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Forum Review

Link Between Macrophage Migration Inhibitory Factor and Cellular Redox Regulation

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is an evolutionary conserved 12.5-kDa protein mediator with multiple functions in innate and acquired immunity. Upon leaderless secretion, MIF acts as a typical inflammatory cytokine, but there is no structural homology between MIF and any of the known cytokine protein families. Also, MIF is unique among cytokines in that it exhibits certain endocrine properties and has enzymatic activity. The catalytic thiol-protein oxidoreductase (TPOR) activity of MIF is mediated by a Cys-Ala-Leu-Cys active site between residues 57 and 60 that can undergo reversible intramolecular disulfide formation. Such a redox motif is typically found in TPORs of the thioredoxin (Trx) family of proteins. MIF seems to act as a disulfide reductase, and structure-function analyses of the redox site indicate that this activity is not only observed *in vitro*, but plays a role in cellular redox homeostasis, apoptosis inhibition, MIF-mediated monocyte/macrophage activation, and possibly the modulation of the activity of MIF-binding proteins. In this *Forum* review, the biochemical and biological evidence for a role of the TPOR activity for various MIF functions is summarized and discussed. In particular, the marked functional homologies with Trx proteins, the MIF redox/MHC II link, and recent attempts to discern the intra- versus extracellular roles of the MIF TPOR activity are dealt with. *Antioxid. Redox Signal.* 7, 1234–1248.

INTRODUCTION

IDENTIFIED FOUR DECADES AGO as a lymphocyte-derived immune activity, macrophage migration inhibitory factor (MIF) was one of the first soluble immune mediators (16, 31, 32, 49) to be discovered ever. In those early studies, the migration inhibitory properties of MIF toward macrophages and its role in cellular immunity, in particular, delayed-type hypersensitivity reactions, were primarily investigated. The molecular entity responsible for the discovered MIF activities remained obscure for almost another quarter of a century until MIF was eventually cloned in 1989 from T cells (151).

Today, it is known that MIF is not only produced by T lymphocytes to regulate macrophage migration, but is a prominent product of the macrophage as well (22). Moreover, MIF expression is not restricted to T cells and macrophages. Other immune cells, as well as some endocrine and parenchymal cells, express and secrete MIF (9, 92). Together, these and other observations have shown that MIF is a pleiotropic inflammatory cytokine and endocrine factor with various functions in innate and acquired immunity (21, 113). As known for several other inflammatory cytokines, MIF can aggravate inflammatory processes under pathophysiological conditions. Thus, MIF was consequently identified to be a pivotal mediator of acute and chronic inflammatory conditions, such as septic shock, colitis, rheumatoid arthritis, acute respiratory distress syndrome, atherosclerosis, pancreatitis, uveitis, as well as tumorigenesis. Our current understanding of these important physiological roles of MIF in homeostasis and immunity and its pathophysiological significance in a number of disease conditions has been summarized and discussed in several excellent recent review articles (12, 17, 18, 20, 21, 30, 35, 83, 88, 92, 97, 104, 120, 150).

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In this *Forum* review, we will focus on the catalytic thiol-protein oxidoreductase (TPOR) activity of MIF and its role for the biological effects of MIF and for MIF-mediated signal transduction. We will also discuss differences in the intra- versus extracellular roles of the TPOR activity of MIF, and its importance for protein–protein interactions involving MIF and its binding partners. We view the reviewing of these recent advancements in understanding MIF's role as a TPOR as important, as these studies are suggestive of an important role of the TPOR activity for MIF's immunological and cellular effects. Also, in spite of the numerous recent MIF review articles, there has been no review appropriately addressing the oxidoreductase activity of MIF.

OVERVIEW OF MIF STRUCTURE

MIF is a 12.5-kDa polypeptide that does not belong to any of the known cytokine protein or cytokine receptor classes (151). MIF does not have an N-terminal signal peptide, and it is secreted by a leaderless export pathway (41). With the exception of the processing of the N-terminal methionine residue, no posttranslational modifications of MIF have been identified (10). X-ray analysis indicates that MIF forms a homotrimer (132, 133), but structural analysis of MIF in solution rather suggests that MIF forms a monomer or dimer at physiological concentrations. Alternatively, an equilibrium of the three oligomeric species may exist between which MIF may switch depending on the surrounding solvent and binding partner parameters (8, 48, 96, 99, 140). The MIF monomer consists of six (or seven) β-strands, of which four form a β-sheet that is flanked by two perpendicular α-helices. In the trimer, the central β-sheet is extended by intermolecular interaction with β-strands from the adjacent subunits. One of the helices exhibits a marked amphiphilic nature. An observed channel or pore structure in the center of the trimer does not appear to be functionally relevant (132, 133).

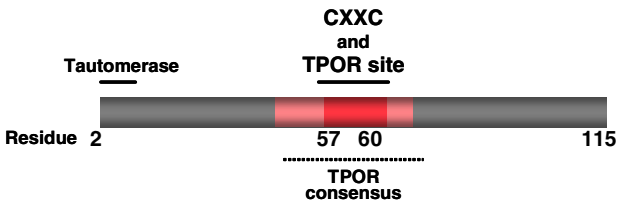
Given that MIF is a relatively small protein mediator, it is not surprising that it exhibits no domain structure. However, two conserved sequence motives have been identified. The motives have been found to represent local catalytic centers of MIF that are responsible for two distinct catalytic activities, a tautomerase/isomerase and a TPOR activity (see next section). With respect to its structure, it is worth mentioning that MIF has a striking three-dimensional (3D) architectural homology with certain bacterial tautomerases, as well as human D-dopachrome tautomerase (DDT), that also share with MIF the catalytic tautomerase site. However, there is only a marginal sequence homology between MIF and these proteins. The functional implications of the observed 3D structural homology are currently unclear. In addition, MIF shares a high sequence and 3D structural homology with glycosylation inhibition factor (GIF). In fact, both proteins have an almost identical sequence and structure, and it is believed today that they are identical proteins. However, functional differences between MIF and GIF arise from specific posttranslational modifications that GIF can undergo in T suppressor cells. A number of recent reviews have comprehensively summarized our knowledge on the further structural features of MIF, including its gene structure and N- and C-terminal structure-function analysis (12, 21, 34, 52, 89, 92), and we will therefore not discuss these issues further in the current review.

MIF AS AN ENZYME AND IDENTIFICATION OF ITS TPOR ACTIVITY

As mentioned above, MIF does not have domains, but only possesses two conserved local sequence motives. When searching for an uncovered D-dopachrome-converting isoenzyme of melanin biosynthesis from bovine lenses, Rorsman and colleagues identified MIF to be capable of catalyzing the conversion of the non–naturally occurring D-isomer of 2-carboxy-2,3-dihydroindole-5,6-quinone (dopachrome) into 5,6-dihydroxyindole-2-carboxylic acid. They concluded that MIF had catalytic activity as an isomerase/tautomerase *in vitro* and later discovered that also phenylpyruvate could be converted by MIF *in vitro* into its tautomer/isomer (114, 115). That MIF can act as an isomerase/tautomerase became convincingly clear, when almost at the same time the elucidation of the structure of rat MIF by x-ray crystallographic analysis revealed that MIF not only shared with certain isomerases/tautomerases, such as 5-carboxymethyl-2-hydroxymuconate isomerase and 4-oxalocrotonate tautomerase, a conserved N-terminal proline residue, but also had a striking 3D architectural similarity to these enzymes (133). In addition, although there was no significant sequence homology between MIF and the bacterial isomerases/tautomerases, MIF was found to have a 27% sequence homology as well as high structural similarity to the DDT/phenylpyruvate tautomerase 2 (PPT2) (130). The structural studies, as well as subsequent mutational analyses, demonstrated that the main structural feature responsible for the isomerase/tautomerase activity is a conserved basic N-terminal proline residue at position 2 together with neighboring aromatic residues which reside in part within the C-terminal end of MIF (8, 91, 105, 128, 133, 137). Despite overwhelming evidence on the significance of the tautomerase activity of MIF *in vitro*, its physiological role has been controversial, as no physiological substrate has yet been identified and because tautomerase-dead mutants still exert MIF-like biological activities in certain immunological assays (61, 114, 115, 134). Thus, it has been proposed that the tautomerase activity of MIF could be due to divergent evolutionary processes and might not be needed in the elaborated immune defense systems that MIF is involved in nowadays.

Almost simultaneously with the discovery of MIF's tautomerase activity, a second sequence motif located within the center of the MIF molecule was identified by Kleemann, Bernhagen and colleagues and was found to mediate catalytic activity (11, 77). This was the so-called Cys-Xaa-Xaa-Cys (CXXC) motif. In the case of MIF, the CXXC is a Cys-Ala-Leu-Cys (CALC) motif (Fig. 1A). The CXXC region is typically found in the thioredoxin (Trx) superfamily of TPORs. In addition to several Trx species, redox-regulating proteins such as glutaredoxin (Grx), peroxiredoxin (PAG), protein disulfide isomerase (PDI), and disulfide bond proteins (Dsb) belong to the TPOR family (40, 44, 45, 65–69, 107, 152). Although the residues between the two neighboring cysteines vary between the family members, these proteins all share additional, conserved residues upstream of the CXXC motif.

A



B

MIF/TPOR	Motif						
Human MIF	DQ	**L**	MA	**F**	GGSSEP	**C**ALC	SLHSIGKI
Mouse MIF	DQ	**L**	MT	**F**	SGTNDP	**C**ALC	SLHSIGKI
Rat MIF	DQ	**L**	MT	**F**	SGTSDP	**C**ALC	SLHSIGKI
Bovine MIF	DQ	**L**	MT	**F**	GGSSEP	**C**ALC	SLHSIGKI
Arabidopsis MIF	SV	P	MS	**F**	GGTEDP	AAYG	ELVSIGGL
Brugia malayi-MIF	GQ	**A**	MV	**F**	GGSEDP	**C**AV**C**	VLKSIGCV
Wuchereria bancrofti-MIF	GQ	P	MV	M	GGSEDP	**C**PV**C**	VLKSIGCV
Human Thioredoxin	LV	**V**	VD	**F**	SATW	**C**GP**C**	KMIKPFFH
Human Glutaredoxin	GK	**V**	VV	**F**	IKPT	**C**PY**C**	RRAQEILS
DsbA	PQ	**V**	LE	**F**	FSFF	**C**PH**C**	YQFEEYLH
PDI precursor/prolyl 4-hydroxylase β-subunit	VF	**V**	E	**F**	YAPW	**C**GH**C**	KQLAPIWD

FIG. 1. **(A)** Scheme of human MIF and localization of its CXXC motif. The CXXC motif is located between residues 57 and 60. The TPOR consensus region is also indicated (for details, see B). For overview, the N-terminal tautomerase site is depicted. Numbering of amino acids refers to the translated cDNA sequence. Note that the N-terminal methionine residue of MIF is processed in all cells examined so far. (**B**) Similarity between the extended TPOR consensus region of several MIF orthologues and members of the Trx protein family. The extended TPOR region according to Ellis *et al.* (39) was analyzed. Conserved residues are presented in bold letters.

These are a phenylalanine five to seven residues upstream of the N-terminal cysteine of CXXC and a leucine or valine another two or three residues further upstream (39). MIF fulfils these extended TPOR motif parameters in having a Phe at position −7 of the CXXC and a Leu residue at −10 (39, 77).

TPORS

TPORs are enzymes involved in disulfide-mediated oxidation–reduction and folding reactions, and their catalytic activity is based on the formation or reduction of a catalytic disulfide bridge between the two vicinal cysteines of the CXXC region. More recent evidence suggests that also additional cysteine residues such as Cys^{62} and Cys^{69} of human Trx may contribute to the redox activity of TPORs (147). Depending on the overall 3D structure of the protein and the residues in the vicinity of the CXXC region, TPORs can have a more reducing or oxidizing redox potential. For example, Trx has a strongly reducing potential of approximately −270 mV and thus primarily acts as a disulfide reductase, whereas PDI has an oxidizing potential of approximately −100 mV and mainly assists in disulfide-mediated protein folding processes (3).

THE CXXC MOTIF, CXXC DISULFIDE BOND FORMATION, AND CATALYTIC TPOR ACTIVITY OF MIF

The CALC motif of MIF is well conserved through all mammalian MIF orthologues (Fig. 1B). Also, the parasite proteins *B. malayi* MIF and *W. bancrofti* MIF have a similar motif, with CAVC and CPVC sequences, respectively. Moreover, although not identical, the residues between the CXXC cysteines in MIF are somewhat similar in size and hydrophobicity to those in other TPORs (77) (Fig. 1B). Of note, the MIFs exhibit almost identical β-turn propensities around 9% in their CXXC regions when compared with TPORs such as Grx or DsbA (77, 127). It is believed that disulfide formation between the CXXC cysteines of the catalytic site of a TPOR is accompanied by the corresponding formation of a β-turn. Together, this suggests that disulfide formation may occur at the CXXC site of MIF and that MIF might be able to catalyze CXXC-mediated oxidation–reduction reactions. This notion is strongly supported by *S*-alkylation experiments in combination with mass spectrometric analysis of natively folded recombinant human MIF (rMIF) and demonstrated that under oxidizing conditions a significant portion of MIF can form a disulfide structure involving Cys residues 57 and 60, *i.e.,* those cysteines constituting the CXXC site (77). Near-UV circular dichroism spectropolarimetry reveals a characteristic maximum at ~280 nm and thus provides additional evidence that disulfide formation occurs upon oxidation of MIF.

As discussed above, MIF forms oligomeric structures, but it has not yet been clarified whether the monomer, dimer, or trimer is the physiologically relevant species, or whether there is an equilibrium between these species in solution. The x-ray crystallographic analyses unanimously show that all three Cys residues of MIF exist in their reduced thiol forms in the crystallized trimer (129, 132, 133). Biochemical and cross-linking studies of MIF further indicate that none of the Cys residues is involved in an intermolecular disulfide bond (10, 96, 151). Yet the biochemical solution methods argue for

the formation of an intramolecular disulfide bond between Cys^{57} and Cys^{60} under appropriate conditions (77). This apparent contradiction may be resolved if one considers the different preparation procedures and MIF concentrations used. For the x-ray crystallographic analyses, MIF was prepared from *E. coli* overexpression systems under reducing conditions and crystallization was achieved from a solution of ~10 mg/ml (132, 133). In contrast, the biochemical solution analyses involved a renaturation procedure during which *E. coli*-derived MIF is refolded under controlled oxidizing conditions. Also, renaturation is performed at more physiological MIF concentrations of ~300 μg/ml, and biologically active rMIF at concentrations between 1 and 100 ng/ml is derived from such preparations by simple dilution (10, 22, 36, 77). Thus, it appears that MIF can form an intramolecular CXXC disulfide bond, when it is refolded under controlled oxidizing conditions, as they prevail in the circulation or within distinct subcellular compartments. The enzyme responsible in the cell for reducing/rereducing MIF's CXXC thiol groups is unknown.

The CXXC consensus motif of typical TPORs such as Trx lies in a so-called Trx fold, where it is located at the N-terminus of an α-helix. Although the overall 3D structure of the MIF monomer shows a remote resemblance to the Trx monomer, MIF is not structurally homologous to the TPOR proteins. Also, the CALC redox motif of MIF lies at the N-terminus of a β-strand element with Cys^{57} located in the preceding loop rather than in a Trx-like fold. As MIF exhibits CXXC-dependent TPOR activity and is able to catalyze the reduction of insulin and small-molecular-weight compound disulfides similar to Trx family proteins (see below for details), it appears that during catalysis the structure of MIF may be partially unfolded or changed. In this respect, it should be remembered that mono- or dimeric MIF could represent the relevant structure in solution, and that those species may have slightly different conformational properties (96). Interestingly, thioredoxin reductase, another member of the TPOR family, does not contain an apparent Trx fold structure, but otherwise shares similar redox properties with the TPOR proteins, indicating that it may undergo a marked conformational change during catalysis (145). The active conformation could be a 'relaxed' conformation, as substrates for the redox activity of Trx have been found to bind to the active-site cleft in extended strand structure (37). The fact that TPOR-derived small peptide sequences have TPOR protein-like catalytic properties as shown for Trx, Grx, and PDI peptides (19, 124–126, 154) further indicates that partially unfolded, altered, or relaxed conformational elements are involved in CXXC redox catalysis. Of note, MIF-derived 10- and 16-amino acid peptides were identified that undergo disulfide formation of their CXXC cysteines and that exhibit catalytic TPOR activity (102). Thus, upon interaction with its redox substrates, MIF may undergo a conformational change that may allow for disulfide bond formation within its CXXC site and concomitant reduction of the substrate.

MIF exhibits catalytic TPOR activity *in vitro*, apparently acting primarily as a disulfide reductase. It can reduce protein and peptide disulfides as shown for the substrates insulin and a cysteinylated insulin peptide (77, 78, 109). Glutathione (GSH) and dihydrolipoamide act as cosubstrates for the insulin-reducing activity (78). MIF can also reduce small-molecular-weight disulfides such as 2-hydroxyethyldisulfide (2-HED) (77). The cosubstrate profile utilized by MIF suggests that MIF's redox activity properties could be more similar to those of Grx than Trx. In support of this notion is the finding that MIF-derived redox-active peptides can form mixed disulfides with cysteine (102), as well as the observation that the catalytic TPOR activity is fully dependent on the presence of Cys^{60}, whereas the Cys^{57} mutant shows partial rest activity. C60SMIF exhibits essentially no catalytic activity in the 2-HED and insulin reduction assays [5% and 14% of wild-type (wt) MIF, respectively]. Similarly, the double mutant C57SC60SMIF has only 20% of the insulin reduction activity of wtMIF. In contrast, C57SMIF retains >50% of the activity of wtMIF in both assays. The latter is indicative of a monothiol mechanism as has been proposed for Grx (40, 93, 116).

The actual redox potential of MIF's redox site is unknown, but the redox potential of the MIF-derived CXXC-spanning peptide MIF(50–65) with an E'_0 value of −258 mV suggests that also the redox potential of full-length MIF is reducing rather than oxidizing (102). In line with this notion, MIF is able to reduce 2-HED (see above). TPOR-derived small peptide sequences encompassing the CXXC motifs of the parent proteins have been studied extensively, and data on their redox potentials have in part been useful in predicting the redox potentials of the corresponding full-length proteins (19, 125, 126). In addition to its CXXC cysteine residues at positions 57 and 60, MIF has a third cysteine at position 81, but extensive structure-activity studies of C81SMIF in comparison with wtMIF and the catalytic center mutants C60SMIF and C57SMIF suggest that this residue is not involved in the oxidoreductase activity of MIF (79). It should be noted that PPT2 (also alternatively termed DDT, according to its functions as phenylpyruvate tautomerase 2 and D-dopachrome tautomerase, respectively), which shares 27% sequence identity with MIF and is highly similar in its 3D architecture (130), but has no CXXC motif due to the lack of a cysteine residue at position 60, is devoid of any catalytic TPOR activity (79).

Compared with Trx and Grx, MIF has a relatively low catalytic activity in the insulin disulfide reduction and 2-HED assays, respectively. This could mean that MIF is a low-efficiency redox catalyst such as the peroxiredoxins (see also below). Alternatively, MIF may rather act as a donor of proteinaceous reducing equivalents through its reduced CXXC thiol groups. Such a mechanism has also been suggested for the antiapoptotic activity of Trx (72). Thus, MIF could be both a low-efficiency redox catalyst and thiol group donor.

BIOLOGICAL EVIDENCE FOR PARTICIPATION OF MIF IN CELLULAR REDOX REGULATION

Over the past few years, a wealth of evidence has become available that demonstrates that MIF plays a role in cellular redox regulation. As MIF had been found to preferentially utilize the physiological cosubstrates GSH and dihydrolipoamide as reducing agents in the insulin reduction assay

and as GSH and lipoamide are potent antioxidants and regulators of cellular oxidative stress (5, 40, 55, 76, 122), it was surmised already several years ago that MIF could be involved in cellular redox regulation. Cellular MIF levels are induced by hydrogen peroxide (H_2O_2), and MIF is secreted upon H_2O_2 stimulation from epithelial cells, macrophages, and neonatal cardiac myocytes (47, 78). Of note, in cardiac myocytes MIF was identified as a key "factor induced by oxidative stress" (FSO) (47, 78). That the production of MIF is controlled by the cellular redox state was also demonstrated *in vivo*. Peritoneal macrophages from rodents produce significantly less MIF upon lipopolysaccharide or phorbol ester stimulation, when the animals are previously injected with vitamin E, a treatment that results in a marked increase of the cellular α-tocopherol content and decrease of cellular oxidative stress (119).

Not only is MIF an indicator of oxidative stress situations of the cell, upon which it is produced and secreted at increased rates, but it also seems to participate directly in modulating the cellular response to redox stress. H_2O_2 treatment of cells may lead to changes in the cellular GSH/GSSG ratio with a resultant decrease in the GSH pool. It was thus of interest whether MIF could modulate cellular GSH levels. By applying an inducible Tet-Off cell system that allows for the defined enhancement of intracellular MIF levels in HeLa cells subjected to oxidative stress, it was demonstrated that MIF leads to a significant increase of the cellular GSH concentration and thus participates in the cellular response that leads to an elevation of cellular antioxidants upon stress (101).

Jung and colleagues have found one possible mechanism by which this kind of regulation may occur. They identified specific protein complexes between MIF and PAG, a thiol-specific cellular antioxidant protein (75). PAG belongs to the protein family of peroxiredoxins. Peroxiredoxins are a subfamily of low-efficiency peroxidases that lack prosthetic groups or catalytically active heteroatoms, but use thiols as reductants. Their peroxidatic activity is due to a conserved cysteine. In mammals, the peroxiredoxins appear to be responsible for the redox regulation of diverse metabolic processes. As there are substantial differences in the cosubstrate requirements of the peroxiredoxins of pathogenic microorganisms and their mammalian host, mammalian peroxiredoxins prominently inhibit the antioxidant defense of pathogens, thereby contributing to the innate immune response of the host (42, 64, 112). The interaction between PAG and MIF involves a mixed disulfide, with Cys^{173} of PAG involved in disulfide bond formation. The Cys residue of MIF participating in this complex formation has not been identified, but it may be speculated that it is Cys^{60}. Cys^{173} of PAG and the disulfide bond-mediated interaction between MIF and PAG also mutually influence the enzymatic activities of these two proteins. However, as MIF inhibits rather than promotes the antioxidant activity of PAG, it appears that regulation of the cellular redox stress response by MIF is complex. MIF does not simply enhance the antioxidant response by promoting the activity of a prominent cellular antioxidant such as PAG. Conversely, as peroxiredoxins are mainly specific for CXXC-type proteins (43), it could also be argued that PAG, which regulates cellular signaling pathways among other processes, "picks" MIF to influence cell signaling.

Nevertheless, together the identified disulfide-dependent PAG–MIF interaction confirms that MIF can interact with cellular proteins carrying susceptible disulfide bonds or thiols. In the case of insulin (see above), MIF interacts with a disulfide bond-carrying protein and reduces its disulfide structure. It is unknown whether mixed protein disulfides are an intermediate in this reaction. As for PAG, MIF interacts with a reactive thiol (Cys^{173}; or it may be argued that PAG, through its reactive thiol group, interacts with MIF) to form a mixed disulfide. Although so far measured only *in vitro*, the former process could have physiological relevance as well, as MIF colocalizes with insulin in secretory granules in the β-cells of the pancreatic islets (144). PAG is only one of eight proteins currently known to interact with MIF. In addition to PAG and insulin, at least one other protein, namely, hepatopoietin (HPO), is directly involved in MIF's redox-modulating activities. Whereas insulin is to be viewed as a substrate of MIF's disulfide reductase activity, PAG and HPO could be regulatory targets of MIF's redox-modulating functions. Figure 2 depicts the currently identified protein interactions that MIF can be involved in and highlights those that are assumed to play a role in redox regulation and/or are mediated through redox/CXXC mechanisms.

MIF's role in cellular redox regulation appears to be connected with cell signaling processes. Direct evidence for this

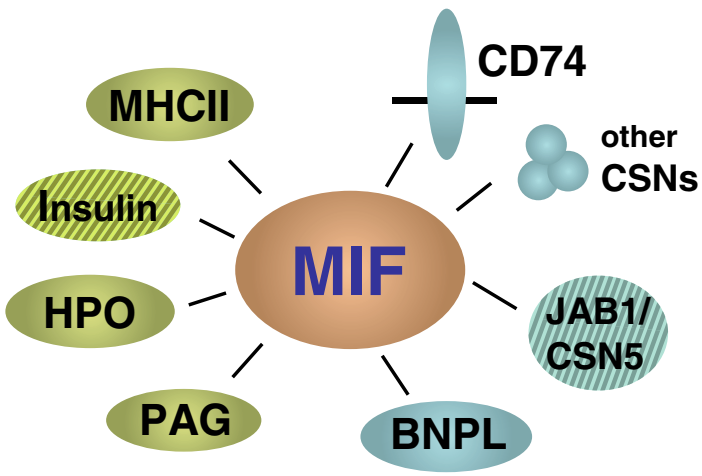


FIG. 2. Schematic of currently known MIF-binding proteins. The interacting proteins shown in green are redox proteins or proteins for which the interaction with MIF may be directly connected with redox regulation. Insulin is depicted as a hatched green symbol, as the evidence for a redox activity of MIF toward insulin is so far mainly *in vitro*-based. All other proteins identified to interact with MIF are depicted in blue. For these proteins, an impact of MIF's redox activity has not yet been shown. JAB1 is depicted as a hatched blue symbol to indicate that JAB1 might be subject to MIF- or Trx-based redox regulation. However, this indication is so far based on observations that a redox-dead mutant of MIF does not inhibit certain JAB1 effects, that Trx binds to JAB1, and that MIF may influence JAB1 through HPO.

notion comes from the recent identification of the intracellular interaction between MIF and the COP9 signalosome (CSN) component c-Jun activation domain binding protein-1 (JAB1)/CSN5 (80). JAB1 is both a transcriptional coactivator of the activator protein-1 (AP-1) transcriptional pathway (28) and a component of the CSN. The CSN is a multiprotein complex that modulates the ubiquitin-proteasome protein degradation pathway through interaction with cullin-dependent E3 ligases (26, 148, 149, 153). CSN enhances the degradation of critical cell regulators like the tumor suppressor p53 (6), the cell-cycle inhibitor p27 (138), or the transcription factors Id1 and 3 (13). Regulation of SCF-E3 ligases by CSN is dependent on JAB1/CSN5, which acts as a so-called deneddylase (29, 94). By deneddylating cullins, JAB1 promotes the cycling rate of the degradation machinery, resulting in an enhanced degradation of SCF-E3 ligase substrates (94, 153). In addition, JAB1, probably in its CSN-associated form, directly participates in regulating signal transduction components such as integrins, c-Jun, protein kinase D, or c-Jun N-terminal kinase (JNK) (7, 14, 60, 80, 100, 123, 139, 142).

MIF inhibits JAB1-mediated AP-1 activation and counterregulates JAB1-dependent p27 degradation and G1 cell-cycle arrest. Precisely how MIF's redox activity may impose on JAB1 and the CSN is unclear. Binding of MIF to JAB1 is dependent on the sequence region 50–65 of MIF, but there is no requirement for the presence of an intact CXXC motif for successful binding as both a bis-serine variant of MIF(50–65) and the redox-dead mutant C60SMIF can bind to JAB1 (80). Yet the JAB1-antagonistic effects of MIF appear to be CXXC-dependent as C60SMIF almost completely loses this activity (80). Interestingly, JAB1 was recently identified to also bind to Trx, and Trx antagonizes the same JAB1-activated signaling pathways as MIF (71). JAB1 contains several Cys residues, but it is unknown if they are involved in the interactions with MIF or Trx. Thus, further structure-activity studies with MIF CXXC mutants and examination of such mutants in additional MIF-JAB1-dependent cellular processes are needed to clarify whether MIF's redox activity is directly involved in JAB1 modulation or whether the inability of C60SMIF to modulate JAB1 function is due to a conformational effect.

However, that redox regulation of JAB1 could in fact be an important regulatory mechanism to modulate the activities of this CSN component is indicated by yet other observations. JAB1 binds not only to MIF and Trx, but also to another redox-regulatory protein. As recently demonstrated, JAB1 directly interacts with HPO (90) and intriguingly, HPO also binds to MIF (86). HPO is a flavin-linked sulfhydryl oxidase, and the invariant CXXC motif in HPO, which forms an intramolecular disulfide, is essential for its catalytic activity. HPO both exhibits extracellular and intracellular functions and resembles Trx and MIF in this regard. Extracellular HPO triggers the mitogen-activated protein kinase (MAPK) pathway by binding to its specific cell-surface receptor. Intracellular HPO potentiates the AP-1 pathway through JAB1, in a MAPK-independent fashion (90), and colocalizes with the CSN in the nucleus of hepatic cells. HPO-mediated potentiation of AP-1 activity is inhibited by curcumin, a potent inhibitor of a CSN-associated kinase, indicating that HPO-JAB1-regulated AP-1 is controlled by cell signaling events.

Neither HPO dimerization nor its binding to JAB1 is CXXC-dependent. However, similar to MIF, HPO variants with the Cys residues at the active site substituted for Ser do not have sulfhydryl oxidase activity, do not retain the c-Jun phosphorylation-enhancing activity, and fail to potentiate JAB1-mediated AP-1 activation (27). This means that the JAB1-mediated potentiation role of HPO on AP-1 is dependent on its sulfhydryl oxidase activity. Thus, the HPO-JAB1 link provides strong evidence that JAB1 could in fact be regulated by a redox mechanism.

HPO binding to MIF represents another example of MIF interaction with a CXXC-containing protein. Although it is unclear whether MIF-HPO heterodimerization actually occurs by a disulfide mechanism and whether the CXXC cysteines are involved in the binding process, it is evident that these two redox factors mutually modulate their effects on cell signaling. As far as the intracrine effects of HPO are concerned, this cross-regulation occurs through JAB1 (86).

A special kind of MIF-mediated redox-regulatory process has been observed in rat sperm maturation. Eickhoff and colleagues showed that incubation of rat sperm cells with MIF leads to an increase of detectable free thiol groups in the sperm flagellum. At the same time, a decrease in the cellular zinc content is measured (38). As this effect is observed with MIF concentrations in the range of 25–50 ng/ml, it is likely that the MIF-mediated increase in sperm protein thiol groups occurs by a true catalytic process. The release of zinc could be a subsequent step. These data confirm that MIF can act as a redox catalyst *in vivo*. The experiments by Eickhoff and colleagues were performed with exogenously applied rMIF and vesicular MIF.[1] Whereas this affirms a role for extracellular MIF in sperm redox regulation, intracellular MIF likely plays a critical role for these processes, too. In fact, vesicular MIF is probably efficiently shuttled into the sperm cell cytosol.

As also discussed further below, MIF's TPOR activity is critically involved in angiotensin II-induced neuronal firing (131). MIF acts as an inhibitor of the chronotropic actions of angiotensin II in hypothalamic neurons. Of note, this inhibitory function is mediated by the TPOR activity of MIF in a CXXC-dependent manner, as MIF peptide fragment MIF(50–65), but not the bis-serine variant C57SC60SMIF(50–65), fully mimics the inhibitory action of MIF on angiotensin II-stimulated neuronal firing (131). The target proteins and the precise mechanism of how MIF redox regulation of neuronal firing occurs are unknown.

MIF is a pivotal mediator of the innate immune response of the host (21). Importantly, MIF's oxidoreductase activity is likely to play a role in MIF-mediated immune cell functions, and the evidence is as follows: In contrast to wtMIF, the redox-

[1]The same authors previously demonstrated that MIF is located in the epithelial cells of rat epididymis and in the outer dense fibers of rat epididymal spermatozoa. As MIF is secreted by an alternative secretion mode from the apical surface of the epithelial cells in vesicles that pinch off from the plasma membrane, it is suggested that MIF is transported from the epithelial cells to the sperm cells by a vesicle-mediated cell-to-cell transfer mechanism (Eickhoff R, Wilhelm B, Renneberg H, Wennemuth G, Bacher M, Linder D, Bucala R, Seitz J, and Meinhardt A. Purification and characterization of macrophage migration inhibitory factor as a secretory protein from rat epididymis: evidences for alternative release and transfer to spermatozoa. *Mol Med* 7: 27–35, 2001).

dead mutant C60SMIF is unable to activate macrophages to kill leishmanial parasites (77). With respect to the same macrophage-activatory effect, C57SMIF exhibits <70% of the activity of wtMIF (77). Similarly, the redox center mutants C57SMIF and C60SMIF exhibit only ~65% and 37% of the glucocorticoid overriding activity (23) that wtMIF exerts on monocytes/macrophages (79). Although the contribution of MIF's TPOR activity is thus obviously not 100%, it is nevertheless suggested from these data that MIF-mediated redox effects play a marked role in regulating inflammatory monocyte/macrophage functions. The glucocorticoid overriding data (79) in conjunction with structure-activity studies of the 16-meric CXXC-spanning peptide MIF(50–65), of which also cysteine mutants and lactam bridge-cyclized variants were investigated (102), suggest that a CXXC disulfide-dependent conformation could be involved in the receptor docking and activation process. As such a receptor-active conformation might also be induced by a receptor-dependent induced-fit mechanism, redox-independent receptor activation may occur to a certain extent; this could explain the rest activity of the C60SMIF mutant regarding its glucocorticoid overriding capacity.

Modulation of inflammatory cell activity can occur by induction of inflammatory cytokine expression or generation of reactive oxygen species (ROS). Another important mechanism of inflammatory cell control is inducing or inhibiting cell death by apoptosis. In fact, induction of macrophage or T-cell apoptosis is an important step in a variety of inflammatory diseases. For example, macrophage apoptosis can lead to the release of numerous aggressive agents, including ROS and hydrolytic enzymes. On the other hand, inhibition of predestined apoptotic processes can also contribute to an enhancement of an inflammatory situation by prolonging the lifetime of the involved immune cells.

A number of years ago, Trx was surprisingly demonstrated to be an important inhibitor of immune cell apoptosis, and apoptosis inhibition by Trx was found to be mediated by its TPOR activity (4, 72, 110). These findings are based on earlier work showing that oxidation of cellular sulfhydryl groups induces apoptosis in T lymphocytes (121) and that human Trx is identical with adult T-cell leukemia-derived factor that induces interleukin-2 receptor and T-cell proliferation (135). Numerous publications have then confirmed that Trx plays a key role in inhibiting cell apoptosis in a variety of cells and cell stress situations (2, 15, 25, 46, 53, 54, 56, 74, 81, 84, 87, 103, 136). One mechanism by which Trx participates in cellular apoptosis inhibition is through inhibition of apoptosis signal-regulating kinase-1 (118).

Recently, MIF was also found to be a potent inhibitor of apoptosis. Using functional screens, Hudson and colleagues identified MIF as a prominent gene product that bypassed either p53-mediated growth arrest or apoptosis (70). These studies, as well as subsequent investigations by Mitchell *et al.* (98), 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. Studies by Nguyen *et al.* suggest that redox effects could play a role, as MIF reduces prooxidative stress-induced apoptosis in several cell types, including immune cells (101). Similar to Trx and low-molecular-weight thiol compounds such as β-mercaptoethanol, micromolar concentrations of extracellular MIF are able to inhibit oxidative stress-induced apoptosis, but overexpression of intracellular MIF also leads to a marked antiapoptotic effect (72, 101). This suggests that, like Trx, MIF may inhibit apoptosis both by a catalytic mechanism and as a donor of proteinaceous thiol groups. Intriguingly, antiapoptotic effects of MIF through p53 and the cellular redox state may be linked and, moreover, the p53- and MIF-interacting protein JAB1 may be associated with these effects (6, 70, 101). As discussed, JAB1 is a potent regulator of AP-1 transcription, and MIF inhibits this process (80). Although a direct redox link between MIF and JAB1 has not been established, it is worth mentioning that AP-1 transcriptional regulation also occurs by Trx and Ref-1, and that Trx was recently found to interact directly with JAB1 and inhibit its AP-1-promoting activity (62, 71).

Another potential link between the apoptosis-modulating properties of the redox regulators Trx and MIF was demonstrated by Kondo and co-workers in CD4+ T cells (82). Accordingly, MIF and Trx reciprocally control their expression levels upon prooxidative stress-induced apoptosis. MIF levels are reduced upon overexpression of Trx, and Trx levels are enhanced in T cells from MIF knockout mice. This could mean that the antiapoptotic activities of MIF and Trx are at least in part coordinated by an interdependent regulation of the expression levels of these cellular players. It is currently unclear whether MIF and Trx may nevertheless act synergistically during oxidative stress-induced apoptosis and whether other means of regulation, such as posttranslational modifications or interaction with JAB1 or p53, could play a role herein. Of note, GIF, which was shown to be identical to MIF in its sequence and 3D structure, is cysteinylated under certain conditions (146).

MIF and Trx not only share striking similarities with respect to their redox-related antiapoptotic activity and modulation of JAB1 functions, but also are alike as they can both be viewed as secretable cellular enzymes with distinct extracellular functions. It has therefore been proposed to term them "redoxkines" (redox-acting cytokines) or "cytozymes" (enzymes with cytokine functions) (50, 77). In fact, Trx, which was originally defined as a classical intracellular enzyme (69), is now recognized as a cocytokine (107). Interestingly, both Trx and MIF and also truncated Trx are secreted by a leaderless, nonclassical export pathway (41, 108, 117). Once secreted, both MIF and Trx exert a broad spectrum of cytokine activities to modulate the immune and inflammatory response of the host. As discussed above, this includes the enhancement of lymphocyte proliferation, activation of macrophages, induction of cytokines and stress-related gene products, and the modulation of cell migration (21, 63, 107, 141).

THE MIF–CD74–MHC II CONNECTION AND ITS POTENTIAL LINK TO THE REDOX ACTIVITY OF MIF

Both MIF and Trx can act as extracellular mediators. Yet no typical receptor has been identified for either protein. As discussed above, the redox activity of MIF and Trx partici-

pates in the regulation of cell signaling by these proteins, but can probably not account entirely for the extracellular effects. Recently, CD74, which is the cell-surface form of the major histocompatibility complex (MHC) class II-associated invariant chain (Ii), was identified to interact with MIF at the cell surface. 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 on the presence of CD74 (85). Current investigations are aiming to elucidate further the role of CD74 in MIF signaling. Interestingly, the MIF–CD74 interaction could be connected with MIF's TPOR activity. This potential link is suggested by a study from Potolicchio and colleagues, as they found that MIF interacts with certain MHC class II allotypes and, importantly, is involved in the reduction of disulfides of oxidized class II-associated peptide antigens. This suggests that MIF-mediated disulfide reduction could be an important step in antigen processing for HLA class II-restricted T-cell responses (109). As MIF can be secreted and reduction of HLA peptide disulfides occurs at neutral pH, MIF may play a role in antigen processing in the extracellular milieu. It may thus be hypothesized that MIF–CD74 binding occurs in the context of class II peptide disulfide reduction. Whether CD74 itself is a target of MIF's TPOR activity is unknown.

DIFFERENTIAL ROLE OF MIF'S TPOR ACTIVITY IN INTRA- VERSUS EXTRACELLULAR COMPARTMENTS

As MIF acts by both transcellular pathways and intracellular mechanisms, assigning the biological effects of MIF to its redox activity and subcellular localization has been attempted. Due to the fact that most cells exhibit substantial endogenous MIF levels and as overexpression of MIF enhances the pool of GSH in thiol-starved MIF-depleted mammalian cells (101), there seems to be a physiological impact of endogenous MIF on cellular redox homeostasis comparable to dithiol-reducing Trx. As eluded to above, it is still unclear which of the redox-regulatory effects of MIF occur by true catalysis and which are mediated by donation of thiol equivalents.

A potential assignment of these mechanisms to the intra- and extracellular TPOR activity of MIF, respectively, may be suggested by the following observations that were made applying the redox-dead mutant C60SMIF in comparison with wtMIF. Ectopically overexpressed wtMIF markedly inhibits cell apoptosis following prooxidative stress. In contrast, redox-dead C60SMIF overexpressed at a similar rate as wtMIF does not exhibit this capability (101). On the other hand, both wtMIF and C60SMIF protect cells from apoptosis to a similar degree, when the recombinant proteins are exogenously added to the cells. As for Trx, for this effect to occur, long incubation times (overnight) and relatively high concentrations of recombinant wtMIF (rwtMIF) or rC60SMIF are necessary. It has thus been suggested that a mere donor function of proteinaceous thiol groups could be responsible for this latter effect (72, 101). Nevertheless, it remains unclear why C60SMIF shows this effect, as in this mutant the CXXC motif of MIF is disrupted. Although, at first sight, the action of extracellularly added rMIF appears to represent an activity of extracellular MIF, it is well possible that apoptosis inhibition involves a prior cellular uptake of MIF. Indeed, efficient endocytosis of MIF into target cells has been measured (80).

Other data indicate that protection by MIF from prooxidative stress-induced apoptosis could be due in part to MIF-mediated inhibition of a stress-induced JNK activity (101). It has not yet been resolved whether this action represents MIF-dependent redox signaling, but this effect could clearly represent an activity of extracellular MIF.

On the other hand, MIF's influence on p53 is most likely an intracellular function of MIF. MIF inhibits p53 transcriptional activity (70), but the question remains whether MIF's redox activity is involved in this process. Besides many post-translational modifications of p53, p53 is subject to redox regulation under conditions of cellular redox stress. p53 is a zinc-binding protein containing several reactive cysteines, and its major biochemical property, *i.e.,* sequence-specific DNA binding, is dependent upon metal binding and redox regulation (33, 57–59, 111, 143). Furthermore, nitric oxide, thioredoxin reductase, Trx oxidation, and the redox/repair protein Ref-1 have been shown to affect p53 conformation and/or transcriptional activity (24, 73, 95, 106). Interestingly, the nuclear levels of p53 are also increased in response to prolonged and severe hypoxia. Low oxygen conditions cause an increase in the DNA binding and transactivation activity of p53 (51). In this case, the activity and level of p53 can be altered by the redox-sensitive protein hypoxia-inducible factor-1α (1). Thus, MIF's TPOR activity could somehow be involved in the preservation of the p53 redox status, under both normal and stress conditions. There is just as well the possibility that redundantly expressed MIF itself could overstrain donor functions for redox equivalents. At present, there is no evidence that MIF acts directly on the redox-active cysteine groups of p53 to modulate p53 activity (Thiele and Bernhagen, unpublished observations).

MIF's binding to another redox-sensitive protein, PAG, and inhibition of PAG's antioxidant activity by MIF are intracellular functions of MIF as well. The inhibitory effect was shown by a decreasing protection of glutamine synthetase against thiol-specific oxidative inactivation through MIF in a dose-dependent manner. In turn, the binding of MIF to PAG depends on the cellular redox status, which could influence protein conformation (75).

The best evidence for an intracellular role of MIF's TPOR activity *in vivo* comes from microinjection experiments with rMIF and the redox-active MIF-derived peptide MIF(50–65). MIF inhibits the stimulatory actions of angiotensin II on neuronal firing in rat neurons (131). Comparable to the findings in prooxidative stress-induced apoptosis, the inhibitory action of MIF in this neuronal setting is clearly mediated via its TPOR activity. This probably involves a subsequent scavenging of ROS as ectopic overexpression of MIF prevents the increase of ROS levels in neurons after stimulation with angiotensin II. Intracellularly injected MIF(50–65), but not the CXXC-mutated peptide C57SC60S-MIF(50–65), is sufficient for mediating the inhibitory effect on the chronotropic action of angiotensin II in rat neurons (131). DDT, which does not contain a cysteine at position 60 and therefore does not have a CXXC site, is not able to mimic MIF's inhibitory

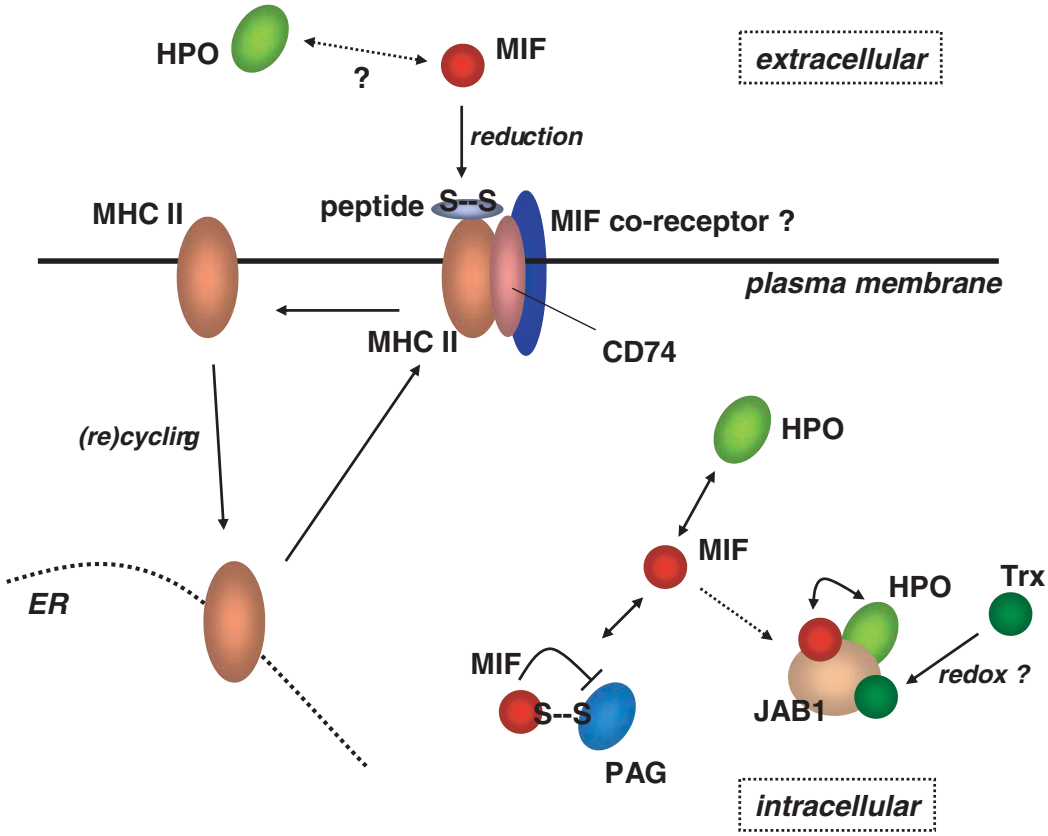


FIG. 3. Schematic summarizing the potential involvement of the MIF redox (TPOR) activity in various cellular processes. The potential role of MIF's TPOR activity is shown for both extra- and intracellular targets of MIF. The cell shown could typically be a macrophage. Involvement of the TPOR activity of MIF in MHC peptide reduction is shown on the extracellular side. Also, in this compartment, studies with MIF CXXC mutants suggest that MIF might affect its membrane receptor by a redox mechanism. On the intracellular side, the interaction of MIF with three (potential) redox targets is depicted. Of these, MIF has been shown to interact with PAG by a heteromeric disulfide, whereas a direct redox-mediated binding to JAB1 and HPO is unclear. MIF may regulate the TPOR activity of HPO through JAB1 and vice versa. S—S stands for an oxidized intra- or intermolecular disulfide. ER, endoplasmic reticulum.

effect in this system. This further affirms that the observed effects can be assigned to the intracellular TPOR activity of MIF. Also, a time-dependent, angiotensin II-mediated increase of MIF expression did not alter MIF levels in the growth media, implying that angiotensin II does not increase MIF secretion from these cells. Lastly, the extracellular application of MIF to neuronal cultures produced no changes in neuronal firing.

CONCLUDING REMARKS

MIF's TPOR activity is not only an *in vitro* function of an evolutionary conserved local sequence site of MIF, but also an intracellular property of this factor that is involved in the regulation of a variety of cellular processes. Whether the extracellular functions of MIF, *i.e.,* its "cytokine" functions, are in part dependent on the redox activity is likely, but additional evidence is clearly needed to discern conformational effects from direct redox-regulatory actions and/or to unravel the molecular details of the link between these mechanisms (Fig. 3).

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ABBREVIATIONS

AP-1, activator protein-1; BNPL, BNIPL, Bcl-2/adenovirus E1B 19 kDA interacting protein 2-like (BNIP-2-like); CALC, CXXC motif of human MIF; CD74, MHC class II-associated invariant chain; COP9, constitutive photomorphogenesis complex; CSN, COP9 signalosome; CXXC, Cys-Xaa-Xaa-Cys redox motif of TPOR proteins; 3D, three-dimensional; DDT, D-dopachrome tautomerase; Dsb, disulfide bond proteins; GIF, glycosylation inhibition factor; Grx, glutaredoxin; GSH, reduced glutathione; GSSG, oxidized glutathione; 2-HED, 2-hydroxyethyldisulfide; H_2O_2, hydrogen peroxide; HPO, hepatopoie-

tin; JAB1/CSN5, c-Jun activation domain binding protein-1/CSN subunit 5; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MHC, major histocompatibility complex; MIF, macrophage migration inhibitory factor; PAG, member of peroxiredoxin family; PDI, protein disulfide isomerase; PPT2, phenylpyruvate tautomerase 2 (alternative name for MIF, when referring to its tautomerase activity); r-, recombinant; ROS, reactive oxygen species; TPOR, thiol-protein oxidoreductase; Trx, thioredoxin; wt, wild-type.

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