ PLA-2 / COX-2 activity ↑ TNF	↑ of PKC, AP-1 and TK activity by MIF ↑ of MIF expression by 10^{-10}–10^{-12} M glucocorticoids in synoviocytes ↑ of PLA-2/COX-2 activity by MIF (Pro-2-based MIF tautomerase activity)
Cancer, tumorigenesis and apoptosis	↓ p53 activity ↓ Redox- and stress-induced apoptosis ↑ Cell proliferation ↓ Cell proliferation	↓ of p53 activity by MIF ↑ of Erk1/2 activity by MIF Subcellular distribution of MIF ↑ of $p27^{Kip1}$ by MIF Interaction of MIF with JAB1/CSN5 and interaction of JAB1/CSN5 with p53 Modulation of JNK activity by MIF
Diabetes	↑ Of glucose-induced insulin secretion ↑ Insulin release ↑ Muscular glycolysis ↑ Catabolic effect of TNF on muscle metabolism	↑ of MIF release by glucose Colocalization of MIF and insulin in secretion granules ↑ of fructose 2,6-bisphosphate by MIF ↑ of MIF expression in adipocytes by costimulation with glucose and insulin
Atherosclerosis	↑ oxLDL uptake ↑ Adhesion molecule expression on endothelial cells	Colocalization of MIF and JAB1/CSN5 ↑ of MIF expression/secretion by oxLDL ↑ of VCAM/ICAM expression by MIF

Table 1. Potential correlations between MIF's mechanism of action, its biological activities and diseases states (from Lue et al. 2002).

1.8. Tissue and cellular distribution of MIF

Cell type	Stimuli	References
Anterior pituitary		
Corticotropic cells	RF, LPS	Bernhagen ***et*** *al.,* 1993; Nishino *et al.,* 1995
Immune system		
Monocytes/macrophages	LPS, TNFα, IFNγ, Glucocorticoids	Calandra et.al 1994
	TSST-1, exotoxin A	Calandra *et al.,* 1998
T cells (TH2> TH I), mast cells	αCD3, PMA/ionomycin, PHA	Bacher *et al.,* 1996;
Eosinophils	PMA, C5a, IL-5	Rossi *et al.,* 1998
HL-60, myelomonocytic	LPS	Nishihira *et al.,* 1996
Adrenal gland		
Cortex-zona glomerulosa, zona fasciculata	LPS	Bacher *et al.,* 1997
Lung		
Bronchial epithelium	LPS	Bacher *et al.,* 1997
Alveolar macrophages		Donnelly *et al.,* 1997
Kidney		
Tubule epithelial cells, proximal tubules	LPS	Imamura, 1996
Glomerular epithelial cells, endothelium, Kupffer cells	LPS	Lan *et al.,* 1996
Tubular epithelial cells	LPS	Lan *et al.,* 1998
Mesangial cells	LPS, PDGF-AB, IFNγ	Tesch *et al.,* 1998
Liver		
Hepatocytes surrounding central veins, Kupffer cells	LPS	Bacher *et al,.* 1997
Skin		
Keratinocytes, sebaceous glands,	LPS, croton oil	Shimizu *et al* 1996/99
outer root sheath of hair follicle,	UV B	Shimizu *et al.,* 1999
epidermal layer, endothelial cells	Acute inflammation	Shimizu *et al.,* 1997
Testis		
Leydig cells		Meinhardt *et al.*1996/1998
Pancreas		
Islet β cells	Glucose	Waeber *et al.,* 1997
Eye		
Corneal epithelial cells		Wistow *et al.,* 1993
Endothelial cells, lens		Matsuda *et al.,* 1996
Iris, ciliary epithelium		Matsuda *et al.,* 1996
Brain		
Cortex, hypothalamus	LPS	Bacher *et al.,* 1998
Glial cells, ependyma, astrocytes		Suzuki *et al.,* 1999
Telencephalon		
Bone		
Neonatal calvaries and osteoblasts,	LPS	Onodera *et al.,* 1996
Fat tissue		
3T3L1 adipocytes	TNF-α	Hirokawa *et al.,* 1997, 1998
Prostate		
Epithelial cells		Frenette *et al.,* 1998 Meyer-Siegler, 1998
Vasculature		
Endothelial cells	LPS	Nishihira *et al.,* 1998

1.9. Aim of the study

Even after 40 years of research, the molecular mechanism of MIF action remains fragmentary understood. Several reports have been published describing the role of MIF in inflammatory diseases, including arthritis, glomerulonephritis, peritonitis, and the delayed-type hypersensitivity reaction (reviewed in Lue et al. 2002). Clinical evidence demonstrates increased MIF expression during inflammatory disease, further supporting the potential role of MIF in inflammatory processes. Development of neutralizing MIF antibodies has proven to be therapeutically effective in numerous animal models of systemic inflammation. This data suggests that blocking MIF activity is a promising approach for preventing inflammation.

Although MIF was shown to interact with several proteins, the biological impact of the discovered interaction has not been fully elucidated so far. Thus, this study aimed to identify new MIF binding proteins and to reveal the effect on MIF activity. For identification of novel MIF-interacting proteins, co-immunoprecipitation and chemical cross-linking should be employed, with subsequent validation of the protein-protein interaction using *in vitro* pull-down assay with recombinant proteins. The physiological relevance of the verified interactions will be explored by several established MIF functional assays, such as chemotaxis, tautomerase assay, glucocorticoids activity assay.

2. ABBREVIATIONS

aa	Amino acid(s)
Amp	Ampicillin
AP-1	Activator protein 1
APS	Ammonium persulphate
bp	Base pair
BSA	Bovine serum albumin
°C	Degree Celsius
CXXS	Cys-Xaa-Xaa-Cys motif
cDNA	Complementary DNA
DAPI	4', 6'-diamino-2-phenylindole, dihydrochloride
DCME	L-dopachrome methylester
DEX	Dexamethasone
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	2'-deoxynucleoside-5'-triphosphates
DTT	Dithiothreitol
E. coli	*Escherichia coli*
et al.	and others
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-Linked-Immunosorbent-Assay
ERK1/2	Extracellular signal-regulated kinases
FCS	Fetal calf serum
g	gram or gravity, depending on the context
GST	Glutathione S-Transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	Histidine
HRP	Horse radish peroxidase
IPTG	Isopropyl β-D-thiogalactopyranoside
JAB1	Jun-activation domain-binding protein 1
JNK	c-Jun N-terminal kinase

kb	Kilo base pair
kD	Kilo Dalton
LB	Luria Bertani medium
LPS	Lipopolysaccharide
M	Molar
MALDI MS	Matrix-assisted laser desorption ionization MS
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemotactic factor 1
mg	Milligram
MES	Morpholinoethane sulfonic acid
MIF	Macrophage migration inhibitory factor
min	Minute
ml	Milliliter
MW	Molecular weight
NaCl	Sodium chloride
NHS	Normal horse serum
NP-40	Nonidet P-40
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBMC	Periferal blood monocyte
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RP S19	Ribosomal protein S19
RT	Room temperature
SDS	Sodiumdodecylsulphate
sec	Second
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TNF-α	Tumor necrosis factor alpha
Tris	Tris(hydroxymethyl)-amino-methane
U	Unit

UV	Ultraviolet
V	Volt
v/v	Volume per volume
w/v	Weight per volume
wt	wild type
µ	Micro
µg	Microgram
µl	Microliter
µM	Micromolar

3. MATERIALS

3.1. Chemicals

Acetic acid	Merck, Darmstadt
Acrylamide 30%	Roth, Karlsruhe
Agarose	Invitrogen, Karlsruhe
Bacto-Tryptone	BD Bioscience, Sparks, USA
Bacto-yeast extract	BD Bioscience, Sparks, USA
Bromophenol blue sodium salt	Sigma-Aldrich, Steinheim
Calcium chloride	Merck, Darmstadt
Chloroform	Merck, Darmstadt
Brilliant Blue G-Colloidal Concentrate	Sigma-Aldrich, Steinheim
Dexamethasone	Sigma Aldrich, Steinheim
2'-Deoxynucleoside 5'-triphosphate	Gibco-BRL, Neu-Isenburg
Dimethyl sulfoxide	Merck, Darmstadt
di-potassium hydrogen phosphate	Merck, Darmstadt
di-sodium hydrogen phosphate	Merck, Darmstadt
1,4-Dithiothreitol	Roche, Mannheim
L-dopachrome methyl ester	Sigma-Aldrich, Steinheim
Ethanol	Sigma-Aldrich, Steinheim
Ethidiumbromide	Roth, Karlsruhe
Ethylene diaminetetraacetic acid disodium salt	Merck, Darmstadt
Formamide	Merck, Darmstadt
Glutathione	Amersham, Freiburg
Glycerol	Merck, Darmstadt
Glycine	Sigma-Aldrich, Steinheim
Guanidine hydrochloride	Sigma-Aldrich, Steinheim
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Roth, Karlsruhe
Igepal CA-630 (NP-40)	Sigma-Aldrich, Steinheim
Isopropylthio-β-D-galactoside	Serva, Heidelberg

Leupeptin	Sigma-Aldrich, Steinheim
Lipopolysaccharide	Sigma-Aldrich, Steinheim
Magnesium chloride	Merck, Darmstadt
Magnesium sulphate	Sigma-Aldrich, Steinheim
β-Mercaptoethanol	AppliChem, Darmstadt
Methanol	Sigma-Aldrich, Steinheim
Morpholinoethane sulfonic acid	Serva, Heidelberg
Non-fat dry milk	Bio-Rad, München
Paraformaldehyde	Merck, Darmstadt
Phenylmethylsulfonyl fluoride	Sigma-Aldrich, Steinheim
Ponceau S	Roth, Karlsruhe
Potassium chloride	Merck, Darmstadt
Rotiphorese Gel 30	Roth, Karlsruhe
Sodium acetate	Roth, Karlsruhe
Sodium azide	Merck, Darmstadt
Sodium chloride	Sigma-Aldrich, Steinheim
Sodium citrate	Merck, Darmstadt
Sodium dodecyl sulfate	Merck, Darmstadt
Sodium periodate	Sigma-Aldrich, Steinheim
N,N,N',N'-Tetramethylethylenediamin	Roth, Karlsruhe
Tris(hydroxymethyl)aminomethane	Roth, Karlsruhe
Triton X-100	Sigma-Aldrich, Steinheim
Tween-20	Roth, Karlsruhe
Urea	Merck, Darmstadt

3.2. Enzymes

Taq Polymerase	Promega, Mannheim
T4 DNA Polymerase	Promega, Mannheim
EcoRI	Promega, Mannheim
XhoI	Promega, Mannheim

NdeI	Promega, Mannheim
T4 DNA Ligase	Promega, Mannheim
DNase	Promega, Mannheim
RNase	Promega, Mannheim

3.3. Antibodies

Antibody	**Manufacturer**	**Dilution**
Primary antibodies		
Rabbit α-rat MIF	(Kim 2003)	1:20,000
Mouse α-MIF	Picower Institute, Manhasset, NY	1:200
Rabbit α-mouse RP S19	own production of this thesis	1:500
α-GST-HRP	Amersham, Freiberg	1:5,000
Rabbit α-mouse Jab-1	Santa Cruz, USA	1:500
α-Biotin-HRP	Amersham, Freiburg	1:1,500
Secondary antibodies		
Goat α-rabbit-HRP	ICN, Ohio, USA	1:10,000
Donkey α-rabbit IgG-Cy3	Chemicon, Hampshire, UK	1:1,000
Donkey α-mouse IgG-FITC	Dianova, Hamburg	1:1,000

3.4. Cells

NIH 3T3 mouse fibroblasts were obtained from the research group of Dr. Oliver Eickelberg, Department of Internal Medicine, University of Giessen.

Human mononuclear cells were isolated from buffy coats kindly provided by the Department of Clinical Immunology and Transfusion Medicine, JLU Giessen (Head: Prof. Gregor Bein).

PC12, a cell line derived from a pheochromocytoma of the rat adrenal medulla, was obtained from the working group of Prof. Wolfgang Kummer, Department of Anatomy and Cell Biology, University of Giessen.

Rat Sertoli and peritubular cells were isolated and kindly provided by Dr. Ruth Müller, Department of Anatomy and Cell Biology, University of Giessen.

3.5. Animals

Adult male Wistar rats were purchased from Charles River Laboratories (Sulzfeld).
Homozygous MIF knockout mice were kindly provided by Dr. Günter Fingerle-Rowson (University Hospital of Cologne, Department of Internal Medicine I).
Normal mice were obtained from University of Marburg, Department of Anatomy and Cell Biology.

3.6. Kits

ECL protein biotinylation module	Amersham, Freiburg
Gel Extraction Kit	Qiagen, Hilden
Maxiprep Plasmid Purification Kit	Genomed GmbH, Löhne
Miniprep Kit	Genomed GmbH, Löhne
PCR Purification Kit	Qiagen, Hilden
ProFound Sulfo-SBED Biotin Label Transfer Kit	Pierce, Rockford, USA
Silver staining Kit	Invitrogen, Karlsruhe
TNF-α ELISA Kit	BD Bioscience, Sparks, USA
QIAX II DNA extraction Kit	Qiagen, Hilden

3.7. Cell Culture Media and Antibiotics

Ampicillin sodium salt	Ratiopharm, Ulm
Bovine serum albumin (endotoxin free)	Invitrogen, Karlsruhe
Dulbecco's Minimal Essential Medium	PAA Laboratories, Cölbe
Fetal calf serum	Invitrogen, Karlsruhe
L-Glutamine	PAA Laboratories, Cölbe
MEM Non Essential Amino Acids	PAA Laboratories, Cölbe
Penicillin/Streptomycin	PAA Laboratories, Cölbe
RPMI 1640 medium	PAA Laboratories, Cölbe
Trypsin	PAA Laboratories, Cölbe
Ultrasaline A	PAA Laboratories, Cölbe

3.8. Equipment

Biofuge Fresco	Heraeus, Hanau
Cell culture incubator	Binder, Tullingen
Clean bench	BDK, Sonnenbühl-Genkingen
Easypet 4420 Pipette	Eppendorf, Hamburg
Electronic balance SPB50	Ohaus, Giessen
Gel Jet Imager 2000	Intas, Göttingen
Heater Block DB-2A	Techne, Cambridge, UK
Horizontal Mini Electrophoresis System	PEQLAB, Erlangen
Microwave oven	Samsung, Schwalbach
Mini centrifuge Galaxy	VWR International
Mini-Rocker Shaker MR-1	PEQLAB, Erlangen
Fluorescent microscope	Carl Zeiss, Jena
PCR system	Biozyme, Oldendor
Potter S homogenizer	B. Braun, Melsungen
Power supply units	Keutz, Reiskirchen
Pre-Cast Gel System	Invitrogen, Karlsruhe
SDS gel electrophoresis chambers	Invitrogen, Karlsruhe
Semi-dry-electroblotter	PEQLAB, Erlangen
Vertical electrophoresis system	PEQLAB, Erlangen
Ultrasonic homogenizer	Bandelin, Berlin
Ultrospec 2100 pro	Biochrom, Cambridge,UK
Confocal laser scanning microscope TCS SP2	Leica, Wetzlar

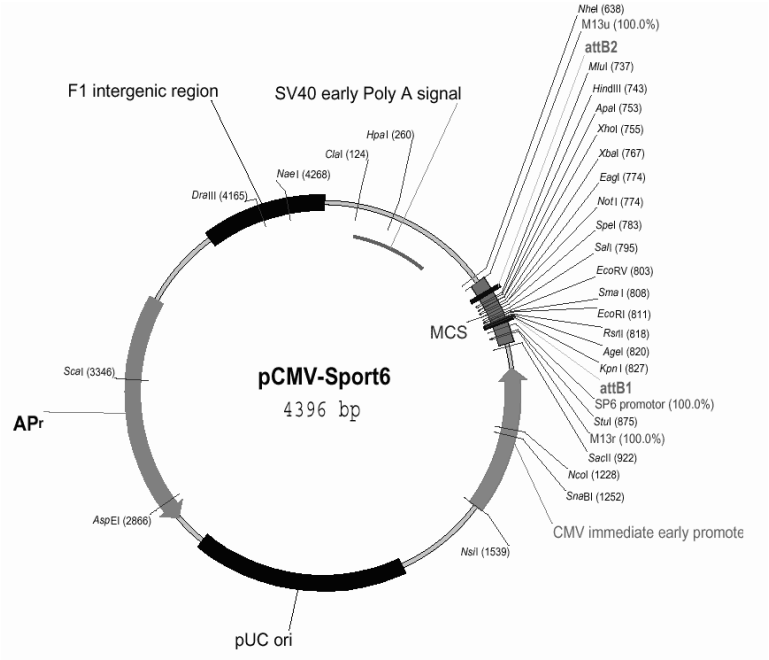
3.9. Miscellaneous

Avidin beads	Pierce, Rockford, USA
Bio-Rad Protein Assay	BioRad, München
Complete Freund's adjuvant	Sigma-Aldrich, Steinheim
DNA High and Low Mass Ladder	Invitrogen, Karlsruhe
DNA Ladder (100bp and 1kb)	Promega, Mannheim

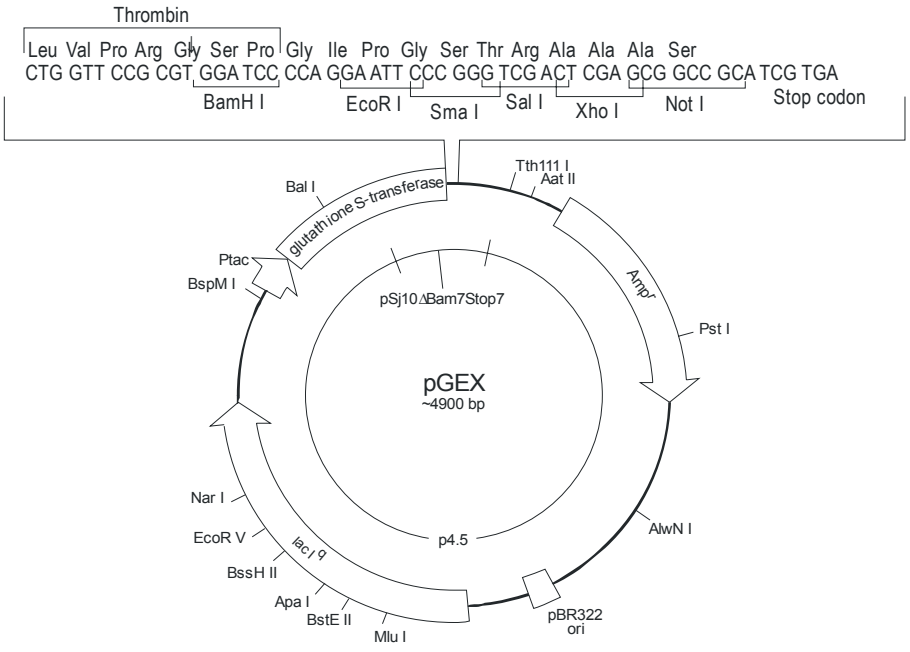
DAPI	Vector, Burlingame,USA
Enhanced chemiluminescence (ECL) reagents	Amersham, Freiburg,
Ficoll-Paque Plus	Amersham, Freiburg
Glutathione Sepharose 4B	Amersham, Freiburg
Hoechst 33342	Sigma-Aldrich, Steinheim
Hybond ECL nitrocellulose membrane	Amersham, Freiburg
Incomplete Freund's adjuvant	Sigma-Aldrich, Steinhaim
NuPAGE 4-12% Novex Bis-Tris gel	Invitrogen, Karlsruhe
Pefabloc SC inhibitor	Serva, Heidelberg
Protein size markers	Invitrogen, Karlsruhe
NAP^{TM}-5 Sephadex G-25 column	Amersham, Freiburg
Sterile plastic ware for cell culture	Sarstedt, Nümbrecht
Streptavidine beads	Novagen, Darmstadt
SYBR Green I Nucleic Acid Gel Stain	Roche Diagnostics, Mannheim
Transwell filter system	Corning, Schiphol, NL
X-ray Hyperfilm	Amersham, Freiburg

3.10. Cloning Plasmids

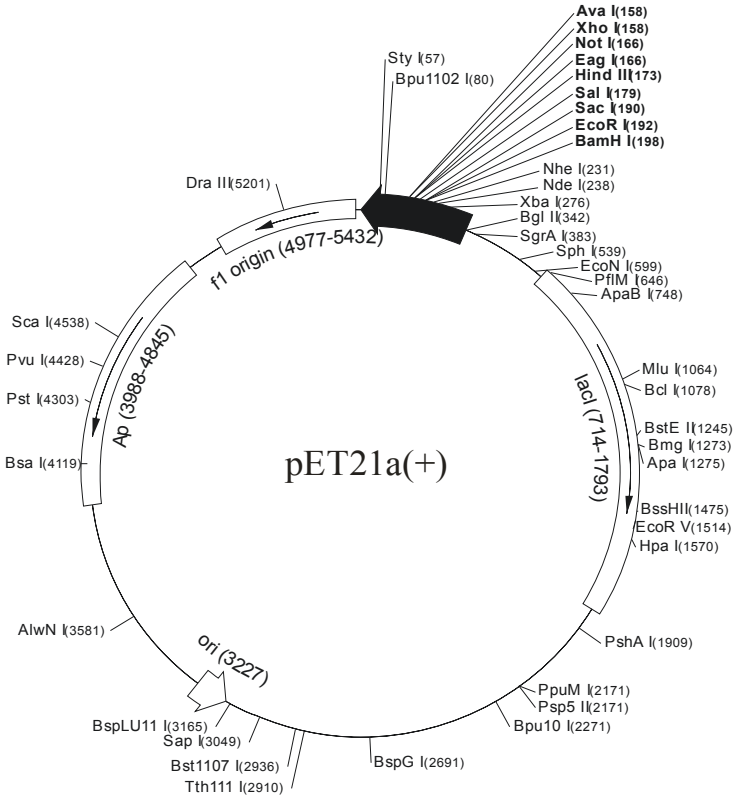
1. pCMV-Sport6 (Deutsches Ressourcenzentrum für Genomforschung,)



2. pGEX-4T-2 vector (Amersham, Freiburg)



3. pET21a(+) (Merck, Bad Soden)



4. METHODS

4.1. Cell culture and tissue preparation

4.1.1. NIH 3T3 cell culture

The cell line was established from disaggregated Swiss albino mouse embryos in 1962. Morphologically, fibroblasts grow adherently as monolayer with contact inhibition. NIH 3T3 mouse fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 2 mM glutamine supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin/streptomycin, 2.7% ultrasaline A and grown in an incubator under 5% CO_2 atmosphere at 37°C. Cells were allowed to grow until 80-90% confluency, washed twice with PBS and then split at a ratio of up to 1:8 every 2 to 4 days by means of detachment using 1 ml Trypsin/EDTA (0.5 g/L Trypsin, 0.2 g/L EDTA) per 75 cm^2 culture flask. Incubation time was 2-3 minutes at 37°C. Trypsin was then inhibited by addition of 7 ml 10% FCS containing DMEM medium, and cells were collected by centrifugation (30 x g for 10 minutes at RT). The resulting cell pellet was resuspended in medium and seeded in new culture flasks.

4.1.2. Isolation of human blood monocytes

Human mononuclear cells were isolated from buffy coats (kindly provided by the Department of Clinical Immunology and Transfusion Medicine, JLU Giessen) by density gradient centrifugation using Ficoll-Paque Plus solution. Buffy coats were diluted 1:1 with Ca^{2+}/Mg^{2+} free PBS, overlaid on the Ficoll solution (15 ml Ficoll solution per 30 ml diluted blood) and centrifuged at 250 x g for 30 min at RT. Isolated leukocytes were washed twice with PBS by mixing one volume of leukocytes with two volumes of PBS and centrifuged at 250 x g for 10 minutes. Washed leukocytes were resuspended in 30 ml RPMI 1640 medium containing 10% FCS, 1% penicillin/streptomycin, 1% L-Glutamine, 1% MEM non essential amino acids and cultured overnight on four 78.5 cm^2 culture dishes at 37°C. Non-adhered cells were discarded and monocytes/macrophages attached to the dish were gently washed twice with 10 ml warm (37°C) PBS, detached with a

rubber policeman and collected in RPMI 1640 medium containing 0.5% endotoxin-free BSA and counted using a hemocytometer. The purity of isolated cells was greater than 90%.

4.1.3. Preparation of testis homogenate

Adult male Wistar rats weigthing 200-250 g and wild-type ($MIF^{+/+}$) and MIF knockout ($MIF^{-/-}$) mice were used for preparation of total testes homogenate. While animals were under deep halothane anesthesia their testes were removed. After removal of the capsules the testes were homogenized in ice-cold buffer (10 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM EDTA and 1 mM leupeptin) using a Potter homogenizator. Cell debris was pellet by centrifugation at 1,000 x g for 10 min, and the resulting supernatant was used as total testis homogenate. Protein concentration was 20 mg/ml as determined by Bradford assay (Bradford 1976).

4.1.4. Isolation of sperm cells from epididymis

For isolation of sperm cells, adult male Wistar rats were anesthetized and killed by CO_2 asphyxiation. Testes and epididymes were removed and the caput region was separated from the rest of the epididymis. The caput segments were cut in small pieces and the epididymal fluid was obtained by rinsing the respective segment in ice-cold buffer (10 mM Tris-HCl pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM Pefabloc SC inhibitor). All successive steps were carried out at 4°C. Caput epididymal sperm cells were separated by centrifugation at 600 x g for 10 min and counted.

4.2. Gel electrophoresis

4.2.1. Agarose gel electrophoresis

Agarose gels (2% to 0.8%) were routinely used to separate DNA fragments ranging in size from 100 bp to 5 kb. The appropriate amount of agarose was dissolved in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA) by heating in a microwave oven. After cooling the gel solution was poured into a gel mold, a comb was inserted in order to

generate wells for the samples. After 30-40 min the comb was removed, and the gel mounted into an electrophoresis chamber filled with 1x TAE buffer. DNA samples and size marker were mixed with an appropriate volume of DNA sample buffer (3% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol FF) and pipetted into the wells. The gels were run at 100V (2-10V/cm gel) until the bromophenol blue and xylene cyanol dyes had migrated considerable distance through the gel. After electrophoresis, the gel was immersed in 1x SYBR green staining solution (1:10,000 in 1x TAE buffer) and incubated for 30 min at RT with gentle shaking. Occasionally, SYBR green staining solution was added directly to the sample (prestaining). The gel was then examined on a 305 nm UV transilluminator and photographed using a gel documentation system.

2.2.2. SDS polyacrylamide gel electrophoresis

Discontinuous sodium-dodecyl-sulphate (SDS) polyacrylamide gel electrophoresis (Laemmli 1970) was performed in order to analyze protein expression in cell lysates or tissue samples. An 18% resolving gel solution (375 mM Tris-HCl pH 8.8, 0.1% SDS, 18% acrylamide, 0.05% APS, 0.05% TEMED) was poured into the assembled gel mold between two glass plates separated by 1 mm thick spacers leaving about 1 cm space for the stacking gel solution (125 mM Tris-HCl pH 6.8, 0.1% SDS, 4% acrylamide, 0.05% APS, 0.1% TEMED). Samples were prepared in 1 x Laemmli SDS gel-loading buffer (62.5 mM Tris pH 6.8, 2% SDS, 5% glycerol, 0.3% bromophenol blue, 0.9% (v/v) β-mercaptoethanol) and boiled for 3 min to denature the proteins. After polymerization of the stacking gel, the comb was removed and the gel mounted in the electrophoresis chamber. Both electrode reservoirs were filled with 1x SDS electrophoresis buffer (25 mM Tris, 1.44% glycine, 0.1% SDS), the wells were cleaned and samples loaded. Electrophoresis was performed at 150 V constant. For the immunoprecipitation samples NuPAGE 4-12% precast gradient gels were used, which were run in 1x MES buffer (50 mM MES, 50 mM Tris, 3.46 mM SDS, 1.025 mM EDTA) at constant 200 V for 35 min. After electrophoresis gels were incubated in fixing solution (7% glacial acetic acid in 40% (v/v) methanol) for 1 h. Staining solution was prepared by mixing 4 parts of 1 x Brilliant Blue G-Colloidal with 1 part methanol, and the gel was incubated for 1h with gentle shaking. The gel was then rinsed for 60 sec with destaining solution I (10% acetic

acid in 25% (v/v) methanol) to reduce the background staining, followed by destaining solution II (25% methanol) until a sufficient destaining level was reached. For documentation purposes the gel was scanned and dried between cellophane on air.

4.2.3. Western blotting

Proteins were separated on a 15% or 18% SDS-PAGE gel and electro-transferred to a nitrocellulose membrane at 100 mA per gel/membrane for 90 min using a semi-dry blot system. After blotting, the membrane was incubated in blocking buffer (5% (w/v) non-fat dry milk in PBS containing 0.1% Tween-20) for 1 hour at RT. Subsequently, the membrane was incubated overnight at RT or 4°C with the first antibody diluted in blocking buffer or as stated in the text. After washing (3 x 10 min) with PBS-Tween, the membrane was incubated for 1 hour at RT with a secondary antibody diluted in blocking buffer. Three washing steps (10 min each) with PBS-Tween were performed before the membrane was incubated with ECL Detection Reagent (1:1 mixture (v/v) of Reagent 1 and Reagent 2) for 60 sec. The membrane was wrapped in plastic foil, exposed to X-ray film for 1-15 min, which was subsequently developed.

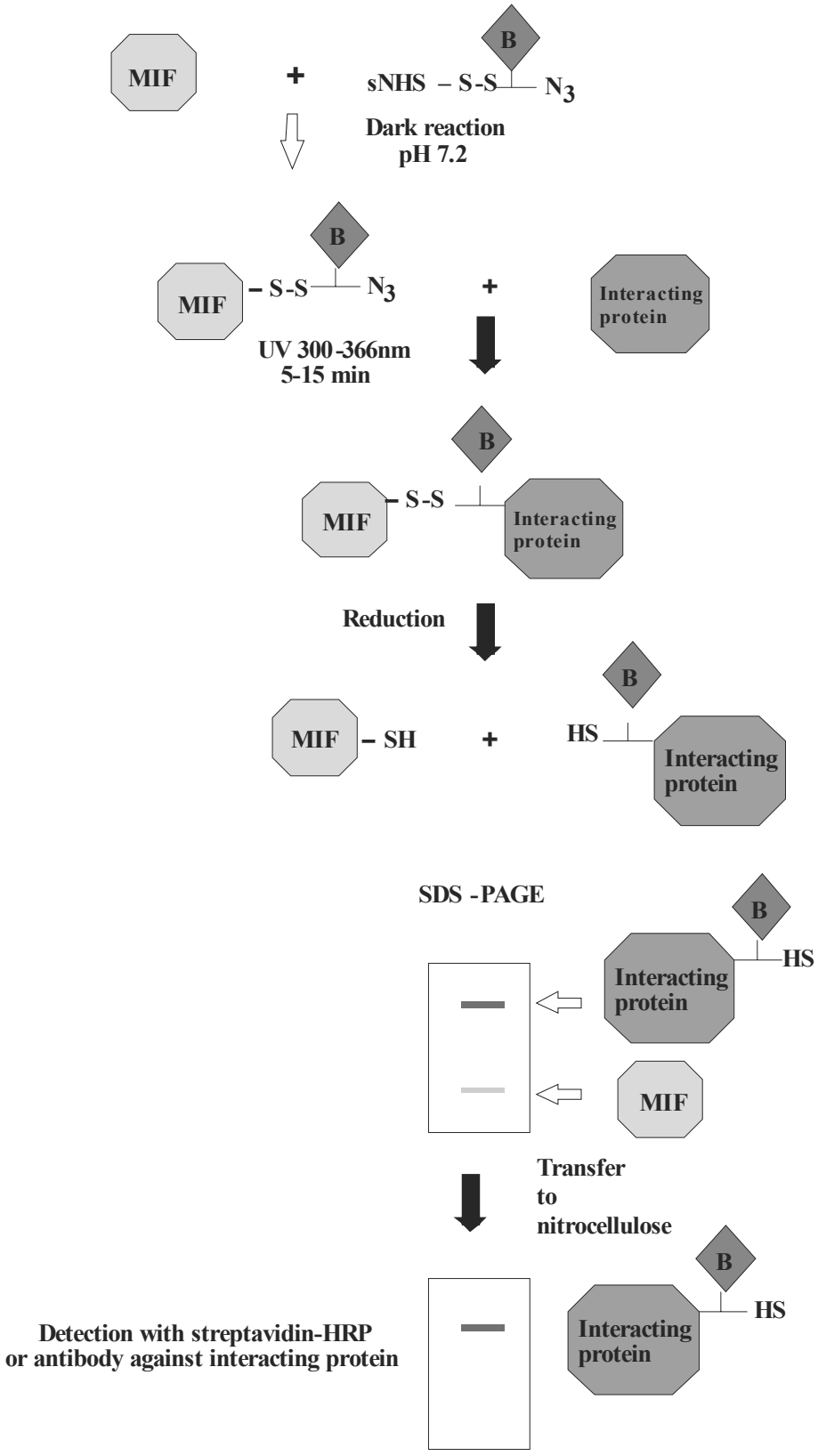
4.3. Far-Western blotting

Total rat testis homogenate (rat TH), mouse TH from normal and homozygous MIF knockout mice, cell lysates from PC12 cell line (rat adrenal medulla), mouse NIH 3T3 fibroblasts, isolated rat Sertoli and peritubular cells and rat epididymal caput sperm cells were separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane using the semi-dry technique (see *4.2.3.*). Membranes were incubated with 1 µM rat recombinant MIF in lectin buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$) or buffer alone at RT overnight. Subsequently, membranes were washed with Tris-buffer (50 m M Tris-HCl pH 7.5, 250 mM NaCl, 3 mM EDTA, 0.05% Tween) and detection of bound MIF was performed using a polyclonal rabbit anti-rat MIF antibody at 1:20,000 dilution in blocking buffer, followed by a goat anti-rabbit peroxidase conjugated secondary antibody at 1:10,000 dilution in blocking buffer and visualized by enhanced chemiluminescence (ECL).

4.4. Cross-linking

A chemical cross-linking method was employed to study protein-protein interactions. For this assay ProFound Sulfo-SBED Biotin Label Transfer Kit was used according to the manufacturer's instructions. The trifunctional cross-linker Sulfo-SBED contains an amino group, a photo-reactive site, a thiol-cleavable disulfide (S-S) linkage and a biotin handle. The biotin tag is transferred from a labeled purified bait protein to a captured prey protein which can be then detected by Western blotting.

Recombinant rat MIF was used as bait protein and derivatized with Sulfo-SBED via its amino groups. 200 µg recombinant rat MIF were incubated with 5-fold molar excess of Sulfo-SBED in 500 µl of buffer 1 (0.1 M PBS, pH 7.2) for 30 min at RT in the dark. After centrifugation at 13,000 x g for 1 min, the sample was applied to a Nap-5 column and elution was performed with 1 ml of buffer 2 (0.1 M PBS, 10 mM Tris pH 7.2, 0.15 M NaCl). Fractions of 100 µl were collected. Labeled Sulfo-MIF (S-MIF) was stored at -20°C until use. $2x10^5$ NIH 3T3 cells per well were seeded in 6 well plate and grown until 80% confluency. The following steps were performed at 4°C. Cells were washed twice with PBS and 66 µl buffer 2 was added per well and cells were collected with a rubber policeman. Cells were lysed by three freeze-thaw cycles in liquid nitrogen, sonicated and centrifuged for 10 min at 10,000 x g to remove cell debris. The cell lysate was applied to 100 µl of a 50% slurry of streptavidin beads for one hour, to deplete it of endogenously biotinylated proteins. 4.79 µg S-MIF bait protein (3.25 µM) was incubated for 15 min in the dark with 22 µl of the precleared cell lysate in a final volume of 123 µl and then exposed to UV light (365 nm) for 15 min, which activates the phenyl-azide moiety of Sulfo-SBED and transfers the biotin tag to the bound prey protein (Scheme 1). The biotinylated proteins were purified via streptavidin beads and released from the S-MIF via cleavage of the disulfide linker by mixing and boiling the sample with Laemmli sample buffer containing β-mercaptoethanol as reducing agent. The biotinylated prey proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane and detected with anti-biotin antibody. For the competition experiment, a 10-fold molar excess of recombinant rat MIF over S-MIF concentration was used.



Scheme 1. Strategy for cross-linking with Sulfo-SBED Biotin Label Transfer

4.5. Immunoprecipitation

Mouse NIH 3T3 fibroblasts were grown in a 75 cm^2 culture flask to 80% confluency, washed twice with ice-cold PBS and incubated on ice with 1 ml of lysis buffer (50 mM Tris-Cl pH 8.0, 150 mM NaCl, 1% IGPAL-630, 1 µM leupeptin, 1 mM PMSF) for 10-15 min with occasional rocking. Cells were scraped out and transferred to an Eppendorf tube, disrupted by passage through a 21 Gauge needle, subjected to sonication (two 10 sec bursts at 200-300 W with a 10 sec cooling period in between) followed by centrifugation at 10,000 x g for 10 min at 4°C. The supernatant was precleared for 1 h by incubation with 30 µl Protein G-Sepharose 4B Fast Flow beads at 4°C on a rotating wheel before incubation with either rabbit anti-rat MIF antibody or rabbit preimmune serum immobilized on 30 µl Protein G-Sepharose beads at 4°C followed for 2h. After extensive washing with lysis buffer (5 x 10 min), immune complexes were collected by centrifugation, resuspended in 20 µl Laemmli sample buffer and boiled for 10 min at 95°C. Immunoprecipitates were separated on a NuPAGE 4-12% Novex Bis-Tris gel and stained with colloidal Coomassie staining solution.

4.6. Cloning, expression and purification of recombinant tagged RP S19

4.6.1. Preparation of competent E. coli and transformation

For the preparation of competent *E. coli* an inoculating loop was used to streak E. coli DH5α directly from a frozen glycerol stock onto an LB agar plate containing no antibiotics. The plate was incubated for 16 hours at 37°C. A single colony was picked and grown in 5 ml SOB medium overnight by shaking (235 rpm/min) at 37°C. 50 ml prewarmed SOB medium (2% (w/v) bactotryptone or peptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl) was inoculated with 0.5 ml from the overnight culture. The cells were grown for 2.5-3.0 hours at 37°C under monitoring culture growth by measuring OD_{600} in a spectrophotometer every 20 min. When the culture had reached an OD of 0.45-0.50, the cells was incubated on ice for 20 min. Cells were harvested by centrifugation at 1075 x g for 15 min at 4°C and the supernatant was decanted. The cells were gently resuspended in 100 ml TFB buffer (10 mM MES, 45 mM $MnCl_2$, 10 mM

$CaCl_2$, 100 mM KCl, pH 6.2) and incubated on ice for 10-15 min. After centrifugation at 1075 x g for 15 min at 4°C, the buffer was decanted. The cells were resuspended gently in 3.9 ml TFB buffer, 140 µl DMSO followed by 5 min incubation on ice. Then 140 µl of 1M DTT was added and incubation continued for 10 min before another 140 µl DMSO were added for 5 min. For transformation, 200 µl of competent cells were transferred to an Eppendorf tube and kept on ice. 3 to 7 µl of ligation reaction mixture containing 25 ng of plasmid was added to the competent cells and incubated on ice for 40 min. The tubes were transferred to a heat block preheated to 42°C for exactly 45 sec and then cooled on ice again. After 2 min of cooling 800 µl of warm (37°C) SOC medium (SOB medium containing 5 mM glucose) was added to each tube. Incubation for 60 min in a shaking incubator allowed the bacteria to recover and to establish antibiotic resistance. 200 or 50 µl of transformed competent cells were plated onto 90 mm LB agar plates containing the appropriate antibiotic (usually 50 µg/ml ampicillin). The plates were stored at RT until the liquid had been absorbed. The plates were inverted and incubated at 37°C overnight. Colonies were analyzed by PCR and mini cultures were prepared in parallel. One colony was picked and resuspended in 50 µl distilled water. 25 µl was used for inoculation of a 5 ml mini culture (SOB medium containing ampicillin) and the other 25 µl were boiled for 5-10 min at 95°C. Finally ten µl was used as a template for the PCR reaction.

4.6.2. Cloning of the expression constructs

Full Length RP S19 cDNA (Mus musculus) clone, IRAKp961E1430Q was obtained from the RZPD (Deutsches Ressourcenzentrum für Genomforschung, www.rzpd.de). Plasmid isolation was performed and the integrity of the insert was validated by DNA sequencing.

The RP S19 cDNA was amplified from this clone by PCR with Pfu polymerase using forward primer 5'-CGAGGAATTCCCATGCCCGGAGTTACTG-3' and reverse primer 5'-CGCCTCGAGTAATGCTTCTTGTTGGC-3' for the glutathione-S-transferase (GST) tag vector (introduced restriction sites are underlined: EcoRI for forward primer and XhoI for reverse primer). The cycle conditions for a standard PCR with Pfu polymerase were: 3 min initial denaturation at 96°C, 31 cycles of denaturation for 45 sec at 96°C, annealing for 40 sec at 61°C, elongation for 40 sec at 73°C and a final extension for 10 min at 72°C.

PCR product and pGEX-4T-2 vector were digested with EcoRI and XhoI and the restricted fragments were recovered from an agarose gel using a QIAEX II DNA extraction kit according to the instruction of the manufacturer. The concentrations of PCR fragment and vector were estimated by agarose gel electrophoresis with low and high DNA mass ladder and 50 ng of insert was used in a standard ligation reaction with 100 ng of linearized pGEX-4T-2 vector. Competent cells were transformed with the ligation reaction and colony PCR was performed to screen for positive clones. One clone was validated by DNA sequencing (Seqlab, Göttingen).

For cloning of the expression clone of His-tagged RP S19, the RP S19 cDNA was amplified again from the IRAKp961E1430Q plasmid by PCR with Pfu polymerase using forward primer 5′-CGCCATATGCCCGGAGTTACTGTAAAA-3′ and reverse primer 5′-GCGAAGCTTATGCTTCTTGTTGGCAGC-3′. Due to an internal NdeI site within the RP S19 cDNA, pET21a(+) vector for expression of His-tagged RP S19 was restricted with NdeI, blunted, restricted with HindIII and ligated to the HindIII restricted PCR fragment. Integrity of both inserts plus flanking regions were validated by DNA sequencing.

4.6.3. Expression and purification of GST-RP S19

RP S19 was expressed as a fusion protein with a GST tag at the amino terminus followed by a thrombin cleavage site. GST occurs naturally as a 26 kD protein, but parental pGEX vectors produce a 29 kD GST fusion protein, thus the apparent molecular weight of the fusion GST-RP S19 protein is 45 kD.

For expression of GST-RP S19 tagged protein, *E. coli* BL21 DE3 competent cells were transformed with the pGEX-RP S19 construct. Positive transformants were inoculated in to 5 ml 2YT medium (1.6% tryptone, 1% yeast extract and 1% NaCl, pH 7.0) containing ampicillin and cultured overnight in a shaker at 37°C. For comparison, bacteria transformed with the parental pGEX plasmid were also prepared.

To optimize expression conditions 50 ml of 2YT medium containing 50 µg/ml ampicillin was inoculated with 500 µl of overnight culture. Cultures were kept at 37°C in a shaking incubator until OD_{600} = 0.5. The culture was split in two equal parts and 1 ml aliquot from each culture was saved and prepared for SDS-PAGE (as described later).

One culture was induced by adding IPTG to a final concentration of 0.5 mM and incubation was continued at 37° with shaking. At different time points of induction (1, 2 and 3 hours), 1 ml from each culture were transferred to a microfuge tube, the OD_{600} was measured and each pellet was prepared for SDS-PAGE. The samples were mixed with Laemmli sample buffer, boiled at 95°C for 3 min, stored on ice and then loaded onto 15% SDS-polyacrylamide gel. The gel was stained with Coomassie.

For large scale expression 5 ml of an overnight culture was inoculated into 500 ml 2YT medium (supplemented with 100 µg/ml ampicillin) in a 2 l Erlenmeyer flask and incubated at 37°C until an OD_{600} of 0.5 was reached. Expression was induced by adding IPTG to a final concentration of 0.5 mM and incubation was continued at 37°C for 3 h. Cells were harvested by centrifugation at 3,000 x g at 4°C for 30 min. The supernatant was discarded and the cell pellet was resuspended in ice-cold PBS (50 µl PBS for each ml of culture). The cells were lysed by sonication (10 short burst of 10 sec followed by intervals of 30 sec for cooling) and a small aliquot was saved after this step. Cell lysates were treated with Triton X-100 to a final concentration of 1% and gently mixed for 30 min to solubilize the fusion protein. Centrifugation at 1200 x g for 10 min at 4°C removed the cell debris and the supernatant was transferred to a new tube. An aliquot of supernatant and pellet was saved for analysis by SDS-PAGE to identify the fraction that contains the fusion protein. The supernatant containing GST-RP S19 was subjected to Glutathione Sepharose 4B chromatography. After washing twice with PBS, GST-tagged RP S19 was eluted with 50 mM Tris-HCl pH 8.0 containing 10 mM glutathione. The purity of the eluted protein was 90% as estimated by SDS-PAGE.

4.6.4. Purification of RP S19-His

RP S19-His was expressed in *E. coli* BL21(DE3) by induction with IPTG (0.5 mM) at 37°C for 3h. Bacteria expressing RP S19-His were resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0), treated with 1mg/ml lysozyme for 30 min on ice, and sonicated (six 10 sec bursts at 200-300W with a 10 sec cooling period between bursts). Lysed cells were centrifuged at 10,000 x g for 30 min at 4°C and the supernatant was incubated with 50% Ni-NTA slurry (1 ml bed volume for 10 ml lysate) at 4°C for 60 min. The matrix was then washed twice with 50 mM NaH_2PO_4

pH 8.0, 300 mM NaCl, 20 mM imidazole, and bound protein was eluted with 50 mM Na_2HPO4, 300 mM NaCl, 250 mM imidazole, pH 8.0 and dialyzed against PBS (pH 7.8) containing 0.5 mM PMSF and 1 mM DTT. The purity of the eluted protein was 95% as estimated by SDS-PAGE.

4.7. Production of polyclonal RP S19 antibody

Prior to immunization about 10 ml of preimmune serum was collected from the selected animals. Four New Zealand White rabbits (approx. 3 kg) were subcutaneously injected with His-tagged RP S19 protein (0.5 mg/animal) diluted in PBS and mixed with the same volume of complete Freund's adjuvant. Animals were injected under the skin of the back at four different locations. After four weeks, the first boost was performed with RP S19 (0.25 mg protein/animal) in incomplete Freund's adjuvant. A second boost was performed with the same amount of protein six weeks after starting the immunization protocol. Ten days after the second boost a blood sample was drawn and the clotted blood was stored at 4°C overnight. Serum was separated from the clot by centrifugation at 10,000 x g for 30 min at 4°C and tested in a Western blot with recombinant RP S19 and a protein extract from NIH 3T3 cells. Two rabbits with immunoglobulins against His-tagged RP S19 were sacrificed, whole blood collected and serum was prepared. Sodium azide was added to the serum sample to a final concentration of 0.02 % (w/v), and aliquots were stored at -20°C until use.

Serum samples were affinity-purified using His-tagged RP S19 immobilized on Ni-NTA according to a published protocol (Jun Gu 1994) with slight modifications. A 1 cm high Ni-NTA column (approx. 2 ml bed volume) was washed with equilibration buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl). One ml of crude antiserum was incubated with the matrix for 1 h at 4°C before the column was washed with 5 column volumes of equilibration buffer, followed by 5 column volumes of wash buffer (50 mM Tris-HCl pH 7.4, 2 M NaCl). To elute the antibody, the column was first incubated with 1 column volume of 4 M $MgCl_2$ for 15 minutes, followed by 1 ml of 4 M $MgCl_2$ solution. The column was allowed to flow and the eluate was collected. The purified anti-RP S19 immunoglobulins were dialyzed against distilled water for 1h and against PBS overnight both at 4°C. After being assesed in a Western blot with recombinant RP S19 protein and

endogenous RP S19 from NIH 3T3 cell lysate, the purified rabbit anti-RP S19 antibody was stored in aliquots at -20°C.

4.8. Biotinylation of wild type rat MIF protein

Recombinant rat MIF was expressed and purified as described previously (Kim 2003). Biotinylation of rat MIF was performed using the ECL protein biotinylation module according to the recommendations of the manufacturer. Nonreacted succinimide ester was separated from biotinylated MIF using Sephadex G-25 columns. The column was equilibrated with 5 ml PBS pH 7.5 containing 1% BSA followed by 20 ml PBS. Two ml solution containing rat MIF (200µg), 8 µl biotinylation reagent and bicarbonate buffer was incubated for 15 minutes and then applied to the column, followed by elution with 5 ml PBS. Ten fractions of 500 µl were collected and the protein concentration was estimated by Bradford assay (Bradford 1976). Biotinylated MIF protein was stored at 4°C prior use.

4.9. *In vitro* pull-down assays

4.9.1. GST-RP S19 pull-down

Biotin-MIF (2.5 µg) was immobilized with 30 µl (50% slurry) of avidin beads by incubation in 500 µl PBS at RT on a rotating wheel for 30 min. In order to completely remove free Biotin-MIF, beads were washed 3x with 1 ml PBS and 3x with 1 ml lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% IGPAL-630). Coated beads were incubated in 500 µl of lysis buffer with increasing amounts of GST-RP S19 (50, 100 and 200 ng) on a rotating wheel at 4°C for 1 h. As a control, uncoated avidin beads were incubated with the same amounts of GST-RP S19 alone. Beads were then washed five times with lysis buffer and finally boiled in Laemmli sample buffer for 5 min. Protein complexes were separated by SDS-PAGE, transferred onto nitrocellulose membrane and detected with anti-GST antibody conjugated with peroxidase.

4.9.2. MIF pull-down

Likewise, RP S19-His (2 µg) was immobilized on Ni-NTA agarose beads, washed with PBS to remove the unbound protein and incubated for 1 h with different amounts of recombinant rat MIF or human MIF or P2A, C60S and Δ4 human MIF mutants. As control, wt and mutants of MIF were incubated with Ni-NTA agarose beads. After extensive washing with lysis buffer as described previously or RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF) in case of human wt MIF and MIF mutants, proteins bound to the beads were boiled in Laemmli sample buffer, resolved on SDS-PAGE and stained with colloidal Coomassie or blotted and probed for RP S19, stripped and reprobed for MIF in the case of the mutants.

4.10. Double immunofluorescence

Immunofluorescence staining was performed on NIH 3T3 mouse fibroblasts to investigate the cellular localization of endogenous MIF and RP S19. Cells were cultured on cover slips until 80% confluency. After washing once with 1x PBS, cells were fixed with ice-cold methanol for 10 min at RT. Blocking was performed with 5% BSA and 10% NHS for 1 h at RT. Double immunostaining with rabbit anti-mouse RP S19 (1:400), followed by donkey anti-rabbit IgG conjugated with Cy3 (1:1,000) and mouse anti-MIF (1:200) followed by donkey anti-mouse IgG conjugated with FITC (1:1,000) was performed. Both primary antibodies were incubated over night at 4°C and both secondary antibodies were incubated for 1 h at RT. Three wash steps were performed using 1x PBS and DAPI was used for nuclear staining. Images were acquired with a confocal laser scanning microscope.

4.11. Dopachrome tautomerase assay

The substrate L-dopachrome methyl ester (DCME) was freshly prepared before each measurement (exactly 5 min before measurement) by mixing equal volumes of 8 mM sodium periodate and 4 mM L-dopa methyl ester and incubating for 5 min at RT .

Enzymatic activity was determined for an assay volume of 800 µl per reaction obtained by mixing 400 µl of PBS containing recombinant rat MIF at a concentration of 1 µM with 400 µl of freshly prepared DCME substrate. In reactions that contained MIF and RP S19 both proteins were preincubated in 400 µl PBS for 1 h before measurement. As control SCGB 2A1-His, a protein of similar size fused to the same tag was used. Tautomerase activity was measured by monitoring the decrease in absorbance at 474 nm with a spectrophotometer over a time period of 1 min after the reaction start. SWIFT II software was used for recording the reaction kinetics.

4.12. Monocyte chemotaxis assay

The chemotaxis assay was performed with human blood monocytes/macrophages (see chapter 4.1.2.) and the Transwell filter system (5.0 µm pore size polycarbonate membrane). For each assay $5x10^4$ cells in 200 µl medium (containing 0.5% BSA) were plated onto one Transwell insert in a 24 well culture plate. In the outer wells was added 500 µl per well medium containing either (1) 0.5% BSA as negative control, (2) 25 ng/ml MCP-1 as positive control, (3) 50 ng/ml human or rat recombinant MIF, (4) 5 fold molar excess of RP S19 over MIF, (5) a combination of MIF and RP S19. After 3 h of incubation, the Transwell inserts were removed and cells that had migrated through the membrane and settled on the bottom of the culture well were fixed by adding paraformaldehyde (final concentration 1.8% w/v). Nuclei were stained with Hoechst 33342. For each membrane (i.e. well), eleven high-power microscopic fields were independently counted by two persons in a blinded fashion. Counting was normalized for individual counting bias. As counting control 2,500 and 5,000 cells were plated directly in the outer well (without membrane). The average number of cells quantified on 11 fields of the 2,500 and 5,000 cell counting control wells was set as 5% and 10% migrated cells, respectively.

4.13. Glucocorticoid overriding assay

Human mononuclear cells were isolated from whole blood by Ficoll density gradient centrifugation and purified by adherence (as described in chapter 4.1.2.). For the

assay, $1x10^6$ cells/ml/well in 12-well plates were preincubated for 1 h with (1) dexamethasone (10^{-9} M), (2) dexamethasone (10^{-9} M) plus recombinant human MIF alone or (3) dexamethasone (10^{-9} M) plus recombinant human MIF plus RP S19 before the addition of 0.1 µg/ml LPS. Cell culture supernatants were collected after 16 h of stimulation, centrifuged and secreted TNF-α was quantified by ELISA according to the instructions of the manufacturer. A 96 well plate was coated with purified anti-human TNF-α antibody diluted 1:250 in 1x assay diluent over night at 4°C and then blocked for 1 h at RT. 100 µl per well of standard was added by employing a 2-fold dilution series of recombinant human TNF-α (500 pg/ml was the highest standard concentration and 3.9 pg/ml the lowest) and the same volume of samples was added to the antibody coated plate and incubated for 2 h at RT. After washing with 1x PBS containing 0.05% Tween-20, 100µl of detection antibody (biotin-conjugated anti-human TNF-α) diluted 1:250 in 1x assay diluent was added to the wells and incubated for 1 h at RT. The wells were then washed with PBS-Tween and 100 µl of Avidin-HRP conjugate diluted in 1x assay diluent was added to the wells for 30 minutes at RT. The wells were washed again and 100µl/well of substrate solution was added and incubated for 15 minutes at RT followed by addition of 50µl/well of stop solution (2N H_2SO_4). The color reaction was recorded at 450 nm and 570 nm using an ELISA plate reader. The values obtained by reading the plate at 570 nm were subtracted from those obtained at 450 nm and data was analyzed.

5. RESULTS

5.1. Identification of MIF interacting proteins

In order to identify proteins that specifically interact with MIF three strategies were employed. Initially, a far-Western experiment was performed to gain information about the spectrum of MIF interacting proteins in different cell lines and tissues. Subsequently, co-immunoprecipitation experiments and MALDI-TOF mass spectrometry was chosen to identify MIF interacting proteins. Finally, an adapted cross-linking method, based on biotin transfer to binding protein, was used to enrich MIF interacting proteins of low abundance.

5.1.1. MIF cross-reactivity

Initially, a far-Western experiment was performed to detect MIF interacting proteins in different tissue and cell types and guide in the choice of the best source for the future experiments. For this purpose, protein extracts were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then incubated overnight with 1 μM of recombinant rat MIF. As a negative control, a second membrane was incubated with buffer alone. As illustrated in Fig. 1A, detection of bound MIF with an anti-MIF antibody resulted in a spectrum of potential MIF interacting proteins between 20 and 90 kD beside the endogenous MIF which was observed at a MW of 12.5 kD. The pattern of cross-reactivity was similar for almost all types of cells investigated, whereas the cross-reactivity of the control membrane was restricted to the endogenous MIF only (Fig. 5B).

Therefore, for convenience and comparison with studies of other groups (Kleemann et al. 2002; Nguyen et al. 2003b) the following co-immunoprecipitation experiments were performed with NIH-3T3 fibroblast cells.

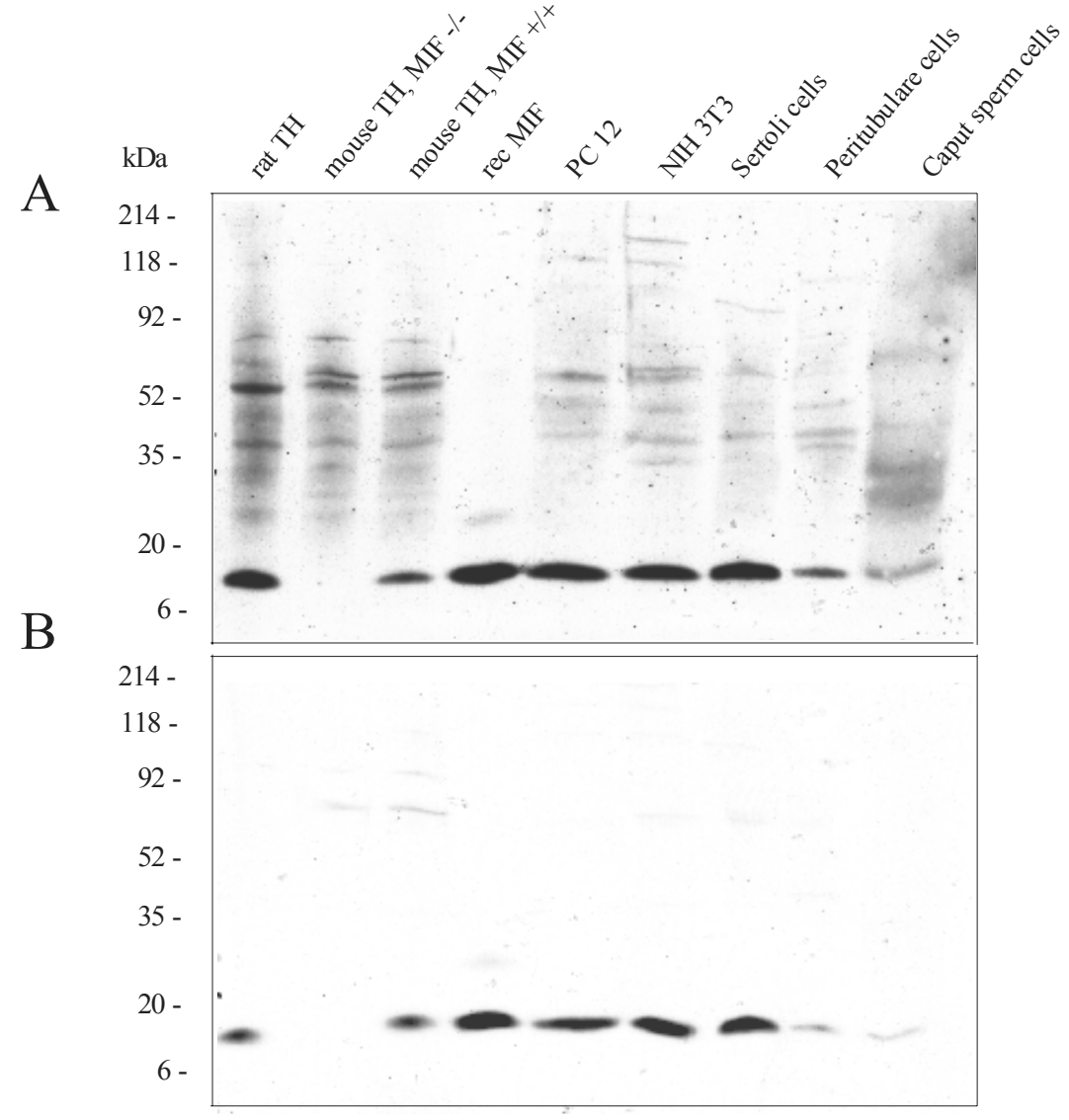


Fig. 5: Cross-reactivity of MIF. Far-Western: protein extracts from rat total testis homogenate (rat TH), mouse total testis homogenate from MIF+/+- and MIF-/- mice, cell lysates from PC12 cells (rat pheochromocytoma cell line), mouse NIH 3T3 fibroblasts (NIH 3T3), Sertoli cells, peritubular cells and rat epididymal caput sperm cells were separated by SDS-PAGE and blots were incubated with 1µM recombinant MIF (A) or with buffer only as a control (B) prior to detection of MIF protein. The molecular weight marker is indicated: [kD].

5.1.2. Co-immunoprecipitation of MIF interacting proteins from NIH 3T3 cells

In order to validate if the anti-MIF antibody can be used for co-immunoprecipitation (Co-IP), an trailer experiment was performed. c-Jun activation domain binding protein-1 (Jab-1), being a known interacting protein for MIF was choosen as a positive control to validate the efficiency of the method. Extracts from mouse NIH 3T3 fibroblast cells were subjected to Co-IP with a polyclonal rabbit anti-rat MIF antibody or with a rabbit IgG polyclonal isotype control antibody which were previously immobilized on protein G-Sepharose beads. The immune complexes were probed for Jab-1, stripped and reprobed for MIF by Western blot analysis. As shown in Fig. 6, anti-MIF antibody efficiently precipitated MIF and Jab-1 from NIH 3T3 cell lysate, demonstrating that the selected antibody is suitable for co-immunoprecipitation experiments.

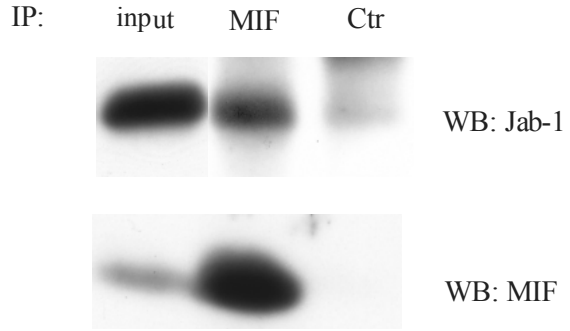


Fig. 6: Co-immunoprecipitation of MIF and Jab-1. Western blot: Co-IP samples obtained with anti-MIF antibody (IP:MIF) and isotype control antibody (IP:Ctr) were analysed by Western blotting with anti-Jab-1 (WM: Jab-1) and anti-MIF (WB: MIF) antibodies. For analysis, 10% of the total NIH 3T3 cell lysate used for immunoprecipitation was loaded.

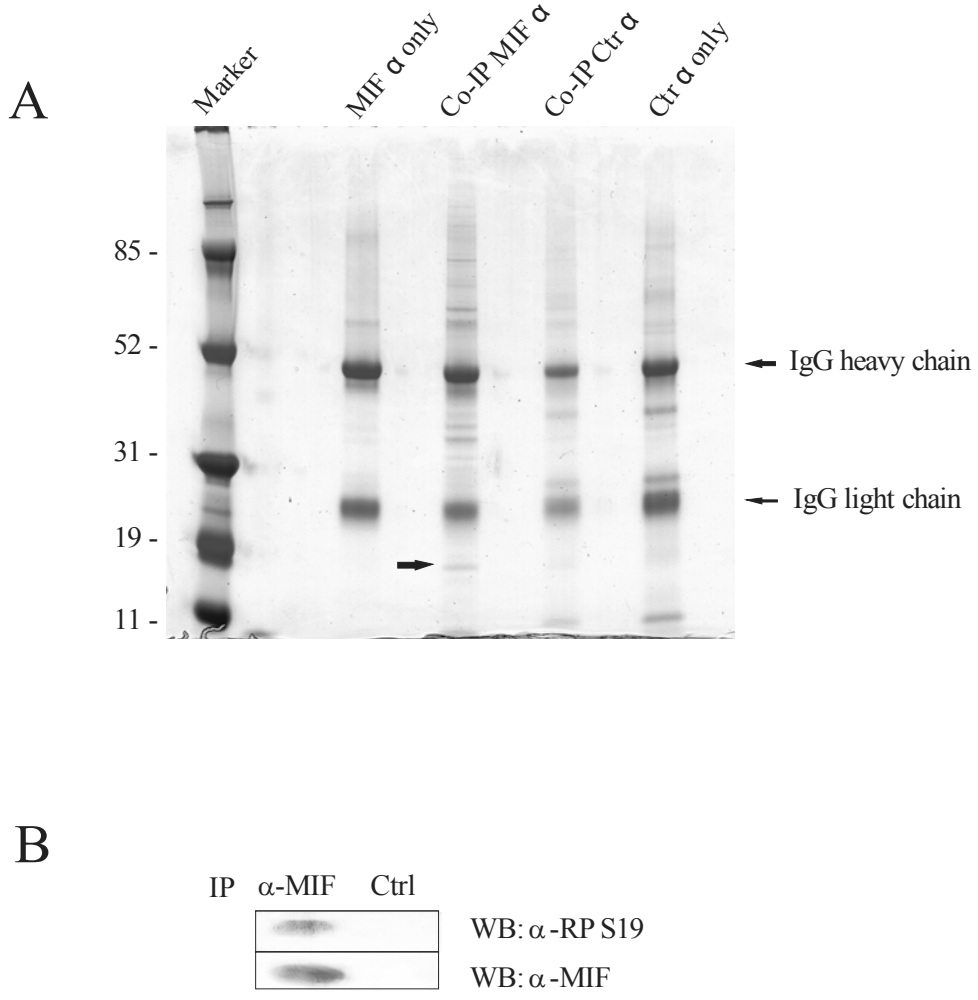


Fig. 7: Co-immunoprecipitation of MIF and RP S19. (A) Extracts from mouse NIH 3T3 fibroblasts were incubated with a polyclonal rabbit anti-rat MIF antibody (Co-IP MIF ab) or with a rabbit IgG polyclonal isotype control antibody (Co-IP Ctrl ab) which were previously immobilized on protein G-Sepharose beads. The immune complexes and the antibody coated beads alone were separated by SDS-PAGE gradient gel (4-12%) and stained with Coomassie blue. The protein band at approximately 16 kD (marked by arrow) was excised from the gel and analysed by tryptic digestion and MALDI-TOF mass spectrometry. **(B)** Western blot analysis of the Co-Ip samples (IP anti MIF and IP ctrl). The blot was probed with anti RP S19 antibody (upper panel), stripped after detection and re-probed with the anti-MIF antibody (lower panel).

The co-immunoprecipitation experiment was repeated with the same cell lysate (NIH 3T3), the immune complexes were again separated by SDS PAGE and the gel was stained with colloidal Coomassie blue solution (Fig. 7A). Beside the immunoglobulin light and heavy chains, several specific bands appeared in the anti-MIF IP sample compared to the control IP. One of this specific bands at around 16 kD was excised from the gel and analyzed by tryptic-in-gel digestion followed by MALDI-TOF mass spectrometry and subsequently identified as ribosomal protein S19 (RP S19). To confirm the result, the IP samples were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti RP S19 antibody, stripped and re-probed with anti MIF antibody (Fig. 7B). Both proteins were only detected in the co-immunoprecipitation sample with the anti-MIF antibody, thus confirming the result obtained by MALDI-TOF mass spectrometry, whilst the control IP was negative.

5.1.3. Enrichment of MIF interacting proteins by cross-linking

Cross-linking experiments were performed to enrich MIF interacting proteins of low abundance as observed in Co-IP. After depletion of endogenously biotinylated proteins by streptavidin agarose beads, the MIF-Sulfo-SBED protein (S-MIF) was added to NIH 3T3 cell lysates and samples were treated with UV light resulting in biotin-labeling of interacting proteins. Several potential MIF interacting proteins with molecular masses between 40 and 60 kD were visualized via biotin detection (Fig. 8A). As a positive control for the specificity of the cross-linking method Jab-1, was detected in total and precleared cell lysate at comparable levels (Fig. 8B, lanes 1 and 2) suggesting that endogenous Jab-1 is not biotinylated and does not bind unspecifically to the straptavidin matrix. Jab-1 was biotinylated after the cross-linking experiment and subsequent biotin purification (Fig. 8B, lane 3). In a competition experiment, addition of 10-fold molar excess of recombinant MIF over the S-MIF concentration resulted in a clear decrease of biotin labeled proteins, indicating the specificity of the protein-protein interaction (Fig. 8A, lane 4). As a result of the competition experiment, biotin-labeled Jab-1 was substantially decreased, validating the specificity of Jab-1-S-MIF (Fig. 8B, lane 4).

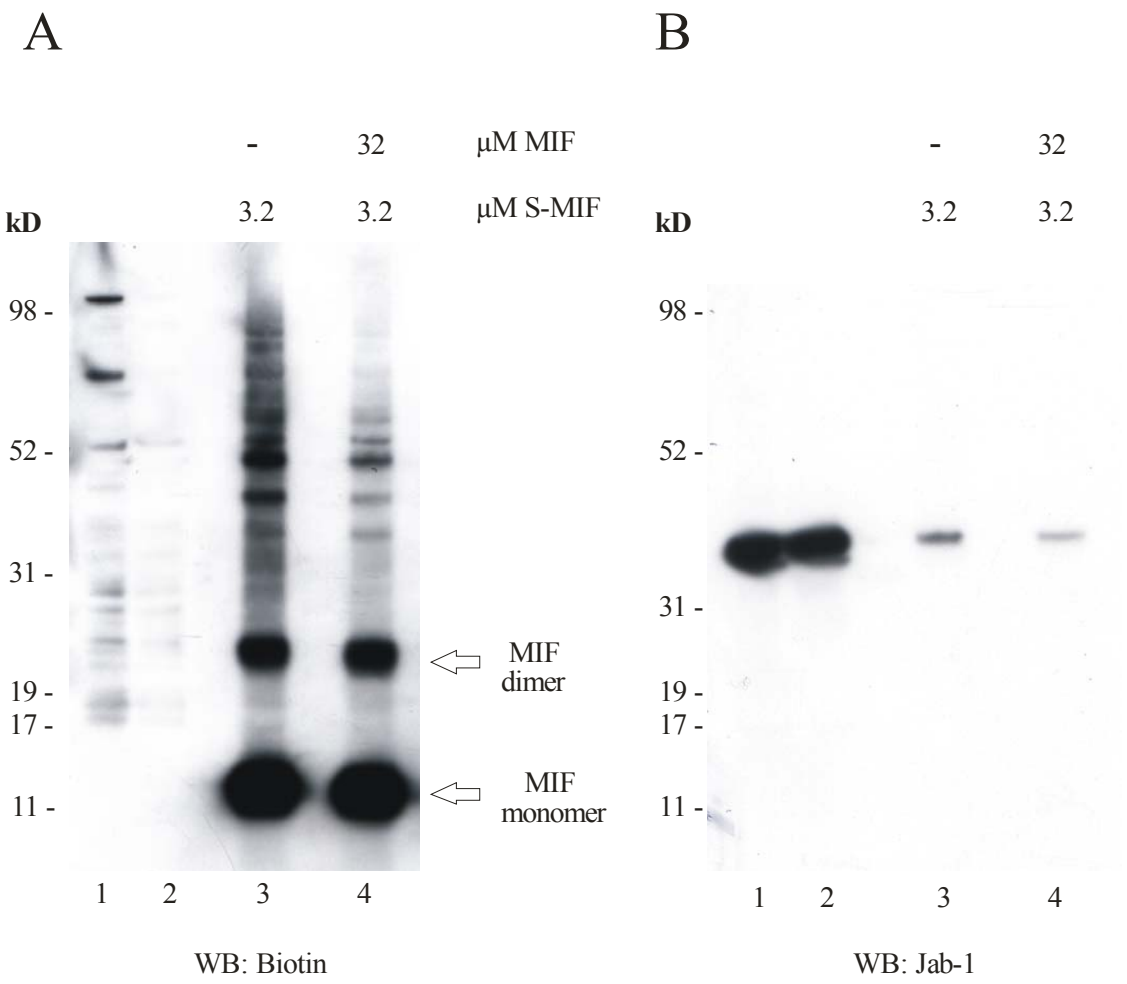


Fig. 8: Detection of MIF interacting proteins by cross-linking. (A) Detection of biotinylated proteins: Lane (1): total cell lysate; lane (2): biotin precleared cell lysate; lane (3): purified biotinylated proteins after cross-linking with 3.2 µM S-MIF; lane (4): purified biotinylated proteins obtained after competition with 32 µM recombinant MIF (10 fold molar excess over the S-MIF concentration) and cross-linking. **(B)** Jab-1 detection. The same membrane was stripped and reprobed for Jab-1.

5.2. Cloning, expression, and purification of GST-RP S19

The coding sequence of RP S19 amplified from IRAKp961E1430Q vector was cloned into the bacterial expression vector pGEX-4T-2. The obtained construct (RP S19-pGEX) was used to transform E. coli BL21(DE3) bacterial cells for the expression of GST-tagged RP S19. In order to screen for positive clones, colony PCR was performed using transformed bacteria as template with primers for amplifying RP S19 (Fig. 9B).

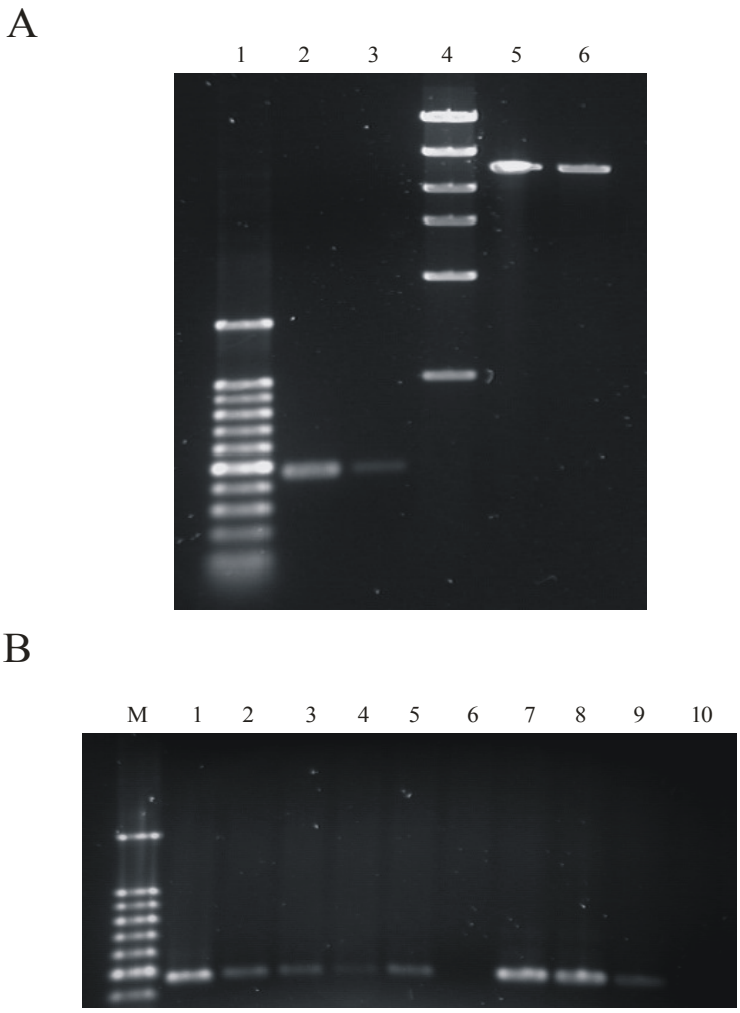


Fig. 9: Preparation of an expression construct and screening for positive transformants. (A) RP S19 digested PCR product (lanes 2 and 3) and linearised pGEX-4T-2 vector (lanes 5 and 6) used in a ligation reaction to generate RP S19-pGEX

construct. Lanes (1) and (4) were loaded with 100bp and 1kb markers, respectively. **(B)** RP S19 PCR products obtained after screening for positive transformants using the transformed bacterial cells as a template and primers for RP S19 amplification. (M) = 100 bp marker.

One clone (Fig. 9B, lane 1) from the successfully transformed bacteria was selected for protein expression. In order to determine the optimal condition for protein expression, bacterial RP S19 protein expression was induced with 0.5 mM IPTG at 37°C for different time points. As shown in Fig. 10, the fusion protein became the major bacterial protein after 1h of induction and the maximum expression of RP S19-GST was observed after 2h.

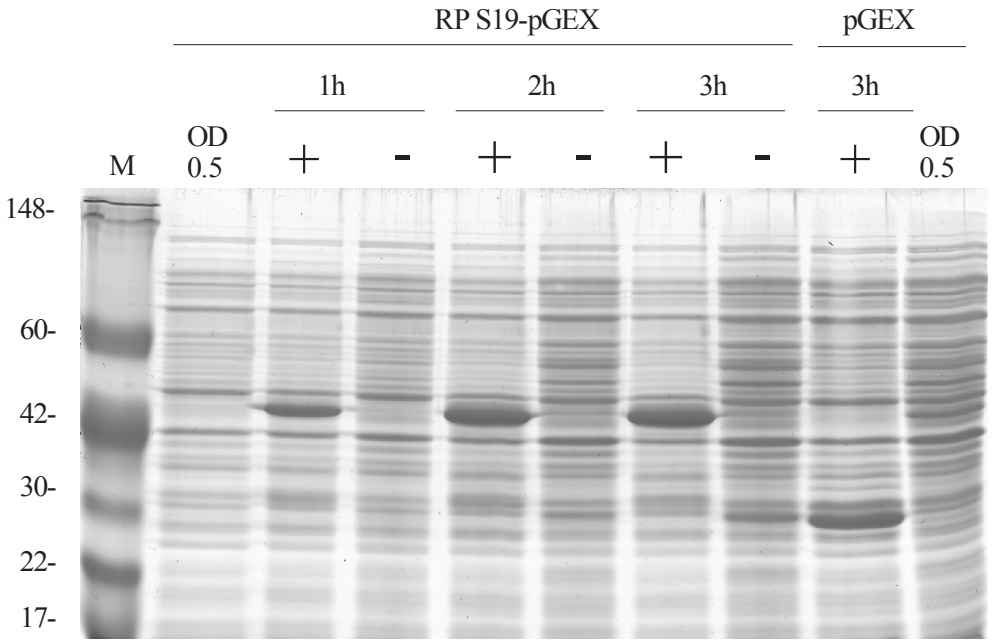


Fig. 10: Expression of GST-tagged RP S19 in *E. coli*. A 500 ml culture of E. coli BL21 (DE3), carrying the RP S19 expression plasmid (RP S19-pGEX), was induced (+) with 0.5 mM IPTG at 37°C. Bacteria transformed with empty vector, pGEX, served as control. At three time points (1, 2 and 3 h), cells were harvested, lysed in SDS Laemmli buffer and separated by SDS-PAGE. After electrophoresis, the gel was stained with colloidal Coomassie blue. (M) = molecular weight marker [kD].

Using the established expression conditions, a preparative culture was produced and GST-RP S19 protein was affinity purified using a Glutathione agarose column. Eluted fractions were collected and the purity of each fraction was validated by SDS-PAGE and subsequent Coomassie staining of the gel (Fig. 11).

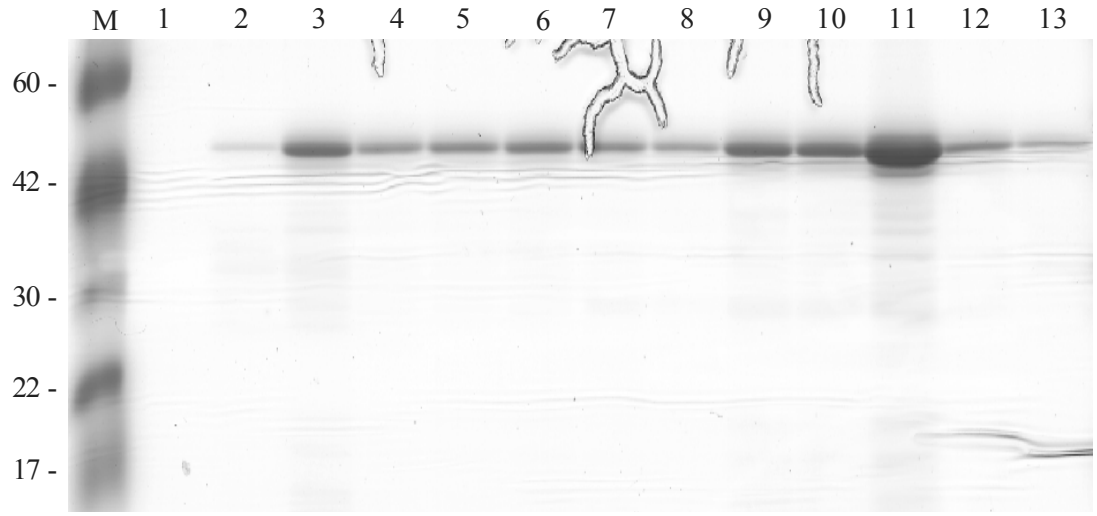


Fig. 11: Purification of GST-tagged RP S19. An aliquot (2 µl) from each eluted fraction (lane 1 to 13) was separated by 15% SDS-PAGE and stained with Coomassie.

5.3. RP S19-His purification and antibody production

His-tagged RP S19 protein was expressed in E. coli and affinity purified on a Ni-NTA column. The recombinant protein was then used in a standard immunization protocol to reise polyclonal antibody against RP S19. Rabbit serum was subsequently affinity purified using a Ni-NTA column packed with His-tagged RP S19. The specificity of the full serum and purified polyclonal RP S19 antibody was tested by Western blot analysis with recombinant and endogenous expressed RP S19 protein in NIH 3T3 cells. As shown in Fig. 12, the range of unspecific reactivity (A) was considerably reduced after affinity purification of the serum (B). Due to addition of the His tag, the recombinant protein exhibited a higher molecular weight than the native protein.

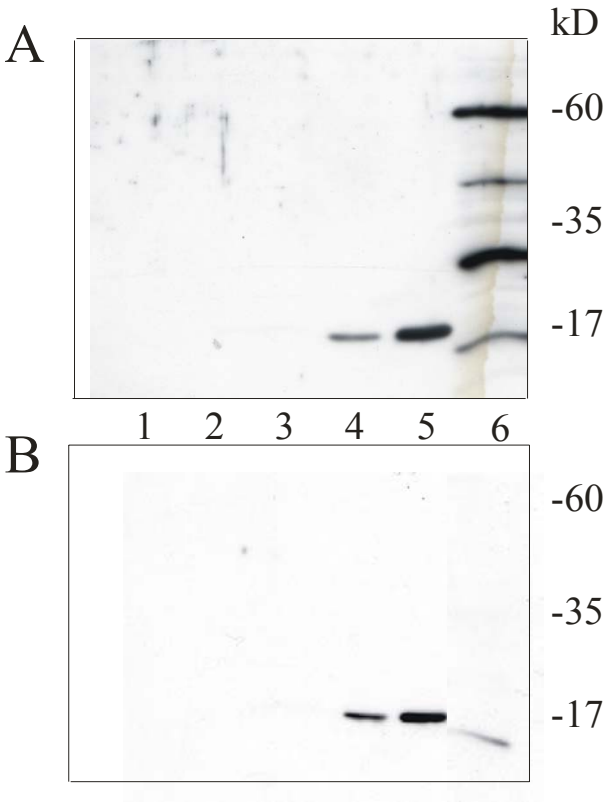


Fig. 12: Purification of polyclonal RP S19 antibody. Lanes (1) to (5):1, 4, 16, 64 and 256 ng of recombinant RP S19-His, respectively; lane (6): 60 µg NIH 3T3 cell lysate were separated on a 15% SDS gel and electro-transferred onto a nitrocellulose membrane. **(A)**. The blot was incubated with full serum from immunized rabbits. **(B)**. The blot was incubated with the affinity purified antibody against RP S19 protein.

5.4. Interaction of MIF with RP S19 *in vitro*

To analyze whether the observed interaction between MIF and RP S19 occurs by direct protein-protein binding or is mediated by linking co-factors, pull-down experiments with recombinant MIF and RP S19 were performed.

5.4.1. Pull-down of GST-RP S19 with biotinylated MIF

For this pull-down experiment, biotin-MIF was immobilized on avidin beads (Fig. 19) and then incubated with increasing amounts (0.05, 0.1 or 0.2 µg) of RP S19-GST (lanes 4-6). As a control avidin beads were directly incubated with the same amounts of RP S19-GST (lanes 1-3). In this case, no unspecific binding of RP S19-GST to avidin beads was observed. In contrast, biotin-MIF loaded beads recovered RP S19-GST in a dose dependent manner (lanes 4 to 6) suggesting a direct interaction of both molecules *in vitro*.

To determine if the observed interaction was indeed specific, a competition experiment was performed using recombinant rat MIF and β-lactoglobulin, respectively. Addition of 35-fold molar excess of recombinant MIF over RP S19 concentration resulted in a clear competition of the biotin-MIF-RP S19-GST interaction (Fig. 13, lane 7), which was not observed when β-lactoglobulin, was used as a competitor (Fig. 13, lane 8).

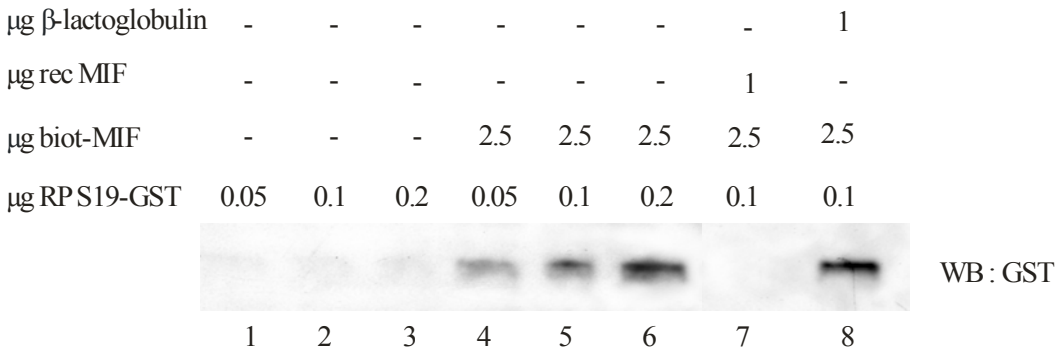


Fig. 13: Interaction of biotin-MIF with RP S19-GST. Biotin-MIF immobilized on avidin beads was incubated with increasing amounts of RP S19-GST (lanes 4-6). As control, unloaded avidin beads were directly incubated with the same amounts of RP S19-GST (lanes 1-3). A competition experiment was performed with 1 µg of unlabeled MIF (lane 7). As a control for the competition experiment, β-lactoglobulin was used as competitor (lane 8). Detection of recovered RP S19-GST was performed using Western blot analysis with an anti-GST antibody.

5.4.2. Pull-down of recombinant MIF with His-tagged RP S19

In a second pull-down experiment His-tagged mouse RP S19 was immobilized on Ni-NTA agarose beads and incubated with either increasing amounts (0.5, 1 or 2 µg) of recombinant rat MIF or 2 µg of recombinant human MIF (Fig. 14). Protein complexes bound to the beads were separated by SDS-PAGE and then stained with Coomassie blue. Unloaded Ni-NTA beads were incubated with recombinant rat MIF in which case, no unspecific binding of MIF was observed (lanes 5-7). In contrast, RP S19-His loaded beads recovered recombinant rat MIF in a dose dependent manner (lanes 1-3) suggesting a direct interaction of both molecules *in vitro*. Interestingly, recombinant human and rat MIF exhibited a similar affinity to mouse RP S19-His (lane 4). Taken together the results of both pull-down studies support the hypothesis of a direct interaction between MIF and RP S19 *in vitro*.

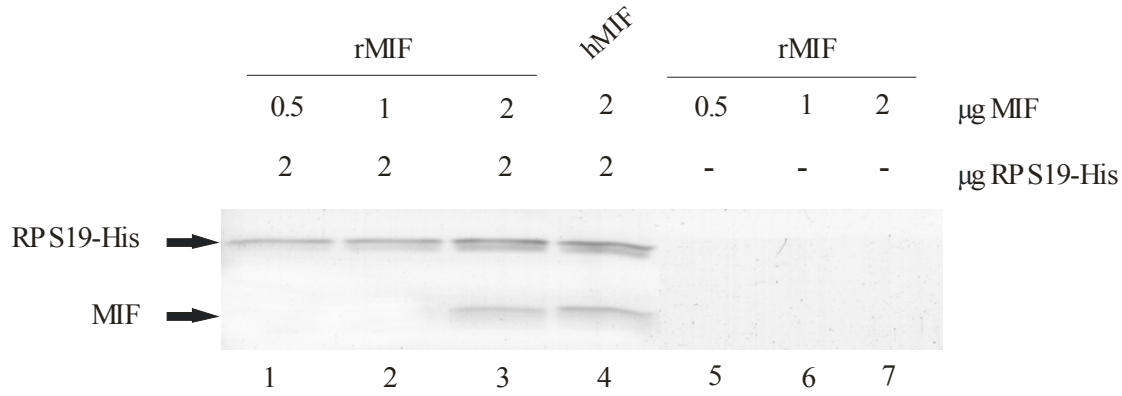
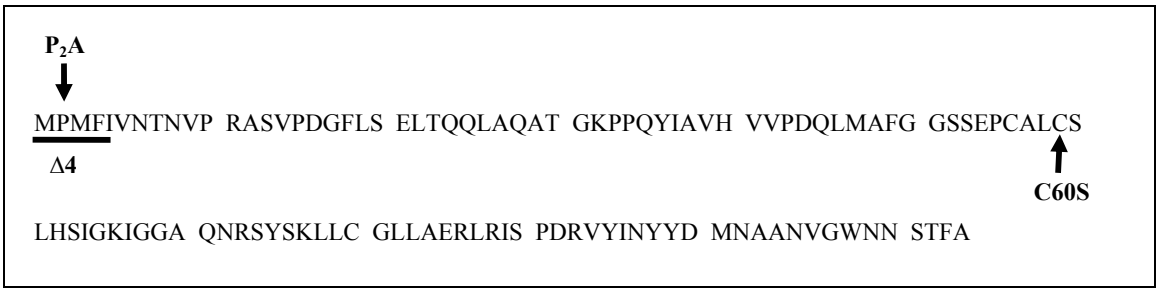


Fig. 14: Binding of His-tagged RP S19 to rat and human MIF. His-tagged RP S19 was immobilized on Ni-NTA-agarose beads and incubated with different amounts of recombinant rat MIF (lane 1-3) or with human MIF (lane 4), respectively. As a control recombinant rat MIF was directly incubated with Ni-NTA-beads (lane 5-7). The immobilized proteins were resolved by SDS-PAGE and stained with Coomassie.

5.4.3. Interaction of RP S19 with MIF mutants

Our previous studies showed a strong specific affinity of mouse recombinant RP S19 to recombinant human MIF (see 4.4.2.). To determine domains essential for interaction of MIF and RP S19, pull-down experiments with recombinant mouse RP S19-His and three different human MIF mutants (P2AMIF, Δ4MIF and C60SMIF) were performed. The position of the amino acid substitution or deletion is described in Fig. 15 (upper panel). The sequences of the wt human MIF and mutants of MIF were confirmed by MALDI-ToF MS (Fig. 15 lower panel).

His-tagged RP S19 was loaded on Ni-NTA agarose beads and incubated with either 1 µg of recombinant human MIF, or P2A, Δ4MIF and C60S MIF mutants. The binding of RP S19 to the wt MIF or to the selected MIF mutants was analyzed by immunoblotting using anti-RP S19 and anti-MIF antibodies, respectively.



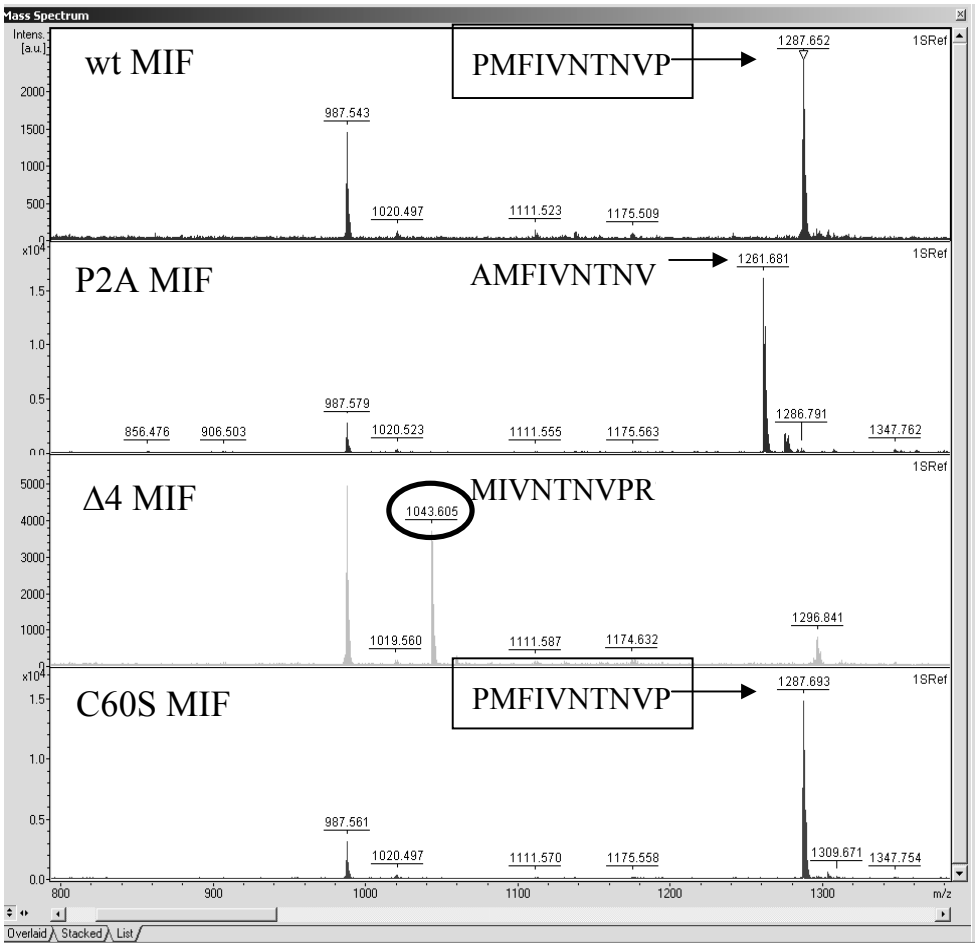


Fig. 15: Characteristics of human MIF and mutant species. The upper panel shows MIF amino acid sequence. Substituted or deleted amino acids are indicated. P2A mutant has the first proline exchanged to alanine, Δ4 mutant is a deletion mutant lacking the first 4 amino acids and the C60S mutant has the cysteine at the position 60 mutated to serine. The lower panel shows the MALDI-ToF mass spectra of the tryptic peptides of wt MIF and MIF mutants.

As shown in Fig. 16, human wt MIF and Δ4MIF mutant exhibited similar levels of interaction with RP S19, whereas the human P2A and C60S mutants did not display any affinity to mouse RP S19-His protein.

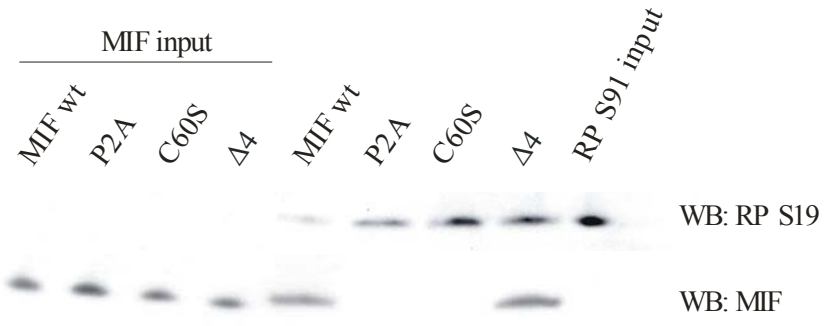


Fig. 16: Interaction of MIF mutants with RP S19. His-tagged RPS19 was immobilized on Ni-NTA-agarose beads and then incubated with 1 µg of human MIF wild type (wt), P2A, C60S and Δ4 MIF mutants. The immobilized protein complexes were resolved by SDS-PAGE, and the blot was probed for RP S19 (upper panel), stripped and reprobed for MIF (lower panel). For analysis 100 ng of MIF protein and 200 ng of RP S19-His were loaded.

5.5. Cellular localization of endogenous MIF and RP S19

The cellular localization of endogenous RP S19 and MIF in mouse NIH 3T3 cells was investigated by immunofluorescence microscopy. For immunostaining an antibody raised in rabbit against mouse RP S19 and an antibody raised in mouse against MIF were used. Both proteins were localized in the cytoplasm of the cells with weak/diffuse nuclear staining for RP S19 (Fig. 17) demonstrating that endogenous MIF and RP S19 occupy the same cellular compartment.

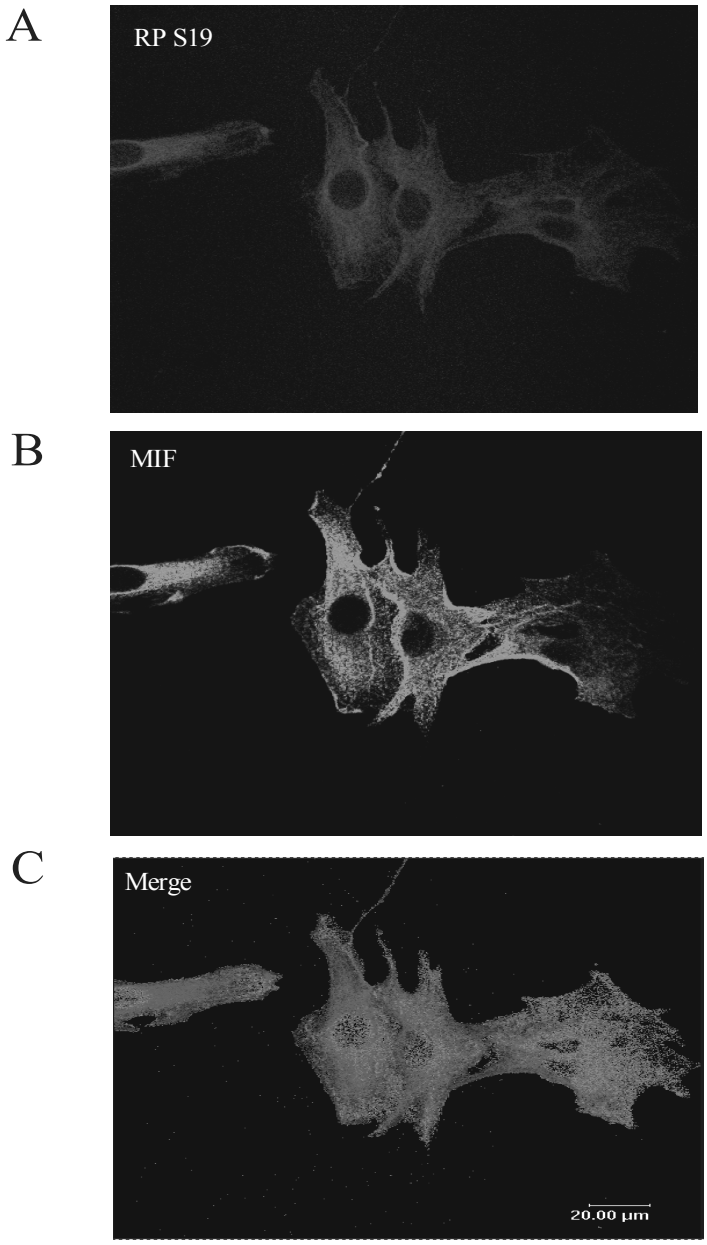


Fig. 17: Cellular localization of MIF and RP S19 in NIH 3T3 fibroblasts. Double immunostaining with rabbit anti-mouse RP S19 followed by donkey anti-rabbit IgG conjugated with Cy3 **(A)** and mouse anti-MIF followed by donkey anti-mouse IgG conjugated with FITC **(B)** was performed. Panel **(C)** shows the merged image of the two upper panels. Blue colour indicates co-localization. Images were taken with a confocal laser scanning microscope (Leica TCS SP2).

5.6. Effect of RP S19 on MIF tautomerase enzymatic activity

Tautomerase activity of MIF was shown to play an important role in several biological functions of the cytokine (Lubetsky et al. 2002). Thus, we wanted to investigate the effect of RP S19-MIF interaction on MIF tautomerase activity.

To determine if RP S19 modulates MIF tautomerase activity, tautomeric conversion of L-dopachrome methyl ester to 5,6-dihydroxyindole-2-carboxylic acid by MIF was measured in the absence or presence of His-tagged RP S19. One representative experiment is shown in Fig. 18. In a control experiment secretoglobin (SCGB) 2A1, a recombinant His-tagged protein of 13 kD replaced His-tagged RP S19.

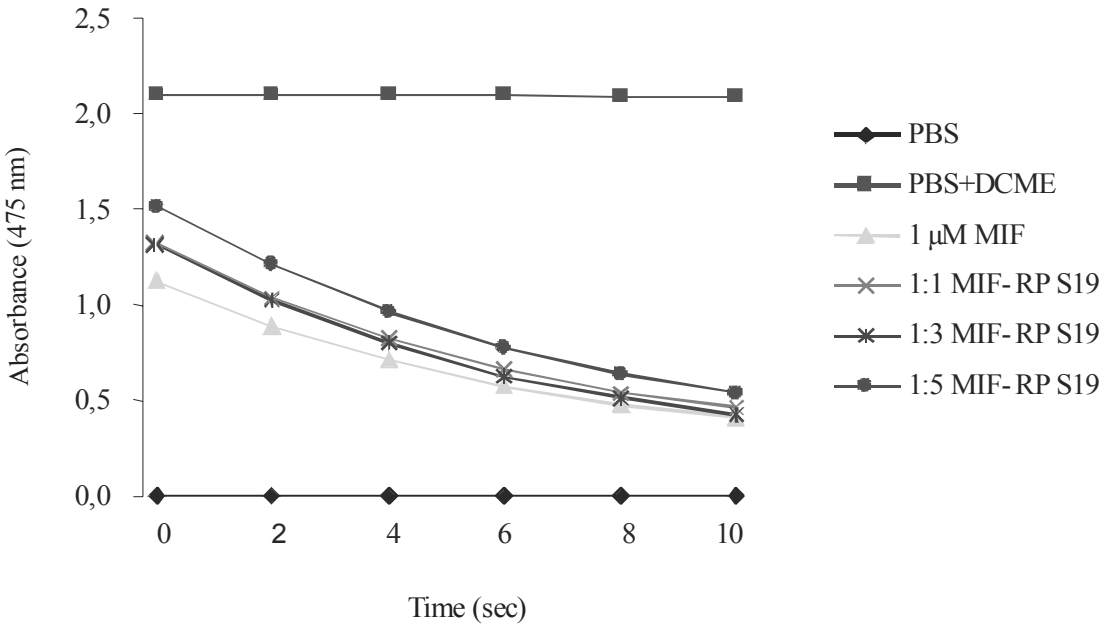


Fig. 18: Tautomerase activity of MIF in the presence of RP S19: MIF catalyzes tautomerisation of L-dopachrome-methyl-ester (DCME) to 5,6-dihydroxyindole-2-carboxylic acid. Reaction kinetics was spectrophotometrically recorded at 475 nm.

Recombinant His-tagged RP S19 at 5 fold molar excess over MIF concentration decreased the tautomerase activity of MIF by 40%, whereas His-tagged secretoglobin 2A1, a His-tagged protein with no enzymatic activities, had no influence on the reaction (Fig. 19). In conclusion, RP S19 displayed a moderate inhibitory effect on MIF tautomerase activity.

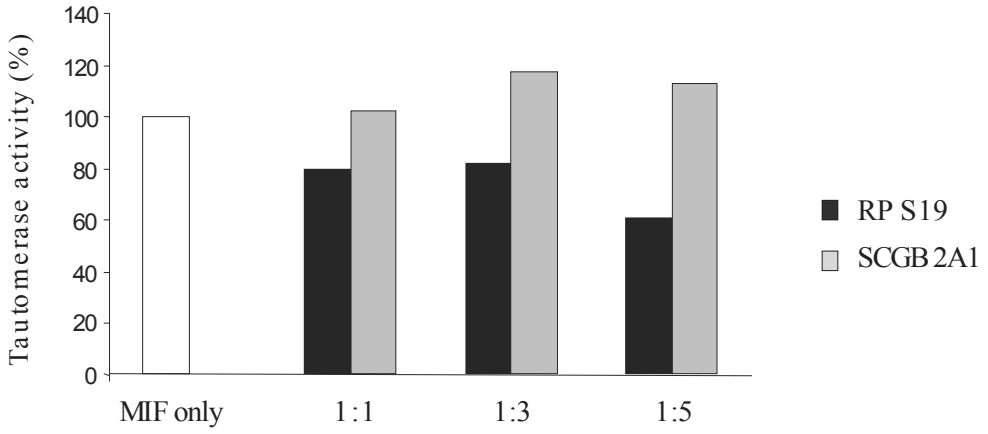


Fig. 19: MIF tautomerase activity is affected by of RP S19: Decrease in absorbance from 0 to 4 sec was converted and defined as tautomerase activity. The activity of MIF alone was set to 100%. The assay was performed with 1 µM of recombinant MIF alone or preincubated with 1-, 3- or 5-fold molar excess (1:1, 1:3, 1:5) of His-tagged RPS19 (black bars) or His-tagged SCGB 2A1 (grey bars) for one hour.

5.7. Modulation of MIF-induced monocyte migration by RP S19

Since MIF-RP S19 interaction exhibited an effect on MIF tautomerase activity, although not being statistically significant, we wanted to investigate if RP S19 is capable to modulate other functions of MIF.

MIF was characterized to inhibit the chemotaxis and the random migration of macrophages against monocyte chemoattractant protein-1 (MCP-1). Therefore, we raised the question if the inhibitory action of MIF is not in fact an chemoattractant effect which MIF exerts on macrophages.

To characterize the chemotactic ability of MIF migration assays were performed using isolated human blood monocytes. In our experimental setup the basal/random level of migrated cells was 2.4% on average, whereas 25 ng/ml of MCP-1 used as positive control increased the migration level to 6%. After addition of 50 ng/ml human MIF the number of migrating cells increased to 6.4% (Fig. 20). Thus, MIF and MCP-1 exhibited similar effects on monocyte migration suggesting that MIF displays a previously unknown chemotactic activity.

Next, we wanted to investigate the effect of RP S19 on the chemotactic activity of MIF. Addition of RP S19 (5 fold molar excess of RP S19 over MIF concentration) alone to the lower chamber had no significant effect on macrophage migration. In contrast, addition of five fold molar excess of RP S19 over MIF concentration inhibited human MIF chemotactic activity from 6.4% to 3.4%. Therefore, we conclude that binding of RP S19 to MIF negatively modulates its ability to attract human monocytes *in vitro*.

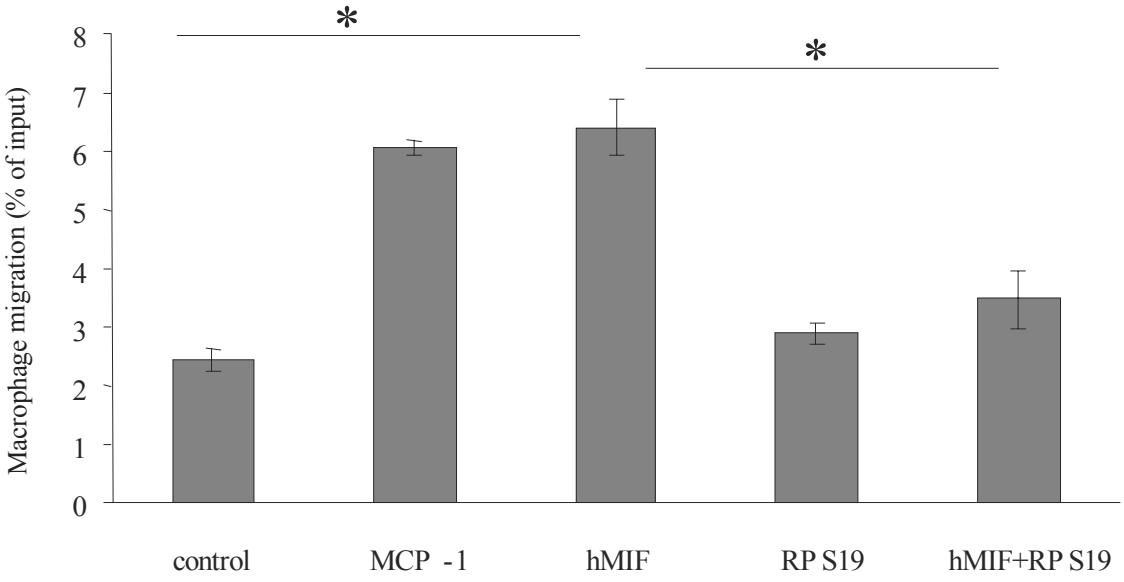


Fig. 20: Effects of RP S19 on MIF-induced monocyte migration. The chemotactic capacity of human MIF (hMIF), His-tagged RP S19, hMIF combined with RP S19 on isolated human blood monocytes was examined. MCP-1 was used as positive control and RPMI medium containing 0.5% BSA as negative control. Data are means ± SEM of four independent experiments. Assays were performed in duplicates and two investigators in a blinded fashion counted migrated monocytes individually. Statistically significant differences (nonparametric impaired Mann-Whitney Test ($p < 0.05$) are marked by asterix.

5.8. Effect of RP S19 on MIF glucocorticoid overriding activity

The glucocorticoid dexamethasone, is known to inhibit LPS-stimulated secretion of the proinflamatory cytokine tumor necrosis factor α (TNFα) by monocytes /macrophages, thus exerting a strong anti-inflammatory effect. MIF is capable to abolish dexamethasone inhibition of TNF production, therefore overriding the anti-inflammatory action of glucocorticoids. In this context, we raised the question if RP S19 interferes with the well-established MIF action on glucocorticoid mediated TNF production.

Human peripheral blood monocyte cells were isolate (see section 2.1.2.) and

sequentially incubated with either (1) dexamethasone (10^{-9} M) or (2) dexamethasone (10^{-9} M) plus increasing amounts of MIF alone or (3) dexamethasone (10^{-9} M) and MIF together with 5 fold molar excess of RP S19 prior to the addition of LPS (0.1µg/ml). After six hours of conditioning, the secreted TNF-α in the cell culture medium was quantified by ELISA with a detection range of 3.9 to 500 pg/ml (Fig. 21)

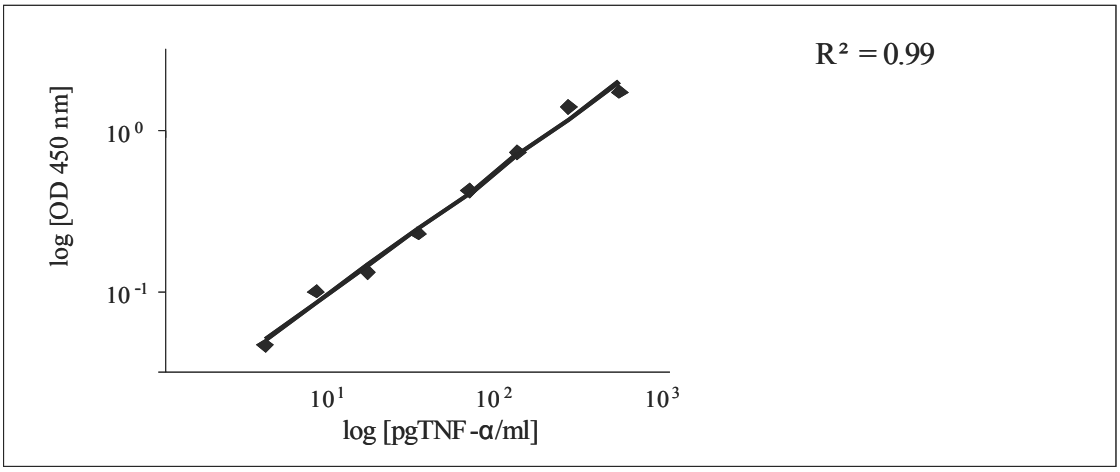


Fig. 21: Calibration courve of human TNF-α ELISA

As shown in Fig. 22, dexamethasone completely abolished the TNF-α secretion by monocyte/macrophages stimulated with LPS. Pretreatment of the cells with increasing amounts of recombinant MIF before addition of dexamethasone resulted in a decrease of the inhibition effect on TNF-α secretion. In contrast, preincubation of MIF with 5 fold molar excess of RP S19 was efficient in antagonizing MIF-dependent inhibition of dexamethasone activity, whereas RP S19 alone did not exhibit any effect on TNF- α production. In summary, RP S19 was identified as a protein with the capacity to neutralize the pro-inflammatory effects of MIF.

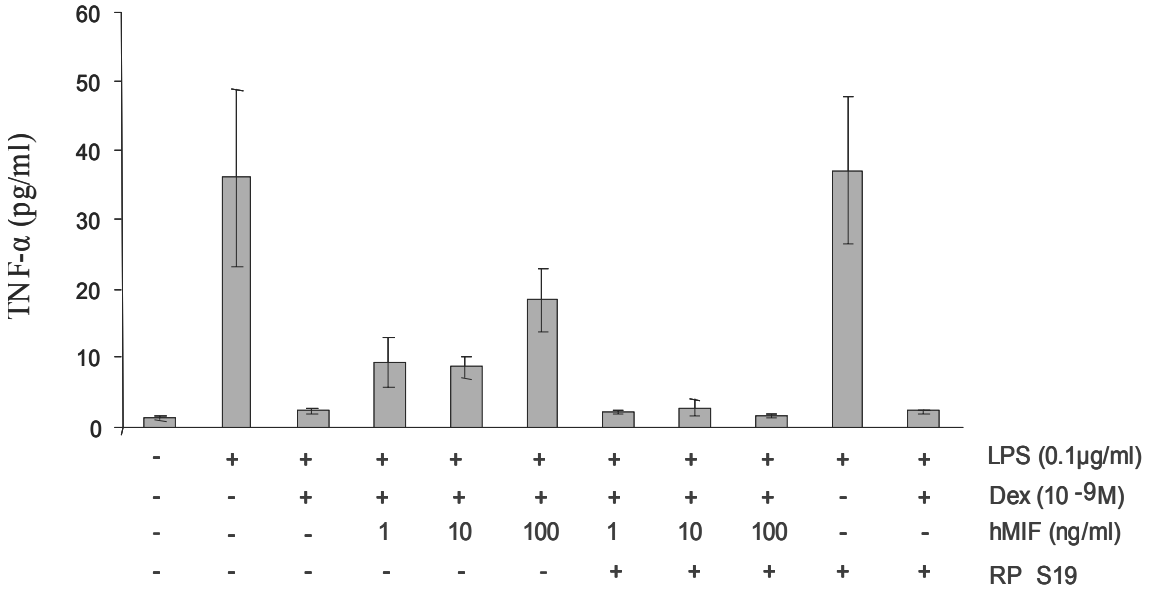


Fig. 22: Effect of RP S19 on MIF's glucocorticoid overriding activity. Peripheral mononuclear blood cells (PMBCs) were purified from blood of normal human volunteers by adherence to plastic. The cells were incubated sequentially with (1) dexamethasone (10^{-9} M) or (2) dexamethasone (10^{-9} M) plus increasing amounts of MIF alone or (3) dexamethasone (10^{-9} M) and MIF together with 5 fold molar excess of RP S19 prior to the addition of LPS (0.1µg/ml). Conditioned medium was collected after 16 h and assayed for TNFα by specific ELISA. Values are means ±S.D. (n = 3). Statistically significant differences are indicated by asterisks ($p \leq 0.05$, Student's t test, independent variable).

6. DISCUSSION

Macrophage migration inhibitory factor (MIF) was identified four decades ago as one of the first soluble immune mediators discovered (Bloom and Bennett 1966; David 1966). As a lymphocyte-derived activity it was initially implicated in delayed-type hypersensitivity reactions and found to inhibit the random migration of lymphocytes (David 1966). More recently, it has been shown also to actively recruit leukocytes (Lan et al. 1997) and hepatocellular carcinoma cells (Ren et al. 2003). MIF was shown to be a prominent product of macrophages (Calandra et al. 1994) and to be expressed in a large number of endocrine and parenchymal cells, like anterior pituitary cells (Bernhagen et al. 1993). MIF is a critical mediator of a number of immune and inflammatory diseases including septic shock (Bozza et al. 1999; Calandra et al. 2000), rheumatoid arthritis (Mikulowska et al. 1997), inflammatory lung diseases (Donnelly et al. 1997) and cancer (Chesney et al. 1999). Thus, it can be considered as an important pleiotropic master regulator that is neither a typical cytokine nor a typical chemokine. Therefore, it was proposed to consider MIF as a member of the heterogeneous family of chemokine-like function (Degryse and de Virgilio 2003).

In an attempt to discover proteins that interact with MIF, Jung and co-workers identified specific protein complexes between MIF and proliferation-associated gene (PAG), a thiol-specific cellular antioxidant protein (Jung et al. 2001). The interaction between PAG and MIF occurs via disulfide bond between Cys^{173} of PAG and a yet not identified Cys residue of MIF resulting in the inhibition of PAG's antioxidant activity. The identification of disulfide-dependent PAG-MIF interaction shows that MIF can interact with cellular proteins carrying susceptible disulfide bonds or thiols. MIF was also shown to colocalize with insulin in secretory granules in the β-cells of the pancreatic islets (Waeber et al. 1997) which can be enzymatically reduced by MIF *in vitro* (Kleemann et al. 1998a). In addition to PAG and insulin, another protein, hepatopoeitin (HPO) is directly involved in MIF's redox-modulating function by direct interaction (Li et al. 2004). A connection between MIF's role in cellular redox regulation and cell signaling processes was identified when MIF was found to interact with COP9 signalosome (CSN) component c-Jun activation domain binding protein-1 (JAB1)/CSN5

(Kleemann et al. 2000a). JAB1 is a transcriptional co-activator of the activator protein-1 (AP-1) (Claret et al. 1996) and component of the CSN. MIF inhibits JAB1-mediated AP-1 activation and counterregulates JAB1-dependent p27 degradation and G1 cell-cycle arrest (Kleemann et al. 2000a). The JAB1-antogonistic effect of MIF appears to be dependent of the presence of the CXXC motif of MIF (Kleemann et al. 2000a). As recently demonstrated, JAB1 directly interacts with HPO (Lu et al. 2002) and interestingly, HPO also binds MIF (Li et al. 2004). Although MIF acts also as an extracellular mediator, no typical receptor has been identified. CD74, a cell-surface form of the major histocompatibility complex (MHC) class II-associated invariant chain was found to interact with MIF at the cell surface (Leng et al. 2003). Although CD74 does not constitute a typical receptor with a signal-transducing domain, it was demonstrated that MIF-mediated enhancement of cell proliferation and MAPK activation is in part dependent of the presence of CD74 (Leng et al. 2003). In addition, MIF was found to interact with BNIPL, an apoptosis associated protein and it was suggested that BNIPL could be involved in governing cell proliferation (Shen et al. 2003). The same study shows that overexpression of BNIPL suppresses Hep3B cell growth, suggesting that BNIPL could inhibit MIF-mediated tumor cell proliferation.

Although several proteins are described as MIF interacting partners (Tabe 2), the exact mechanism of action of MIF remains incompletely elucidated. To confine a continued proinflammatory response, which, if generalized, can lead to shock, the careful control of local MIF levels and/or bioactivity may provide an important step in managing the outcome of an immune response. Therefore, the research focus was set on the identification of MIF interacting proteins that are able to compromise MIF activity.

Table 2: Known proteins that interact with MIF

Name	General function	Effect of the interaction
JAB1/CSN 5	Transcriptional co-activator of the AP 1 Binds and promotes degradation of cell-cycle inhibitor p27Kip1 Binds to glucocorticoid and progesterone receptors Degrades the cell cycle inhibitor KIP1 and the tumour suppressor p53	MIF inhibits JAB1-mediated AP-1 activation MIF reduces JNK activity stimulated by JAB1 MIF inhibits phoshorylation of c-Jun activated by JAB1
PAG	It is a thiol-specific cellular antioxidant protein Posesses conserved cysteine groups and use thiols as reductants	PAG reduces the D-dopachrome tautomerase activity of MIF PAG-MIF interaction is dependent on the redox status
BNIPL	Can cause apoptosis mediated by an apoptosis-inducing BCH domain	MIF suppresses the antioxidant activity of PAG BNIPL could inhibit MIF-mediated tumor cell proliferation.
CD74	Role in the transport of MHC class II proteins from the endoplasmic reticulum to the Golgi complex Accessory role in immune cell stimulation which require an interaction with CD44	CD74 expression is required for MIF-mediated ERK-1/2 phosphorylation, PGE2 production, and cell proliferation.
HPO	It is a liver-specific regeneration augmenter	Both HPO and MIF could bind to JAB1 and modulate the AP-1 pathway.

6.1. Identification of proteins that interact with MIF

In order to identify proteins that specifically interact with MIF, a systematic approach was employed.

To select a promising source for the identification of MIF interacting proteins, a far-western experiment was performed with proteins from different tissues and cell types. Although the technique is not suitable for direct identification of possible interacting proteins, it enables an overview of MIF immune-reactivity in the tissues and cell types selected for the study. The far-western approach resulted in a broad but similar spectrum of MIF reactivity in all cell types and tissues analyzed. These results suggest that MIF possesses a ubiquitous spectrum of potential interacting proteins that is not restricted to specific cell types and tissues. Since the NIH 3T3 fibroblast cell line was employed in studies on MIF functions, receptor and on cellular up-take (Kleemann et al. 2002; Nguyen et al. 2003a), it was selected for further experiments.

In previous studies, co-immunoprecipitation (Co-IP) was shown to provide an efficient approach for the detection of protein-protein interactions (Qoronfleh et al. 2003). Therefore, this method was employed to identify MIF interacting proteins in NIH 3T3 cell extracts. Initially, a "proof of concept" experiment was performed. Using the generated rabbit polyclonal antibody against MIF, Co-IP was performed to investigate if this antibody is able to co-precipitate MIF together with JAB1, a protein that was shown to interact with MIF (Kleemann et al. 2000a). By this, anti-MIF antibody was proven to efficiently precipitate MIF, and Jab-1 was found to co-precipitate in NIH 3T3 cell extracts, demonstrating that the selected antibody is suitable for co-immunoprecipitation experiments. Co-IP samples were then separated by SDS gel electrophoresis and immunoprecipitated proteins were visualized by Coomassie staining. Beside immunoglobuline light and heavy chains, several specific bands appeared in the anti-MIF IP sample. One of these specific bands, with an apparent molecular weight of 16 kD, was excised from the gel and analyzed by tryptic in-gel digestion followed by MALDI-TOF mass spectrometry and identified as ribosomal protein S19 (RP S19). To confirm this result, IP samples were then analysed by Western blotting using anti RP S19 and anti-MIF antibodies. Both proteins were only detected in the co-immunoprecipitation sample with anti-MIF antibody, thus confirming the specificity of the immunoprecipitation. In

conclusion, RP S19 was identified as a novel MIF-interacting protein.

Although co-immunoprecipitation provides a robust technique for the identification of protein-protein interactions, its application is not suitable for the detection of low abundant interacting proteins. Furthermore, the presence of high amount of immunoglobuline in the IP sample limits the protein quantity for final detection. Therefore, a chemical cross-linking method based on the transfer of biotin was employed to enrich MIF interacting proteins of low abundance. The method allows covalent labeling with a biotin tag of proteins which come in a very close proximity to the "bait" protein. To confirm that this method is eligible for this study, a control experiment using NIH 3T3 cell extracts was performed. Western blotting identified Jab-1 as one of the purified proteins. This result confirms the applicability of the cross-linking method for the detection of MIF interacting proteins *in vitro*. Several proteins with an apparent molecular weight between 15 and 55 kDa were specifically visualized by biotin detection in the sample incubated with Sulfo-MIF confirming the specificity of the label transfer process. In summary, the application of the cross-linking technique resulted in the enrichment and detection of additional potential MIF interacting proteins, which were not observed using the co-immunoprecipitation approach. Thus, the cross-linking technique provides a promising strategy for the identification of low abundant MIF interacting proteins.

6.2. MIF directly interacts with RP S19 *in vitro*

In order to confirm the interaction between RP S19 and MIF *in vitro*, RP S19 was expressed as a His or GST tagged fusion protein. Pull-down experiments with rat or human MIF and GST-RP S19 and His-tagged RP S19 proved that the interaction between MIF and RP S19 occurs directly without the need for a co-factor.

This is the first report on the identification of a ribosomal protein interacting with the cytokine MIF. Ribosomes are complex macromolecular machines that are responsible for protein synthesis in every living cell. However, various individual ribosomal proteins and also translational initiation and elongation factors have been found to play roles in regulating cell growth (Marechal et al. 1997), transformation (Wilson et al. 1994) and cell

death (Horino et al. 1998), leading to the hypothesis that components of the translational apparatus can act as multifunctional proteins. Previous studies have shown that several ribosomal proteins, including RP S19, possess extraribosomal functions (Wool 1996), supporting the hypothesis that the transition of the ribosome from a RNA to a RNP (ribonucleoprotein) complex occurred by recruiting preexisting proteins. Some of these extraribosomal functions include malignant transformation (Kastan 1993), regulation of development (Cramton and Laski 1994; Fisher et al. 1990; Hart et al. 1993), and DNA repair (Grabowski et al. 1991). In this context, RP S19 was found to be required for chromatin condensation (Etter et al. 1994). RP S19 was also shown to interact with fibroblast groth factor 2 (FGF2) (Soulet et al. 2001), contributing to the idea of extra cellular function. In the same study, RP S19 was found predominantly in the cytosolic compartment of NIH 3T3 cells and it lacks a nuclear localization sequence (Soulet et al. 2001). RP S19 is implicated in different human diseases. In rheumatoid arthritis synovial tissue, the monocyte chemotactic factor was shown to correspond to free oligomers of RP S19 intermolecularly cross-linked by a transglutaminase-catalyzed reaction (Nishiura et al. 1996). In Diamond-Blackfan anaemia, a congenital erythroblastopenia, the gene encoding RP S19 is mutated and the mutations were associated with clinical features that suggested a function for RP S19 in erythropoiesis and embryogenesis (Amaldi and Pierandrei-Amaldi 1990; Matsson et al. 1999). All of the patients with RP S19 gene mutations were heterozygotes, suggesting that homozygous abnormality of the RP S19 gene is embryonic lethal, and that erythropoiesis needs a higher level of functional RP S19 molecules than other cellular systems (Matsson et al. 2004).

MIF was characterized to exhibit its functions via specific amino acid sequences. Pro^2 was identified to be essential for tautomerase activity of MIF (Bendrat et al. 1997; Kleemann et al. 2000b; Swope et al. 1998). The sequence motif Cys^{57}-Ala-Leu-Cys^{60} (CALC) is a characteristic feature for thiol-protein oxidoreductase activity (Takahashi and Creighton 1996) and mutation of either of these cysteines changes the redox potential of MIF (Kleemann et al. 1998a; Kleemann et al. 1998b). Thus, it was investigated if the functional domains of MIF are relevant for the binding to RP S19. Pull-down experiments were performed with RP S19 and wt MIF and different MIF mutants: (1) P2A: Pro^2 is substituted by Ala; (2) C60S: Cys^{60} is substituted by Ser; (3) Δ4: the first

four N-terminal amino acids are removed. The sequences of these mutants were validated by MALDI-TOF MS. The P2A MIF and the Δ4 MIF mutants do not exhibit tautomerase activity, whereas the C60S MIF mutant does not possess oxidoreductase activity. The pull-down assays showed that the Δ4 MIF mutant showed similar binding affinity to RP S19 as the wild type MIF does, while the P2A- and C60S MIF mutants did not bind to the RP S19. In previous studies, far-UV CD spectroscopy analysis revealed that the spectra of P2A MIF, Δ4 MIF (Kleemann et al. 2000b) and C60S MIF mutants (Kleemann et al. 1999) were similar to the spectrum of wt MIF so that gross conformational integrity is not significantly affected by the introduced mutations. Thus, the presumtion that conformational modifications could prevent MIF mutants to interact with RP S19 can be disregarded. Since the C60S MIF mutant did not interact with RP S19, it can be concluded that Cys^{60} is essential for a direct interaction. In contrast, it was surprising that the Δ4 MIF mutant exhibited binding capacity to RP S19 similar to that of wt MIF, whereas the P2A MIF mutant did not bind to RP S19. A similar phenomenon was observed in studies on the enzymatic and immunologic functions of MIF. It was shown that the Δ4 MIF and P2A MIF mutants behave unequal in terms of glucocorticoid-antagonism and oxidoreductase activity (Kleemann et al. 2000b). Thus, the identification of RP S19 binding sites for MIF might provide an explanation for the drastic difference in binding capacity of the P2A mutant compared with wt MIF and Δ4MIF mutant.

6.3. MIF and RP S19 co-localize in the cytoplasm

In several cell types endogenous MIF was shown to be targeted to the cytosol (Kleemann et al. 2000a), while exogenous added recombinant MIFis taken up by cells and found in the cytoplasm and the lysosomal compartment (Kleemann et al. 2002). For RP S19, cellular localization studies have shown endogenous RP S19 to be present in the cytoplasm of NIH 3T3 fibroblasts (Soulet et al. 2001), while ectopically expressed RP S19 shows predominantly nucleolar localization in transfected Cos-7 fibroblast cells (Da Costa et al. 2003). The majority of the ribosome biogenesis takes place in a special compartment, the nucleolus but the final maturation of the small subunit occurs in the cytoplasm, a mechanism to prevent assembly of functional ribosomes within the nucleus.

This explains why ectopically expressed RP S19 was found predominantly in the nucleolus, while the endogenous RP S19 was found in the cytoplasmic extract. In the present study, double immunostaining performed on NIH 3T3 cells revealed that endogenous MIF and RP S19 co-localize in the cytoplasm of these cells. Since the MIF-RP S19 interaction was found in cytoplasmic extracts of NIH 3T3 cells, the immunohistochemical analysis confirms that the MIF-RP S19 interaction likely takes place in the cytoplasm of fibroblast cells.

6.4. RP S19 negatively modulates MIF tautomerase activity

The previous results demonstrate that RP S19 directly binds to MIF and that both proteins co-localize in the cytoplasm of NIH 3T3 cells. In order to assess the functional implications of this protein-protein interaction, RP S19 was tested in several MIF's functional assays.

The three-dimensional structure of MIF reveals that MIF shares a similar structure and active site to several bacterial enzymes 4-oxalocrotonate tautomerase and 5-carboxymethyl-2-hydroxymuconate isomerase (Sun et al. 1996a). MIF possesses the unusual ability to catalyze the tautomerization of the non-physiological substrates D-dopachrome and L-dopachrome methyl ester into their corresponding indole derivatives (Rosengren et al. 1996; Zhang et al. 1995). However, physiological substrates for the tautomerase activity have not been identified yet. Recently, small-molecule inhibitors that interact with the active tautomerase/isomerase pocket of MIF have been shown to inhibit its cytokine function (Lubetsky et al. 1999; Orita et al. 2002; Stamps et al. 1998). One compound, (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (known as ISO-1), inhibits the tautomerase activity of MIF and its cytokine actions on Phospholipase A_2 (PLA_2) activity, and also reverses glucocorticoid-inhibited TNF release (Lubetsky et al. 2002). More recently, this compound was reported to be active *in vivo* models improving survival in lipopolysaccharide models of endotoxic shock- and caecal ligation and puncture-induced models of septic shock (Al-Abed et al. 2005). ISO-1 has also been reported to be active in a model of diabetes mellitus-like pancreatic islet inflammation induced by streptozotocin (Cvetkovic et al. 2005).

In order to study if RP S19 affects the tautomerase activity of MIF, *in vitro* experiments with recombinant RP S19 and MIF were performed. The data show a dose-dependent inhibition of MIF's tautomerase activity by RP S19. A five fold molar excess of RP S19 over the MIF concentration repressed MIF tautomerase activity by 40%, while secretoglobin 2A1, a protein of similar molecular weight as RP S19 used as a control, had no influence on the tautomerase activity of MIF. If the inhibitory effect of RP S19 on MIF's tautomerase activity is relevant for the modulation of MIF-dependent pro-inflammatory events, remains to be investigated. In conclusion, the data show that RP S19 specifically represses MIF tautomerase activity, suggesting that RP S19-MIF interaction might play a role in the regulation of physiological MIF functions.

6.5. RP S19 prevents the pro-inflammatory action of MIF

Glucocorticoids are among the most effective anti-inflammatory substances known, acting through various mechanisms to inhibit inflammation (Tjandra et al. 1996). In contrast to other pro-inflammatory cytokines that are generally suppressed by glucocorticoids, MIF expression and secretion by macrophages, T cells and certain endocrine cells in response to varying concentrations of glucocorticoids is bimodal: it is increased in response to physiological concentrations of glucocorticoids (Calandra et al. 1995; Leech et al. 1999; Waeber et al. 1997), whereas higher concentrations of glucocorticoids do not induce MIF secretion. This feature closely mirrors bimodal physiological regulation of immune function by glucocorticoids.

The present study proved that RP S19 negatively modulate the tautomerase activity of MIF. Other studies identified a mechanism of inhibiting MIF pro-inflammatory activities by targeting its tautomerase activity (Kleemann et al. 2000b). ISO-1, the above mentioned small molecule inhibitor of MIF tautomerase activity, inhibits tumor necrosis factor α (TNF α) release from macrophages isolated from LPS treated wild type mice but has no effect on cytokine release from MIF-deficient macrophages. LPS, a gram negative bacterial cell wall component, is an inflammatory mediator that induces the release of TNFα from macrophage cell lines or primary monocytes (Bendrat et al. 1997; Roger et al. 2005). Glucocorticoids such as

dexamethasone exert anti-inflammatory effects by inhibiting TNFα release, whereas exogenously applied MIF counteracts this anti-inflammatory activity (Bucala 1996; Calandra and Bucala 1995). These studies demonstrate that the administration of recombinant MIF together with dexamethasone in mice completely blocks the protective effects of glucocorticoids on LPS lethality (Calandra et al. 1995).

In order to assess the impact of RP S19 on pro-inflammatory functions of MIF, a study was conduced in order to answer the question if RP S19 is capable to modulate the counter-regulatory activity of MIF on glucocorticoid-mediated immunosuppression. The results unequivocally show that RP S19 counteracts MIF's overriding effect on glucocorticoid inhibition of cytokine production leading to lower TNFα production in dexamethasone-treated and LPS-activated monocytes. A connection between MIF's glucocorticoid overriding action and its tautomerase activity was identified. It was shown that the N-terminal proline is essential for MIF's tautomerase activity and that its substitution with alanine abolishes this enzymatic activity (Lubetsky et al. 1999; Stamps et al. 1998). Interestingly, this MIF mutant is less efficient in counterregulating glucocorticoid-mediated immunosuppression (Kleemann et al. 2000b). In addition, other studies support the idea that MIF enzymatic activity is directly linked to its pro-inflammatory function (Al-Abed et al. 2005; Onodera et al. 2000; Swope et al. 1998; Zang et al. 2002) and low molecular weight inhibitors of MIF's enzymatic activity were developed in order to block the pro-inflammatory function (Lubetsky et al. 2002; Senter et al. 2002). It was shown that ISO-1 leads to abrogation of MIF glucocorticoid-overriding capacity. Current data suggest that neutralization of the pro-inflammatory activity of MIF would be highly beneficial in the treatment of several inflammatory disorders (Calandra and Roger 2003; Riedemann et al. 2003). This assertion is supported by the substantial therapeutic effects of MIF-specific antibodies in several models of inflammatory and autoimmune diseases. Administration of neutralizing anti-MIF antibodies has proven therapeutically effective in numerous animal models of systemic inflammation, including gram-negative, gram-positive, and polymicrobial sepsis, arthritis, and autoimmune diabetes (Bernhagen et al. 1993; Bozza et al. 1999; Calandra et al. 2000; Lan et al. 2000; Murakami et al. 2002).

Intracellular MIF plays a critical role in mediating cellular response to pathways

activated by LPS (Roger et al. 2001) and is required for basal expression of Toll like receptor 4 (TLR4), the endotoxine receptor. Accordingly, inhibition of MIF by RP S19 would suppress the endotoxin response in macrophages. Thus, RP S19 recapitulates the phenotype of MIF deficient macrophages which are hyporesponsive to endotoxin (Bozza et al. 1999; Roger et al. 2001), and is associated with decreased TNFα production in response to LPS. Thus, RP S19 is the first identified endogenous compound capable to modulate MIF's tautomerase activity and its proinflammatory function.

6.6. RP S19 blocks MIF-induced monocyte migration

It is known that chemokines are secreted by damaged or inflamed tissue and act as chemoattractants for specific types of white blood cells, causing these cells to become polarized and migrate toward the source of the attractant. As a result, large numbers of white blood cells enter the affected tissue. Once the monocytes leave the blood stream, they become activated and transformed to macrophages, which phagocytose and digest invading microorganisms and foreign bodies as well as damaged senescent cells.

MIF was initially identified as a soluble factor produced by activated T lymphocytes that could inhibit the random migration of macrophages (Bloom and Bennett 1966; David 1966). In the mentioned experiment, the supernatant of sensitized peritoneal T cells prevented the migration of macrophages out of a capillary tube. However, looking from a different angle this activity could likewise be that of a chemokine that attracts the macrophages to the capillary rather than simply blocking migration out of it. Several studies support the idea of inhibitory effects of MIF on both random migration and chemotaxis of human monocytes (Bloom and Bennett 1966; David 1966; Weiser et al. 1989). MIF was recognized to be associated with immune cell activation (Metz and Bucala 1997; Swope and Lolis 1999), and influences the migration and proliferation of various cell types, predominantly monocytes and macrophages (Lacey et al. 2003). Recent evidence suggests an important role for MIF in the progression of atherosclerosis (Burger-Kentischer et al. 2006; Morand et al. 2006) and restenosis (Chen et al. 2004). MIF also acts as a chemoattractant for vascular smooth muscle cells (Schrans-Stassen et al. 2005) and freshly separated rat keratinocytes (Abe et

al. 2000). It was shown that MIF is also abundantly produced by monocytes/macrophages (Onodera et al. 1997), and acts in an autocrine/paracrine fashion to up-regulate and sustain cell response to concurrent, activating stimuli (Bozza et al. 1999; Mitchell et al. 2002).

In this context, it was intended to specify the mode of MIF action on monocyte/macrophage migration and to explore if RP S19 is capable to modulate MIF's chemotactic activity. Previous studies have shown that the monocyte chemotactic protein 1 (MCP-1) possesses a strong chemoattractant effect on monocytes and macrophages (Kakizaki et al. 1995; Segerer et al. 2000). MIF was shown to abrogate MCP-1-induced monocyte-derived macrophage migration when the cells were pre-incubated with MIF prior to the investigation of their migration to MCP-1 (Hermanowski-Vosatka et al. 1999). However, the mode of MIF's action on macrophage migration arrest has not yet been analyzed in detail. In this context, it was investigated whether the inhibitory effect of MIF on macrophage migration is in fact a chemoattractant activity. Using peripheral blood monocyte cells (PBMC), and a modified experimental setup, PBMC chemotaxis was investigated by using MCP-1 or MIF as chemoattractants. MIF displayed a strong chemoattractant effect on PBMCs which was comparable to that of MCP-1. Thus, the effect of MIF on the migration of PBMCs is in fact not an inhibitory one but a distinct chemoattractant. In conclusion, the results suggest that MIF rather acts as a chemokine that recruits macrophage to the sites of inflammation than as a migration inhibitor.

RP S19 was reported to homologously cross-link by a transglutaminase-catalysed reaction (Nishiura et al. 1996) and being released as a dimer into the extracellular milieu during apoptosis (Nishiura et al. 1996). The homodimer functions as a monocyte-selective chemoattractant *in vitro* and recruits circulating monocytes to the apoptotic lesion *in vivo* by means of binding to the C5a receptor, while the monomer has no chemoattractant capacity (Nishiura et al. 2005). The recruited monocytes clear the apoptotic cells by phagocytosis and rapidly translocate to the regional lymph nodes via lymphatics to present potential non-self antigens derived from apoptotic cells to T cells. By promoting the clearance of apoptotic cells, the RP S19 dimer could play a role in preventing tissue damage, inflammation and autoimmune reactions. Interestingly, the RP S19 dimer but not C5a behaves as an antagonist of the C5a receptor on

polymorphonuclear leukocytes (Shrestha et al. 2003).

In the present study, it was examined whether the RP S19 monomer is capable of modulating MIF's chemoattractant activity. The migration assays revealed that RP S19 significantly inhibits MIF's chemoattractant activity on PBMCs, whereas RP S19 alone did not influence the migration of PBMCs. Thus, the high chemotactic responsiveness of macrophages to MIF can be attenuated by the addition of RP S19.

If the discovered inhibiting effect of RP S19 on MIF chemoattractant activity is functionally relevant for MIF-dependent proinflammatory events remains to be demonstrated by *in vivo* studies. In summary, MIF might contribute to the immune response by recruiting macrophages to the sites of inflammation, whereas RP S19 monomer could modulate macrophage migration by attenuating MIF's chemoattractant activity.

7. SUMMARY

Macrophage migration inhibitory factor (MIF) is a naturally occurring immune modulator which is synthesized by various cell types and originally has been named by its ability to inhibit the random migration of human monocytes. More recently, MIF was shown to override the immunosuppressive effects of glucocorticoids and to stimulate pro-inflammatory cytokine expression such as TNF-α in leukocytes. Accumulating research over the past ten years revealed an important role for MIF in normal and pathological immune function. However, the molecular mechanism of MIF action and its modulation in normal and diseased state remain poorly understood. To gain more insight into MIF's biological role, the research focus of this thesis was put on the identification of MIF interacting proteins with the ability to modulate relevant MIF functions.

The present study demonstrates that the ribosomal protein S19 (RP S19) interacts with the cytokine MIF and that both proteins are co-localized in the cytoplasm of NIH 3T3 cells. The interaction was found by co-immunoprecipitation in extracts of NIH3T3 cells. Direct interaction of MIF and RP S19 was verified *in vitro* by pull-down assays following cloning and expression of recombinant proteins. To identify crucial domains for MIF-RP S19 interaction, further pull-down assays were performed with wild type (wt) MIF and Δ4MIF, P2AMIF and C60SMIF mutants. Human wtMIF and Δ4MIF mutant exhibited similar levels of interaction with RP S19, whereas the human P2A- and C60SMIF mutants did not display any affinity to RP S19.

In order to evaluate the functional role of this interaction, the effect of RP S19 on relevant MIF functions was further investigated. RP S19 was found to inhibit MIF's tautomerase enzymatic activity in a dose-dependent manner and to counteract the overriding effect of MIF on glucocorticoid inhibition of TNF-α production under LPS stimulation. Several studies support the idea that the tautomerase activity of MIF is directly linked to its pro-inflammatory functions and that the attenuation of the tautomerase activity by synthetic inhibitors results in the abrogation of MIF's glucocorticoid-overriding activity. Thus this study has identified RP S19 as the first endogenous molecule capable of modulating both MIF's tautomerase activity and its pro-

inflammatory function by influencing glucocorticoid sensitivity.

In investigations of other authors, MIF was shown to abrogate monocyte chemotactic protein (MCP) 1-induced macrophage migration, emphasizing the original view that MIF exerts an inhibitory effect on monocyte/macrophage migration. In this context, our study intended to specify the mode of MIF's action on monocyte/macrophage migration and to explore if RP S19 is capable to modulate the migration inhibiting activity of MIF. Surprisingly, MIF displayed a previously unknown strong chemoattractant effect on peripheral blood monocytes, suggesting that MIF acts rather as a chemokine to recruit macrophages to sites of inflammation than as a migration inhibitor of those cells which already have been attracted by other factors. The chemoattractant activity of MIF is significantly inhibited by RP S19, whereas RP S19 alone as a control did not influence monocyte migration. Thus, the high chemotactic responsiveness of macrophages to MIF can be attenuated by the addition of RP S19.

For a number of ribosomal proteins, including RP S19, also extraribosomal functions have been reported beside the participation in protein synthesis. RP S19 released by necrotic or apoptotic cells in inflammatory lesions could serve to control the outcome and magnitude of an inflammatory response. Future studies will aim to investigate if RP S19 could be used in animal models or in pre-clinical applications as a potential new tool to inhibit the pro-inflammatory functions of MIF.

8. ZUSAMMENFASSUNG

Der Makrophagen-Migrations-Inhibitionsfaktor (MIF) ist ein endogener Modulator des Immunsystems und wird von einer Vielzahl von Zelltypen synthetisiert. MIF verdankt seinen Namen der Fähigkeit, die ungerichtete Migration von humanen Monozyten *in vitro* zu hemmen. Neuere Untersuchungen zeigen, dass MIF die immunsuppressiven Wirkungen von Glukokortikoiden aufheben und die Synthese von proinflammatorischen Zytokinen, wie z.B. TNF-α, in Leukozyten stimulieren kann. Obwohl in den vergangenen zehn Jahren viele Studien gezeigt haben, dass MIF eine entscheidende Rolle bei einer ganzen Reihe von normalen und pathophysiologischen Immunprozessen spielt, wurden die zugrunde liegenden molekularen Mechanismen nur unzureichend aufgeklärt. Um die biologische Rolle von MIF besser verstehen zu können, sollten in dieser Arbeit neue Proteine identifiziert werden, die mit MIF interagieren und dessen Funktionen modulieren können.

Es wurde nachgewiesen, dass MIF mit dem ribosomalen Protein S19 (RP S19) interagiert, und das beide Proteine im Zytoplasma von NIH 3T3 Zellen lokalisiert sind. Die Interaktion von MIF und RP S19 *in vivo* konnte durch Koimmunpräzipitation identifiziert und über *in vitro pull-down* Experimente validiert werden. Die *in vitro* Versuche mit rekombinantem MIF und RP S19 machten zudem deutlich, das MIF und RP S19 direkt miteinander interagieren, d.h. ohne die Beteiligung möglicher Kofaktoren. Durch *in vitro* pull-down Experimente mit rekombinantem Wildtyp MIF (wtMIF) und den MIF-Mutanten Δ4MIF, P2AMIF und C60SMIF wurden Aminosäuren im MIF Polypeptid identifiziert, die für die Interaktion mit RP S19 notwendig sind. Hierbei zeigten wtMIF und Δ4MIF eine vergleichbare Bindungsaffinität zu RP S19, wohingegen die MIF-Mutanten P2AMIF und C60SMIF nicht mehr mit RP S19 interagieren konnten.

In nachfolgenden Studien wurde die funktionelle Bedeutung der MIF/RP S19 Interaktion eingehender charakterisiert. So konnte zunächst gezeigt werden, daß RP S19 die Tautomeraseaktivität von MIF dosisabhäng hemmt. Weiterhin wurde nachgewiesen, daß Glukokortikoide die Ausschüttung von TNFα nach LPS-Induktion humaner Blutmonozyten inhibiert und MIF diese Wirkung aufheben kann (*glucocorticoid*

overriding effect), allerdings nicht in Anwesenheit von RP S19. Verschiedene Studien weisen auf einen ursächlichen Zusammenhang zwischen der Tautomeraseaktivität und pro-inflammatorischen Wirkungen von MIF hin, da synthetische Inhibitoren der Tautomeraseaktivität den *glucocorticoid overriding effect* neutralisieren können. Somit konnte über die funktionelle Charakterisierung der MIF/RP S19 Interaktion erstmalig ein endogener Modulator der Tautomeraseaktivität von MIF und folglich der proinflammatorischen Eigenschaften von MIF identifiziert werden.

In Untersuchungen anderer Autoren wurde ein inhibitorischer Effekt von MIF auf die *monocyte chemotactic protein* (MCP) 1-vermittelte Migration von Makrophagen beobachtet, was frühere Befunde zur Migrationsinhibiton von Makrophagen durch MIF bestätigte. Daher wurde die MIF-abhängige Migration von Monozyten/Makrophagen eingehender charakterisiert und der Einfluss von RP S19 darauf untersucht. Überraschenderweise zeigte sich, dass MIF ein starkes Chemoattraktant für periphere Blutmonozyten darstellt. Folglich weist MIF keine antimigratorischen Eigenschaften auf, sondern fungiert eher als ein Zytokin, das Monozyten zum Ort der Entzündung aktiv rekrutiert. Dagegen wurde bei gleichzeitiger Applikation von MIF und RP S19 eine signifikante Hemmung der chemoattraktiven Eigenschaften von MIF nachgewiesen, wohingegen RP S19 allein keinen Einfluss auf die Migration von Monozyten ausübt.

Für verschiedene ribosomale Proteine, einschließlich RP S19, konnten - neben ihrer Bedeutung in der Ribosomenbiogenese und der Proteinbiosynthese - auch extraribosomale Funktionen nachgewiesen werden. Als Folge von Entzündungsprozessen könnte die Freisetzung von RP S19 aus nekrotischen und apoptotischen Zellen den Verlauf und das Ausmaß von Entzündungsreaktionen beeinflussen. Zukünftige Untersuchungen werden zum Ziel haben, den therapeutische Nutzen von RP S19 als Inhibitor proinflamatorischer MIF-Funktionen zunächst im Tiermodell und gegebenenfalls in vorklinischen Studien zu testen.

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Ana-Maria DOBRE, Jörg KLUG, Andreas MEINHARDT, RP S19 interacts with macrophage migration inhibitory factor and attenuates its pro-inflammatory function. *NGFN RNAi 2005 GBM Annual Fall Satellite, Berlin, Germany, September 18-21, 2005*

Ana-Maria DOBRE, Jörg KLUG, Suada FRÖHLICH, Regina EICKHOFF, Monica LINDER, Andreas MEINHARDT, Ribosomal protein S19 interacts with macrophage migration inhibitory factor and attenuates its proinflammatory function. *Molecular Andrology, Giessen, Germany, October 7-9, 2005.*

Ana-Maria FILIP, Jörg KLUG, Monica LINDER, Regina EICKHOFF, Andreas MEINHARDT, Identification of Ribosomal Protein S19 (RP S19) as binding partner of Macrophage Migration Inhibitory Factor (MIF): RP S19 attenuates key pro-inflammatory actitivties of MIF, *International symposium functional genomics of infectious diseases and inflammation, Giessen, Germany, June, 30, July 2, 2006*

Publications

Gabriela KRASTEVA, Uwe PFEIL, **Ana-Maria FILIP,** Katrin S. LIPS, Wolfgang KUMMER, Peter KÖNIG, Confinement of caveolin-3 together with eNOS and CHT1 to an apical compartment in ciliated airway epithelial cells in the rat, manuscript in revision, International Journal of Biochemistry and Cell Biology.

Ana-Maria FILIP, Jörg KLUG, Sevil CAYLI, Suada FRÖHLICH, Tamara HENKE, Patrick BULAU, Regina EICKHOFF, Monika LINDER, Henning URLAUB, Jürgen BERNHAGEN, Andreas MEINHARDT, Ribosomal Protein S19 Interacts with Macrophage Migration Inhibitory Factor and attenuates its Pro-Inflammatory Function, manuscript in preparation to be submitted to The Journal of Biological Chemistry in October 2006.

12. EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre: die vorgelegte Dissertation selbstständig, ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt zu haben, die in der Dissertation angegeben sind. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichen Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.

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Giessen, September 2006