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Post-translational regulation of macrophage migration inhibitory factor: Basis for functional fine-tuning

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a chemokine-like protein and an important mediator in the inflammatory response. Unlike most other pro-inflammatory cytokines, a number of cell types constitutively express MIF and secretion occurs from preformed stores. MIF is an evolutionarily conserved protein that shows a remarkable functional diversity, including specific binding to surface CD74 and chemokine receptors and the presence of two intrinsic tautomerase and oxidoreductase activities. Several studies have shown that MIF is subject to post-translational modification, particularly redox-dependent modification of the catalytic proline and cysteine residues. In this review, we summarize and discuss MIF post-translational modifications and their effects on the biological properties of this protein. We propose that the redox-sensitive residues in MIF will be modified at sites of inflammation and that this will add further depth to the functional diversity of this intriguing cytokine.

1. Introduction

Identified in 1966, macrophage migration inhibitory factor (MIF) was initially shown to inhibit the random migration of macrophages upon its release from T lymphocytes during a delayed-type hypersensitivity response [1,2]. Today, MIF is widely recognized as a critical upstream player in the innate immune response, where it triggers and amplifies cytokine production by stimulating the production of pro-inflammatory mediators, such as TNF-α, interferon-γ, interleukins (IL-1β, IL-2, IL-6, IL-8), nitric oxide, prostaglandin E_2 and tissue-degrading matrix metalloproteinases [3–6]. MIF also promotes inflammation by orchestrating leukocyte trafficking [7], inhibiting p53-mediated apoptosis of inflammatory cells sustaining their survival span [8,9], and by counter-regulating the immunosuppressive action of glucocorticoids [10,11]. Furthermore, MIF exhibits tumor growth-promoting properties [12,13].

Given its broad pro-inflammatory activities, it is not surprising that MIF is implicated in acute and chronic inflammatory diseases such as rheumatoid arthritis, asthma, diabetes, sepsis, cancer, atherosclerosis and other cardiovascular diseases [8,14–22]. MIF is released from different immune cell types including monocytes, macrophages, neutrophils, T cells, B cells, dendritic cells, and eosinophils, but secretion can also occur from certain endocrine, endothelial and epithelial cells upon inflammatory stimulation or injury [23,24]. MIF consists of 114 amino acids and has a molecular mass of 12,345 Da, with a 90% sequence homology between human and murine MIF. The sequence of rat and mouse MIF only differ in a single amino acid [25]. More recently, a genetic homolog of MIF, termed D-dopachrome tautomerase (D-DT) or MIF-2, was identified and found to play role in the inflammatory response [26,27].

MIF binds to the chemokine receptors CXCR4 and CXCR2 [28,29] to foster inflammatory and atherogenic monocyte/neutrophil and T cell chemotaxis, respectively, and is therefore classified as a chemokine-like function (CLF) or atypical (ACK) chemokine [30]. In light of the discovery of MIF's chemokine activity, it appears that the eponymous 'migration-inhibitory effect' represents a chemokinetic effect of MIF on random leukocyte motility [30]. MIF also binds to the type II transmembrane protein CD74, which leads to intramembranous cleavage ('RIP') and signaling and/or co-activation of CD44 [31–33], while CD74/CXCR complexes also have been implicated in MIF signaling responses in atherosclerosis [29]. Following receptor activation, MIF facilitates cell proliferation, inhibition of apoptosis and migration of immune cells via the ERK1/2 MAP kinase, Gαi and PI3K/AKT pathway [29,32].

MIF is widely considered to act as a key-regulator in myocardial ischemia/reperfusion injury, where MIF is released in two waves

Abbreviations: GIF, glycosylation inhibiting factor; MIF, macrophage migration inhibitory factor; MPO, myeloperoxidase; TPOR, thiol-protein oxidoreductase

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[34,35]. MIF release during the first wave in the early stage of ischemia is thought to stem from the ischaemic myocardium and to adopt a protective function [34,35]. On a molecular basis, MIF's protective activity is due to its ability to inhibit the apoptosis-inducing c-Jun *N*-terminal kinase (JNK) pathway and to reduce oxidative stress to the myocardium generated during ischemia/reperfusion [36,37]. Importantly, MIF released from the ischaemic heart can induce AMPK activation via binding to CD74, which promotes glucose uptake and protects the heart during ischemia/reperfusion by metabolic pathways [17,38]. The second MIF wave is that of pro-inflammatory MIF released from activated infiltrating immune cells including monocytes [34]. Therefore, MIF can have multiple and sometimes opposing functions depending on the cellular source and the time of release. The mechanisms for regulating MIF function and the mechanistic details of the fine-tuning between cell types and phases are currently unclear.

MIF differs from other pro-inflammatory cytokines by being semi-constitutively expressed and secreted into circulation rather than being regulated by a surge in transcriptional activity in response to inflammatory stimuli [39–41]. MIF forms homo-trimer [42,43] and, unlike other common known cytokines, has two evolutionarily conserved catalytic activities – a tautomerase and a thiol-protein oxidoreductase (TPOR) activity – that are carried out by two distinct catalytic centres (Fig. 1). The TPOR activity of MIF is mediated through a conserved CALC motif containing Cys-57 and Cys-60 [44] and has been shown to catalyse the reduction of insulin and 2-hydroxyethyldisulfide (HED) and to be involved in cellular redox protection [44–46]. The tautomerase activity is facilitated by the conserved *N*-terminal proline, which acts as a catalytic nucleophile at physiological pH due to its unusually low pKa of 5.6 [47].

The function of the tautomerase activity is unknown and a physiological substrate has yet to be identified. However, the region encompassing the *N*-terminal proline is involved in receptor binding and it has been shown that targeting the *N*-terminal proline with small molecule inhibitors can inhibit some of the pro-inflammatory activities of MIF [48–53]. Targeting the tautomerase active site is thus currently being explored as an anti-inflammatory treatment avenue [53].

Apart from pharmacological electrophiles, the *N*-terminal proline has been shown to react with physiological oxidants/electrophiles generated by the neutrophil-derived enzyme myeloperoxidase [54]. Indeed, the inflammatory environment with an array of reactive oxygen and nitrogen species being produced by phagocytic cells makes modifications of redox-sensitive proteins highly likely.

We hypothesize that these post-translational modifications play an important role in the regulation of MIF.

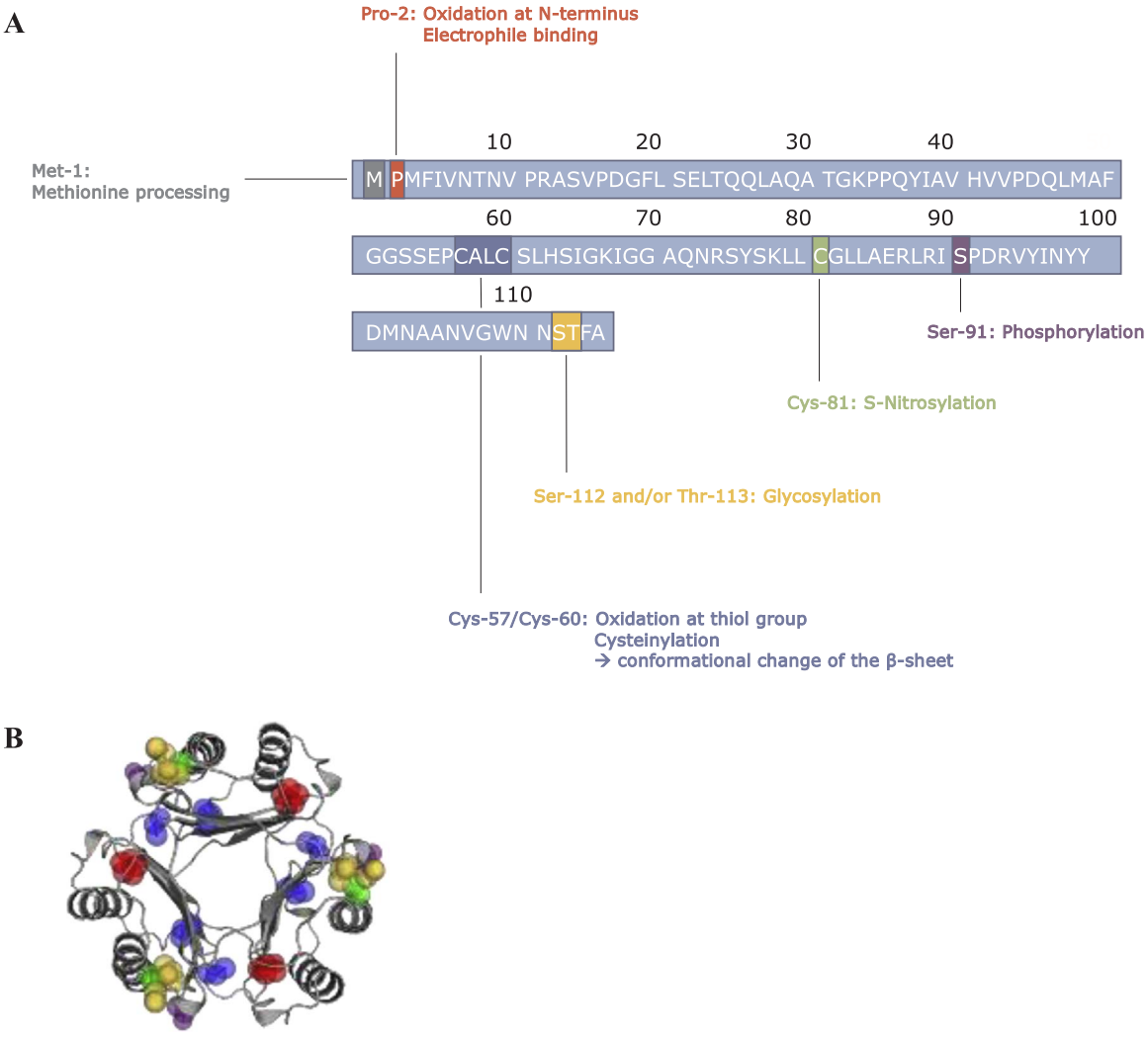


Fig. 1. A) Amino acid sequence of MIF with residues targeted for post-translational modifications highlighted in colour. *Note*: the suggested cysteine-oxidized form is speculative and lacks structural confirmation. **B)** Ribbon structure of the MIF trimer based in the PDB crystal structure 3DJH (1.25 Å resolution) [96] with the side chain susceptible to post-translational modifications shown in coloured spheres: red – Pro-2, blue – Cys-57/Cys-60, green – Cys-81, purple – Ser-91, orange yellow – Ser-112/Thr-113. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

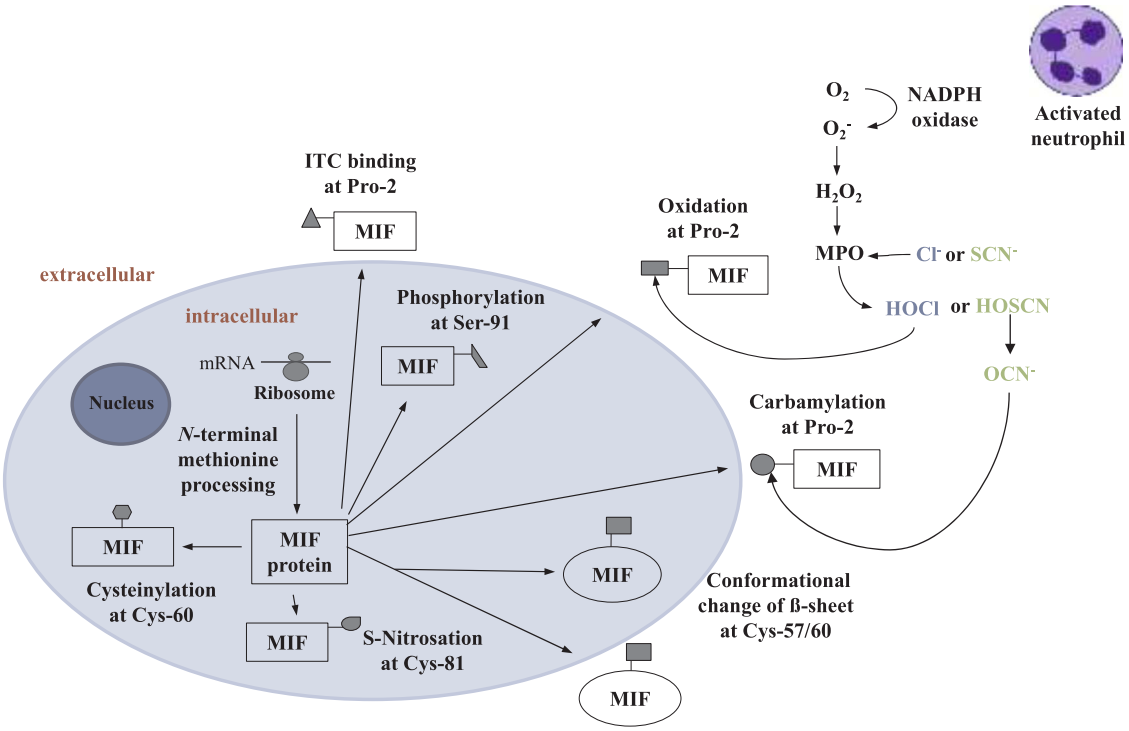


Fig. 2. Post-translational modifications of MIF inside and outside the cell. Posttranslational removal of the *N*-terminal methionine in MIF results in an *N*-terminal proline (Pro-2) in the mature protein. The *N*-terminal proline can be modified by neutrophil-derived oxidants, carbamylation and binding of electrophiles such as isothiocyanate (ITC). MIF can be cysteinylated at Cys-60 and a conformational change can occur at the ß-sheet encompassing Cys-57/60. S-nitrosation and phosphorylation can modify Cys-81and Ser-91, respectively.

2. MIF post-translational modifications

To date, several studies have demonstrated that MIF can be modified both covalently and structurally, and suggested that modifications can change MIF bioactivity. These modifications are discussed in detail below and are summarized in Figs. 1 and 2 and Table 1. In highlighting the variety of modifications, we recommend caution with regards to terminology. Three groups have independently reported MIF oxidation (Dickerhof [54], Thiele [55] and Kassaar [22]), but the modifications occur at different sites in the protein and in response to different oxidizing agents, and are likely to have different biological properties. Therefore, it is too simplistic to describe something as 'oxMIF'. Moreover, the suggested oxidized species reported by Thiele *et al.* lacks structural characterization [55]. Detailed characterization of the oxidation products is essential in preventing confusion and moving the field forward. We recommend that investigators denote the target amino acid and the biochemical nature of the modification, keeping in mind that in any one sample there will be a heterogeneous population of proteins with differing degrees of modification. Accordingly, the species identified could for example be termed 'proline-oxidized MIF' or 'cysteine-oxidized-MIF'.

3. Modification of the N-terminal proline

The removal of the *N*-terminal methionine, encoded for by the translational initiator codon by methionine aminopeptidase (MetAP) is often crucial for the function and stability of proteins. Posttranslational removal of the *N*-terminal methionine in MIF results in an *N*-terminal proline (Pro-2) in the mature protein [25,42], and occurs in almost every cell type including recombinant *E. coli* expressing human MIF. In

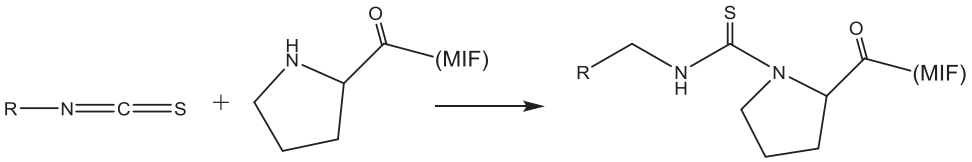
Table 1
Summary of post-translational modifications (PTMs) of MIF and their functional implications.

Site of modification	Chemistry of modification	Biological consequences	References
Met-1	Cleavage of *N*-terminal methionine	Unknown	Weiser et al. [97] Bernhagen et al. [39]
Cys-57/60	Conformational change of ß-sheet comprising Cys-57 and Cys-60	Disease-related isoform of MIF in patients with septicemia, psoriasis, asthma, ulcerative colitis, Crohn's disease, Alzheimer's	Thiele et al. [55] Kassaar et al. [22]
Pro-2	Hypochlorous acid-mediated oxidation to a proline-imine	Loss of tautomerase activity, retention of anti-apoptotic effect on neutrophils	Dickerhof et al. [54]
Pro-2	Covalent binding of isothiocyanates and epicatechin-quinone, carbamylation	Loss of tautomerase activity, conformational change, interference with binding to CD74 receptor	Brown et al. [57] Ouertatani-Sakouhi et al. [56] Dickerhof et al. [61] Dickerhof *et al.* [54]
Cys-60	Cysteinylation at Cys-60	Important role in regulation especially of activated B and T cells	Watarai et al. [77] Nguyen et al. [90]
Cys-81	S-Nitrosation	Increased oxidoreductase activity, increased cardioprotective properties compared to unmodified MIF	Luedike et al. [86] Pohl et al. [87]
Ser-112 and/or Thr-113	S-Glycosylation	Decreased ability to activate ERK1/2 and AKT signaling	Watarai et al. [77]
Ser-91	Phosphorylataion	Diminishing activity of cysteinylated MIF	Watarai et al. [77]

this review, we will number amino acids according to the MIF cDNA sequence, e.g. the *N*-terminal proline residue will be termed Pro-2.

3.1. Modification of the N-terminal proline by dietary electrophiles

While the *N*-terminal proline of MIF has been recognized for some time as a site for the pharmacological targeting of MIF, in 2009 three research groups simultaneously reported that dietary isothiocyanates can covalently modify the *N*-terminal proline of MIF and inhibit tautomerase activity (Reaction Scheme 1) [56–58].



Isothiocyanate MIF Thiourea adduct (Reaction Scheme 1)

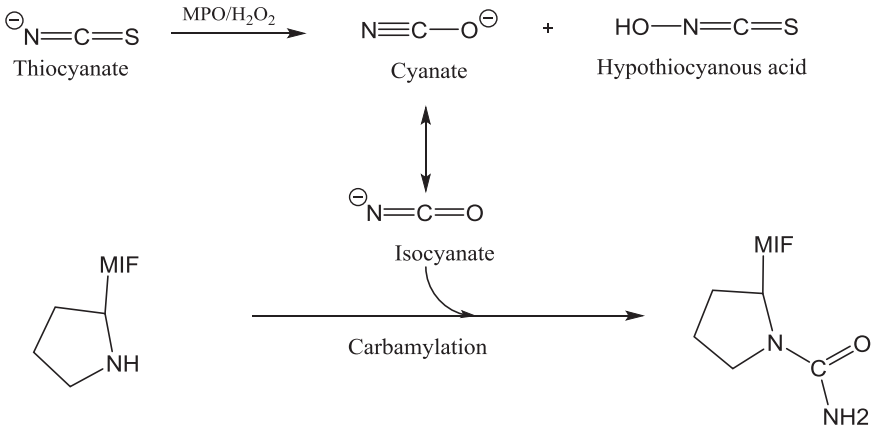
Ouertatani-Sakouhi *et al.* demonstrated that benzyl isothiocyanate did not affect trimerisation of MIF, but interferes with MIF binding to its receptor CD74 [56]. Brown *et al.* showed that MIF plasma levels, as detected by ELISA, were rapidly lowered in human volunteers ingesting phenethyl isothiocyanate-rich watercress suggesting that binding of isothiocyanates induces structural changes that affect its binding to antibodies [57]. Indeed, subsequent crystal structures indicated conformational shifts at the *N*-terminal domain of MIF following isothiocyanate binding [59,60].

Epicatechins, abundant flavonoids in green tea, chocolate and red wine, were also found to inhibit MIF tautomerase activity [61]. Oxidation of the *o*-diphenolic (catechol) moiety in epicatechin to an *o*-quinone, and thus the generation of a potent electrophilic moiety was essential for binding the *N*-terminal proline of MIF. The neutrophil-derived myeloperoxidase (MPO) is able to efficiently oxidize the catechol of epicatechins to a quinone [61,62], suggesting that this post-translational modification of MIF may occur at inflammatory sites where both MIF and MPO are present at high concentrations [63,64]. A range of electrophilic lipids are also generated during inflammation [65] and it would be of interest to examine their ability to react with the *N*-terminal proline and modify MIF function.

3.2. Carbamylation of the N-terminal proline

The *N*-terminal proline of MIF has been shown to become carbamylated by the MPO/H_2O_2/thiocyanate system in vitro (Reaction Scheme 2) [54]. Protein carbamylation was initially identified to be an artefact introduced by denaturation-renaturation, but has since been shown to modulate enzyme activity in different proteins [66,67]. Carbamylation was shown to occur in plasma during uraemia [68–71]. Urea is in equilibrium with cyanate, which can carbamylate lysine residues on proteins to form ε-carbamyllysine (homocitrulline). In 2007, Wang et al. also reported that when MPO oxidizes thiocyanate to hypothiocyanous acid, cyanate is produced as a by-product (Reaction Scheme 2) [72]. MPO-deficient mice had reduced levels of protein carbamylation at sites of inflammation compared to wildtype mice. Wang et al. further proposed that systemic protein carbamylation might contribute to the increased risk of smokers to develop atherosclerotic coronary artery disease (CAD) due to elevated levels of plasma thiocyanates.

MIF is known to be involved in the formation of atherosclerotic plaques [14], where MPO and carbamylated proteins have shown to co-localize [72]. We therefore speculate that MIF will be carbamylated in atherosclerotic plaques. The functional significance of MIF carbamylation is not yet clear.



(Reaction Scheme 2)

3.3. Oxidation of the N-terminal proline by neutrophil-derived hypochlorous acid

Neutrophil MPO uses hydrogen peroxide to oxidize chloride and thiocyanate to hypochlorous acid and hypothiocyanous acid,

respectively [73,74]. Hypochlorous acid reacts most rapidly with cysteine and methionine residues on proteins leading to the formation of cysteine sulfonic acid and methionine sulfoxide [75]. Recently, it was shown that MIF, which is abundant at inflammatory sites, is a target for modifications by MPO-derived hypochlorous acid [54]. There was evidence for methionine sulfoxide and cysteine sulfonic acid formation upon exposure of MIF to hypochlorous acid and stimulated neutrophils as shown by tandem mass spectrometry. Intriguingly, the *N*-terminal proline became oxidized to a proline imine, a novel proline modification of MIF.

The reaction with the proline was facilitated by its unusually low pk_a rendering it a 1000-fold more reactive toward hypochlorous acid than other amines such as *N*-acetyl lysine [76]. While proline imine formation was associated with a loss of tautomerase activity, modified MIF was still able to promote CXCL-8/IL8 production by peripheral blood mononuclear cells and inhibit apoptosis of neutrophils [54]. However, pre-treatment with hypochlorous acid protected the protein from becoming functionally inactivated by larger proline-modifying electrophiles. Therefore, while a possible gain-of-function of the proline imine modification remains to be uncovered, oxidation of MIF's *N*-terminal proline in the presence of activated neutrophils may play a role in maintaining its pro-inflammatory actions during an inflammatory event.

4. Modification of cysteine residues

4.1. Cysteinylation of Cys-60

Watarai *et al.* found a protein that has the same gene structure as MIF and called it glycosylation inhibiting factor (GIF) [77]. For the purpose of this review, we refer to this protein as MIF, because there is only a difference of one amino acid between MIF and GIF, and it has been discussed that this difference could be due to a sequencing error in the GIF sequence suggesting that MIF and GIF are identical proteins [77]. When Watarai et al. purified MIF from supernatants of a human suppressor T (Ts) hybridoma cell line and analyzed the protein by SDS/PAGE, it showed a single band at 13 kDa. Mass spectrometry analysis, however, revealed four different species with molecular mass of 12,345, 12,429, 12,467 and 12,551 Da. While 12,345 Da corresponds to the molecular mass of MIF, the other three species indicated the presence of post-translational modifications. To determine which amino acids had been modified, MS analysis was performed on proteolytic digests of the protein mixture. The increase of molecular mass of 80.0 Da was demonstrated to be a phosphorylation of Ser-91 and the additional 120.0 Da suggested cysteinylation of Cys-60.

To investigate the biological function of these modifications, Watarai et al. carried out an immunosuppressive activity assay. They immunized mice with dinitrophenyl - ovalbumin to elicit an IgE antibody response and found that treatment with cysteinylated MIF suppressed the IgE antibody response, while a mixture of phosphorylated MIF and unmodified MIF did not [77,78]. Previously, it was reported that bioactive derivatives of recombinant human MIF, such as C57A/N106S were able to bind receptors in T suppressor hybridomas and activated T and B cells [79,80]. While cysteinylated MIF was able to compete with C57A/N106S for binding of the receptor in a dose-dependent manner, unmodified MIF was not able to do so, even at a 100-fold excess. Collectively, these results indicate that cysteinylated MIF is the bioactive isoform and unmodified or phosphorylated MIF are silent isoforms.

To get a better insight into the conformational change occurring upon cysteinylation of Cys-60, antibody-binding experiments were performed. An antibody that recognized the peptide region Ala-58 — Arg-74, HG3, was able to bind to cysteinylated MIF, but not unmodified MIF [77]. Interestingly, the HG3 antibody was able to detect unmodified MIF protein upon denaturation. This result indicates that the antibody epitope is concealed in the native protein and can be uncovered by cysteinylation or denaturation.

To further characterize the epitope sensitive to cysteinylation, they designed HG3a and HG3b antibodies directed the β4 strand and α2 helix structures, respectively. Only the HG3b antibody was able to bind to cysteinylated MIF suggesting that cysteinylation of Cys-60 leads to a conformational change of the α2 helix region.

While it is yet to be determined whether cysteinylation of Cys-60 is required for other biological functions of MIF, this study suggests that the modification plays an important role in the regulation especially of B and T cells. This leads to the question whether the MIF receptor microenvironment in lymphocytes differs from that of other cell types, e.g. due to the formation of specific receptor complexes or the association with certain proteoglycan structures.

4.2. S-Nitrosation of Cys-81

S-Nitrosation is a post-translational modification that forms an S-nitrosothiol (SNO) on reactive cysteine residues. S-nitrosothiols are thought to form following the oxidation of nitric oxide (·NO) to N_2O_3 and its reaction with cysteine thiolates or as a result of the recombination of ·NO and thiyl-radicals, metal-catalyzed pathways and trans-nitrosation reactions with other nitrosothiols. S-nitrosation is known to diversify the properties and mechanistic action of certain proteins and can regulate their function [81–83]. Nitric oxide is found to play an important role in the regulation of various biological processes and was reported to be generated during ischemia in the heart [84,85].

Luedike et al. found that MIF is significantly and selectively S-nitrosated at Cys-81 in vitro and in vivo [86]. As mentioned in the introduction, MIF derived from the myocardium has a protective role during ischemia/reperfusion. S-Nitrosated MIF occurred especially in the heart and increased during myocardial ischemia/reperfusion. S-Nitrosation of MIF was associated with an increased oxidoreductase activity compared to unmodified MIF and decreased myocardial apoptosis in mice after ischemia/reperfusion. Collectively, this modification enhanced the cardioprotective properties of MIF providing an example for post-translational regulation of MIF in the setting of ischemia/reperfusion. To support this, Pohl *et al.* showed that S-nitrosation of MIF occurs in the early phase of reperfusion and is able to reduce oxidative stress [87]. The resulting intracellular accumulation of MIF leads to a cardioprotective effect due to reduction of reactive oxygen species. In addition, S-nitrosated MIF showed significantly lower binding to JAB1/CSN5 compared to normal MIF, which suggested that impaired binding is the cause of intracellular MIF accumulation in the early phase of reperfusion [87].

4.3. Uncharacterised modification: 'oxMIF'

Recently, three monoclonal human anti-MIF antibodies (BaxB01, BaxG03, BaxM159) were reported to successfully inhibit pro-inflammatory activities of MIF in vitro and in vivo [88,89]. These antibodies were specific for epitopes within the ß-barrel structure comprising the conserved CALC motif. The same group further reported that these antibodies strictly bind to a conformational isoform of MIF, which was termed 'oxMIF' as it could be generated *ex vivo* by adding oxidized glutathione or L-cystine to recombinant MIF [21,55]. However, although the authors concluded that 'oxMIF' was a redox-dependent conformational isoform of MIF, conformational changes similar to that of the oxidized state of MIF, could also be achieved by denaturing agents, cross-linking with formaldehylde or the addition of 0.2% Procline300 [55].

Although, it seems plausible that glutathionylation or cysteinylation occurs at the CALC motif in the presence of an excess of oxidized glutathione or L-cystine, respectively, the authors reported that 'oxMIF' was not associated with post-translational modifications. In contrast cysteinylation of Cys-60 was observed by Watarai *et al.* for full length

MIF/GIF [77] and by Nguyen et al. for a Cys-60-containing MIF-derived peptide [90]. However, if only one of the cysteine in the CALC motif becomes modified by cysteinylation or glutathionylation, this may be followed by thiol-disulfide exchange with the neighboring reduced cysteine resulting in a disulfide bridge between Cys-57 and −60. More work is needed to elucidate the biochemical nature underlying the formation of this 'oxMIF' species.

Using the Bax159 mAb in an ELISA setting, 'oxMIF' was shown to be elevated in plasma from patients with septicemia, psoriasis, asthma, ulcerative colitis, and Crohn's disease [55]. In plasma samples from healthy donors, 'oxMIF' was not detected, although native MIF was shown to be present. In a follow-up publication by Schinagl *et al.*, 'oxMIF' was identified as a cell surface and cytoplasmic tumor marker that is highly present in ovarian and prostate cancer [21]. The nature of surface anchoring of 'oxMIF' has remained unclear. Cancer cell lines treated with anti-'oxMIF' mAbs and cytotoxic drugs showed a more sensitized reaction to the drugs than cancer cells that were not treated with anti-oxMIF mAbs.

In 2017, Kassaar et al. found an isoform of MIF with an altered electrophoretic mobility compared to native MIF in brains with early stages of Alzheimer's disease [22]. Formation of this isoform also occurred in vitro after prolonged incubation at 37 °C or in response to hydrogen peroxide and S-nitrosoglutathione. The addition of reduced glutathione or dithiothreitol (DTT) prevented the production of this isoform and was therefore considered an oxidized MIF species. Interestingly, the free thiol content of oxidized MIF as well as its oxidoreductase activity was unaltered. The tautomerase activity, on the other hand, was significantly inhibited by this modification. The precise chemical nature of this oxidative modification has also remained unclear.

Taken together, 'oxMIF' is a poorly defined name representing oxidized MIF species that appear to occur in a disease-related manner and that may represent a redox-dependent isoform of MIF (or several isoforms), while anti-'oxMIF'-mAbs ameliorate inflammatory conditions as well as aid chemotherapy. Whether this isoform (or these isoforms) is (are) generated intra- or extracellularly and the molecular basis of its (their) formation is still unknown.

5. Redox-independent modifications

5.1. Glycosylation of Ser-112 and/or Thr-113

O-linked ß-N-acetylglucosamine (O-GlcNAc) is formed by the attachment of ß-N-acetylglucosamine (GlcNAc) to specific serine or threonine residues by *O*-GlcNAc transferase (OGT). O-GlcN-Acylation is known to regulate the bioactivity, stability and protein-protein interactions of certain proteins [91]. Zheng *et al.* demonstrated that extracellular MIF can be O-GlcN-acylated at Ser-112 and/or Thr-113 [92]. Unmodified MIF was shown to induce activation of ERK1/2 and AKT by binding its receptors CD74 and CXCR4, which could be blocked by CXCR4 and CD74 neutralizing antibodies. In contrast, *O*-GlcNAc-MIF was not able to induce phosphorylation of ERK and AKT in glioma cells, but was able to block EGF-induced signaling and tumor cell invasion. This study indicates that extracellular MIF has different receptor specificities and activities depending on its post-translational modification. It was further shown that MIF purified from bacteria was not post-translationally modified, but cellular MIF purified from 293T cells was shown to be O-GlcN-acylated at Ser-112 and/or Thr-113. This leads to the suggestion that the different post-translational modifications are cell-dependent and could also explain the somewhat lower biological activity of recombinant bacterial MIF compared to mammalian-derived MIF in certain cell systems.

5.2. Phosphorylation of Ser-91

Watarai *et al.*, who identified Cys-60 to be cysteinylated, also showed that Ser-91 can be phosphorylated [77]. We have confirmed this modification using in vitro phosphorylation in a cell-free system (H. Fünfzig, H. Lue, J. Bernhagen, unpublished observations). Phosphorylated MIF could not be isolated separately, but a mixture of phosphorylated MIF (30%) and unmodified MIF did not show immunosuppressive effects in contrast to cysteinylated MIF, which was shown to be bioactive in this context. When MIF modified at Ser-91 and Cys-60 was treated with alkaline phosphatase to dephosphorylate Ser-91, bioactivity was found to be enhanced. Therefore, phosphorylation of Ser-91 might play a role in regulating the activity of cysteinylated MIF, but this modification has not been studied further so far.

5.3. Glycation

Kassaar et al. recently identified a glycated MIF isoform present in brains with early stage Alzheimer's disease. Glycation is a post-translational modification of proteins, where sugar molecules, such as glucose, bind to lysine and arginine residues or the *N*-terminal amine [93]. This non-enzymatic glycosylation can lead to functional and conformational changes [94]. Kassaar demonstrated that glucose was the main trigger for MIF glycation in the Alzheimer's disease brain. Glycation inhibited MIF's oxidoreductase and tautomerase activities and glycated MIF had a reduced ability to induce ERK phosphorylation. They also showed that in addition to glycation oxidation is occurring in early stage of Alzheimer's disease brains.

6. Conclusions

MIF has an intriguing *N*-terminal proline residue that acts as nucleophile at physiological pH and is responsible for the intrinsic tautomerase activity of this chemokine-like inflammatory protein toward synthetic substrates. Because a physiological function has not yet been identified for this enzymatic function, the nucleophilic proline has been thought of as an evolutionary remnant. The tautomerase active site has, however, proved to be a useful target for the development of inhibitors that interfere with CD74 binding. Also, we recently found that Pro-2, and thus indirectly the tautomerase cavity, contribute to the MIF-CXCR4 binding interface [95]. The reactivity of this proline with oxidants and electrophiles combined with the potential abundance of these species at inflammatory sites leads us to speculate that the proline has been conserved as a site for post-translational regulation. The disease context, mechanisms and targets of proline-modified MIF are not yet known and further studies are required to identify the extent and nature of modifications of MIF isolated from inflammatory exudate. Another issue to consider is that some MIF modifications may make the protein undetectable by conventional assays, for example, monoclonal antibody dependent-ELISAs due to a concealment of the epitope by modification. The level of active MIF in biological samples may therefore be underestimated. Modification of the proline will also make these forms of MIF insensitive to tautomerase-based inhibitors, a notion with intriguing implications for drug discovery and optimization.

As progress is made in this field, it is likely that the number of identified MIF post-translational modifications will increase and terminology will become an important issue. Recently, the term 'oxMIF' was introduced to refer to a poorly characterized form of the protein that was generated by adding oxidized thiols to recombinant MIF. This is only one type of oxidation and there are clearly going to be a wide variety of potential products formed following exposure to different oxidizing species. Also, since several different residues are susceptible to modifications, there will be considerable heterogeneity in a population of MIF proteins. We recommend caution in the use of simple abbreviations and encourage that the biochemical nature and biological properties of the different MIF post-translational modifications are characterized as extensively as possible.

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Competing interest

All authors declare: no support from any organization for the submitted work; no financial relationship with any organization that might have an interest in the submitted work in the previous three years; no other relationships or activities that could appear to have influence the submitted work.

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