

The Allosteric Regulation of Macrophage Migration Inhibitory Factor

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Table of Contents

Title Page.....	i
Copyright Page.....	ii
Signature Page.....	iii
Curriculum Vitae.....	iv
Acknowledgements.....	viii
Table of Contents.....	ix
List of Tables.....	xii
List of Figures.....	xiii

CHAPTER ONE

Introduction.....	1
The Allosteric Regulation of Proteins in the Design of Therapeutics.....	2
MIF is a Model System for Characterizing Allosteric Protein Regulation.....	3
Targeting MIF in Disease.....	5
Characterizing Allostery in MIF with Solution NMR.....	6
References.....	8

CHAPTER TWO

Regulation of MIF Enzymatic Activity by an Allosteric Site at the Central Solvent

Channel.....	21
Abstract.....	22
Introduction.....	22

Results.....	24
Discussion.....	38
Materials and Methods.....	43
References.....	48
Appendix.....	58

CHAPTER THREE

A Cysteine Variant at an Allosteric Site Alters MIF Dynamics and Biological Function in Homo- and Heterotrimeric Assemblies.....60

Abstract.....	61
Introduction.....	61
Results.....	64
Discussion.....	75
Materials and Methods.....	77
References.....	83
Appendix.....	91

CHAPTER FOUR

Redox-dependent Structure and Dynamics of Macrophage Migration Inhibitory Factor Reveal Sites of Latent Allostery.....92

Abstract.....	93
Introduction.....	93
Results.....	95

Discussion.....	111
Materials and Methods.....	114
References.....	121
Appendix.....	134

CHAPTER FIVE

Redox-dependent Plasticity of oxMIF Facilitates its Interaction with CD74 and Therapeutic

Antibodies.....	135
Abstract.....	136
Introduction.....	136
Results.....	139
Discussion.....	158
Materials and Methods.....	166
References.....	177
Appendix.....	189

CHAPTER SIX

Discussion.....	190
Studies of the Allosteric Regulation of MIF.....	191
Key Findings and Future Directions.....	192
References.....	197

List of Tables

CHAPTER TWO

Table 1. Kinetic parameters for WT MIF and MIF mutants determined from tautomerase assays of 4-HPP.....	26
Appendix Table 1. Data collection and refinement statistics for the Tyr99 and H62 variant crystal structures.....	58
Appendix Table 2. The PDB entries, RMSD values to WT MIF, and resolution of the 19 MIF structures with all three active sites occupied.....	59

CHAPTER THREE

Table 1. Kinetic parameters of the WT MIF and Y99C MIF variants tautomerase activity...	75
Appendix Table 1. Crystallographic Statistics for the Y99C MIF homotrimer.....	91

CHAPTER FOUR

Table 1. Peptides with oxidized methionine residues detected by mass spectrometry.....	105
---	-----

CHAPTER FIVE

Table 1. Peptide mapping MS analysis of modifications in MPO-oxMIF.....	148
Table 2. Peptide mapping MS analysis of modifications in H ₂ O ₂ -MIF.....	149
Table 3. Peptide mapping MS analysis of modifications in MIF.....	149
Table 4. MS/MS analysis of MPO-oxMIF generated with a broad concentration range of MPO-derived HOCl.....	150

List of Figures

CHAPTER ONE

Figure 1. Solution NMR detects the conformational equilibrium of MIF that is not observed by X-ray crystallography.....	8
--	---

CHAPTER TWO

Figure 1. Impact of Tyr99 and His62 mutations on the catalytic activity of MIF.....	25
Figure 2. Secondary structure and stability of Tyr99 variants.....	27
Figure 3. Effect of Tyr99 and His62 mutations at the opening of MIF solvent channel.....	28
Figure 4. Crystallographic analysis of MIF variants.....	29
Figure 5. The role of water-mediated networks in MIF catalysis.....	30
Figure 6. Impacts of allosteric mutations on intra-residue and inter-monomer communication in MIF.....	32
Figure 7. NMR probe of perturbations to the MIF allosteric pathway induced by His62 mutations.....	33
Figure 8. NMR probe of perturbations to the MIF allosteric pathway induced by Tyr99 mutations.....	35
Figure 9. Allosteric reciprocity and sensitivity of the tautomerase and solvent channel sites.....	37
Figure 10. NMR spin relaxation rates for WT MIF.....	38
Figure 11. Active site ligands and the allosteric site Tyr99.....	39
Figure 12. Allosteric pathway(s) influencing MIF activity.....	41

CHAPTER THREE

Figure 1. Schematic of important structural features in MIF.....	63
Figure 2. Verification of novel Y99C/WT mixed trimers.....	65
Figure 3. Sedimentation coefficient distribution for the Y99C MIF homotrimer at different concentrations.....	66
Figure 4. Changes in the MIF structure with mutations at Tyr99.....	67
Figure 5. Secondary structure and stability of Y99C MIF variants.....	68
Figure 6. Structural effects of the Y99C variants by NMR.....	69
Figure 7. Altered dynamics of the Y99C homo- and heterotrimers.....	71
Figure 8. <i>In vivo</i> CD74 activation in the lung by WT MIF and the Y99C MIF homotrimer.....	73
Figure 9. Altered catalytic activity of the Y99C MIF variants.....	74

CHAPTER FOUR

Figure 1. Redox-dependent secondary structure and stability of MIF.....	95
Figure 2. Redox-dependent changes in MIF detected by NMR.....	96
Figure 3. The dynamic profile of MIF is modulated by solution redox conditions.....	99
Figure 4. Sites of μ s – ms flexibility are revealed under oxidizing conditions.....	101
Figure 5. C80 is selectively modified under oxidizing conditions.....	103
Figure 6. Mutations at K66 and C80 cause biophysical changes to the MIF structure.....	106
Figure 7. Mutation of the redox-sensitive K66 and C80 residues alters the redox sensitivity of the MIF structure.....	108
Figure 8. Mutation of the redox-sensitive K66 and C80 residues alters MIF enzymatic and pro-inflammatory function.....	110

Appendix Figure 1. Raw mass spectrometry data and the effects of oxidant concentration...	134
--	-----

CHAPTER FIVE

Figure 1. MIF and oxMIF are produced in isolated human neutrophils.....	140
Figure 2. Hypochlorous acid-modified oxMIF specifically interacts with the antibody ON104.....	142
Figure 3. Intact mass analysis of MIF, MPO-oxMIF, and H ₂ O ₂ -MIF.....	145
Figure 4. Peptide mass analysis of methionine oxidation in MPO-oxMIF.....	146
Figure 5. Peptide mass analysis of cysteine, proline, and tyrosine modifications under various redox conditions.....	147
Figure 6. Structural and dynamic effects of MPO-generated HOCl on MIF by NMR.....	151
Figure 7. Redox-dependent binding of MIF to the CD74 receptor via SPR.....	153
Figure 8. Redox-dependent binding of MIF to HT29 cell-surface expressed CD74.....	154
Figure 9. Additional assays to study the oxMIF-CD74 interaction on the surface of HCT116 and ExpiCHO-S cells.....	156
Figure 10. Endogenous oxMIF binding to CD74 on human neutrophils.....	157
Figure 11. MIF tautomerase assay after treatment with H ₂ O ₂ alone or with the MPO system.....	162
Appendix Figure 1. Validation of 0.5% Proclin300-oxMIF as a standard for oxMIF ELISA.....	189

CHAPTER ONE

Introduction

The Allosteric Regulation of Proteins in the Design of Therapeutics

For decades, drug discovery pipelines have leveraged the structure-function paradigm to site-specifically target proteins for translational outcomes.¹ The first protein structure was solved by X-ray crystallography in 1958,² and coupled with the establishment of the Protein Data Bank³ and development of solution nuclear magnetic resonance (NMR) spectroscopy-based structure determination⁴ in the decades that followed, provided three-dimensional molecular detail that had previously evaded drug discovery efforts. For the first time, the structure of a target protein could guide the rational design of therapeutic molecules, thus revolutionizing drug discovery.⁵ The field of structure-based drug design has largely focused on protein active sites to inhibit biochemical reactions and biomolecular interfaces to preclude protein-ligand interactions. However, current clinically-approved drugs target only a small percentage (~3%) of the human proteome,⁶ necessitating efforts to look beyond the orthosteric sites that are classically targeted and identify new functional hotspots.

Experimental and technological advancements have furthered the premise that proteins are often controlled by specific amino acid residues that transmit chemical information over long molecular distances to induce a functional response.⁷⁻¹² This biological phenomenon is known as allostery. Endogenous and exogenous stimuli, such as changes in pH, solution redox conditions, chemical modifications, and the binding of small molecules, metal ions, partner proteins, or nucleic acids can elicit or alter allosteric signaling to a functional site.¹³ The transmission of these signals is often conferred by intrinsic conformational dynamics and mediated by ‘pathways’ of residues.¹⁴⁻¹⁶ Interruption of these allosteric pathways can alter protein function. Thus, allosterically-driven mechanisms are promising avenues to tune spatial, temporal, and pharmacological properties of proteins via protein engineering.^{17,18} Notably, the elucidation of novel allosteric sites can

significantly broaden the druggable landscape of proteins, and provide opportunities to target proteins once written off as undruggable.¹⁹

MIF is a Model System for Characterizing Allosteric Protein Regulation

Macrophage Migration Inhibitory Factor (MIF) is a ubiquitous immunoregulatory protein most widely recognized for its role in the pathophysiology of inflammation.²⁰ Though classified as a cytokine, MIF diverges from its pro-inflammatory counterparts in that it is constitutively expressed by most cells in the human body, stored in intracellular pools, and then exported in response to stimuli.²¹ Circulating levels of MIF are reported in the low nanomolar range in healthy humans,²² rendering MIF a powerful first line of defense towards infection and injury. MIF initiates several inflammatory signaling cascades through the binding of its cell surface receptors, including its cognate receptor, CD74, and the chemokine receptors CXCR2, CXCR4, and CXCR7.^{21,23,24} MIF binds the extracellular domain of CD74, which in turn recruits the intracellular coreceptor CD44 to subsequently activate the ERK1/2 signaling pathway that promotes inflammation through cell proliferation and migration.²⁵ MIF also initiates the AKT pathway that regulates cell growth and survival, and AMPK signaling involved in cellular homeostasis.²¹ Further, MIF serves to maintain the inflammatory response through the counter-regulation of anti-inflammatory glucocorticoids and suppression p53-mediated apoptosis.^{26,27}

Beyond its receptors, MIF has a large interactome of proteins that it has been shown to bind intracellularly and extracellularly,^{21,28,29} further expanding its biological influence. Many of these interacting proteins are involved in signaling pathways themselves. For example, thioredoxin (TRX) and thioredoxin-interacting protein (TXNIP) are implicated in redox signaling.³⁰ The binding of MIF to TRX inhibited TNF- α production,³⁰ and binding to TXNIP counteracted TXNIP-mediated inhibition of NF- κ B.³¹ JAB1, a subunit of the COP9 signalosome, regulates

cellular proliferation and apoptosis, and binding to MIF inhibits JAB1 activation of JNK.³² p53, a well-known tumor suppressor, is involved in DNA repair pathways, regulating cell division and apoptosis.³³ The direct interaction between MIF and p53 inhibits p53-mediated transcription and apoptosis.³⁴ Beyond signaling, MIF has also been shown to bind SOD1^{35,36} and Amyloid β ,^{37,38} proteins known for misfolding and aggregation that are strongly linked to neurodegenerative diseases. In the case of SOD1, MIF inhibits the accumulation and toxicity of misfolded SOD1, suggesting that MIF may also function as a chaperone. Recently, Cho *et al.* fluorescently-tagged MIF via CRISPR editing, and mapped intracellular interactions via co-immunoprecipitation and mass spectrometry, yielding 99 MIF-interacting proteins.²⁸ Though the majority of the proteins identified have not yet been characterized in complex with MIF, they demonstrate the breadth of the MIF interactome and potential for its expansive biological impact.

Remarkably, MIF is also an enzyme that catalyzes three biochemical reactions via distinct active sites on each of the 12.5 kDa monomeric subunits within its trimeric form. As a tautomerase, its N-terminal Pro1 serves as a nucleophile that interconverts the isomers of several substrates, including phenylpyruvate, hydroxyphenylpyruvate, and D-dopachrome, among others.^{39,40} Though a physiological substrate has not yet been ascertained, and it has been suggested that its tautomerase function may be merely vestigial,⁴¹ inhibition of MIF tautomerase activity by Pro1 mutation or small molecules has been shown to inhibit CD74 binding and activation of downstream signaling.⁴² MIF also functions as a thiol-protein oxidoreductase through its thioredoxin-like CXXC motif, and was demonstrated *in vitro* to catalyze the reduction of insulin and 2-hydroxyethyl disulfide.⁴³ Similar to its tautomerase activity, the physiological relevance of its oxidoreductase activity remains elusive, but mutation of the CXXC cysteines inhibits its immunoregulatory effects, again suggesting a role for enzyme activity in its cytokine functions.

Most recently, MIF was identified as a nuclease.^{44,45} Apoptosis-inducing Factor (AIF) binds and recruits MIF to the nucleus as part of a programmed cell death pathway called parthanatos, where MIF functions as a 3' exonuclease and endonuclease to cleave genomic DNA via a PD-D/E(X)K nuclease motif. The nuclease activity of MIF may contribute to the pathology of disease, such as neurodegeneration⁴⁶ and cancer cell growth and survival,⁴⁴ as a reduction in disease burden was observed upon mutational inhibition in several studies.

From a structural perspective, the ability of MIF to accommodate non-overlapping biochemical activities and protein-protein interactions within its compact 12.5 kDa monomers (or 37.5 kDa obligate trimer) is puzzling. Recently, allostery has emerged as a compelling regulator of its multifunctional effects.⁴⁷ Despite MIF being a well-studied system, the majority of its biological impact is derived from tissue culture or animal studies. The biophysical mechanisms that govern MIF are poorly understood, complicating efforts to resolve the biology of its promiscuous interactions and disparate pro-inflammatory and enzymatic functions. MIF is therefore positioned as a model system for the study of the allosteric regulation of protein function.

Targeting MIF in Disease

Overexpression of MIF has been linked to an overwhelming number of diseases, suggesting the MIF may contribute to their pathology. MIF is upregulated in autoimmune and inflammatory diseases, such as rheumatoid arthritis,⁴⁸ diabetes,⁴⁹ psoriasis,⁵⁰ multiple sclerosis,^{51,52} asthma,⁵³ sepsis,^{54,55} acute respiratory distress syndrome (ARDS),⁵⁶ colitis,⁵⁷ pancreatitis,⁵⁸ Crohn's disease,⁵⁷ and complications of COVID-19.⁵⁹ MIF has also been linked to the pathologies of atherosclerosis,⁶⁰⁻⁶³ cancers,⁶⁴⁻⁶⁹ tumor microenvironments, and neurodegenerative diseases,⁵² including Alzheimer's,^{38,70,71} ALS,^{36,72} and Parkinson's.⁴⁶ Further, increased CATT repeat polymorphisms in the *MIF* promotor have been correlated with increased

expression of MIF, and these CATT repeats as well as a G/C single nucleotide polymorphism are linked to susceptibility and severity of disease.⁷³ As described earlier, the ability of MIF to serve as a nuclease has also been shown to promote cancer survival and neurodegeneration.

Intriguingly, knockout models of MIF and treatment with anti-MIF antibodies have resulted in beneficial and detrimental outcomes, a balancing act that is often observed in translational biology. MIF deletions and neutralization in murine models of sepsis have shown increased survival rates,^{74,75} and a marked decrease in disease severity was reported in models of pancreatitis and osteoarthritis.^{76,77} Conversely, healthy murine models suffer from heightened susceptibility to certain pathogens and cardiac dysfunction when MIF is not present.⁷⁸⁻⁸⁰ Though the role of MIF in disease remains unclear, it has emerged as a compelling target for the development of therapeutics given its broad association. Collectively, the structural, functional, and translational questions surrounding MIF necessitates a biophysical characterization of the allosteric mechanisms that regulate its functions in healthy and disease-relevant conditions, which can then be leveraged for the design of therapeutics that confer selective inhibition, allowing for tunable control over its many functions. These efforts will also be imperative for characterizing and targeting MIF in disease.

Characterizing Allostery in MIF with Solution NMR

Solution NMR is unparalleled in its ability to detect, map, and quantify structural and dynamic properties of proteins with atomic resolution.⁸¹ It is a powerful tool for the study of the biophysical determinants of allostery in protein functional regulation.⁸²⁻⁸⁵ Beyond structure determination, NMR can detect and quantify local structural changes to the protein backbone and side chains, on a per residue basis, in response to environmental stimuli, ligand binding, chemical modification, or mutation.⁸¹ NMR can also identify biochemical properties of residues, such as

post-translational modifications and pK_a . Several methods have been developed to leverage NMR-detected structural perturbations to identify couplings between residues that signify allosteric networks.⁸⁶ Notably, NMR can quantitatively measure protein motions on all biologically relevant timescales, ranging from picoseconds to seconds.^{87,88} Fast dynamics, on the picosecond-to-nanosecond timescale, reflect fluctuations in bond vectors, chemical shift anisotropy, and dipolar interactions between nuclei. These fast dynamics have been shown to contribute to entropically-driven allostery for several systems.^{89,90} Biologically slow dynamics, on the microsecond-to-millisecond timescale, capture conformational fluctuations typically associated with allostery, enzyme catalysis, protein folding, and ligand binding. The sensitivity of NMR allows for the detection of lowly populated (‘excited’) conformational states that are often critical to protein function and allosteric de(activation), but cannot be captured by other structural methods.⁹¹

Structural characterizations of MIF have previously relied on X-ray crystallography and provided little insight given the nearly identical structures of MIF mutants or inhibitor-bound complexes (**Figure 1A**). By contrast, solution NMR reveals vastly different spectra of MIF when perturbed by mutation or inhibitor binding (**Figure 1B**), suggesting that the static X-ray snapshots are not capturing the true conformations of MIF populated in solution, and positioning NMR as an important experimental tool for detecting and characterizing the subtle conformations and motions essential for the propagation of allosteric signals that mediate MIF function. In conjunction with other biophysical and biochemical techniques, as well as functional experiments *in vivo* and *in vitro*, NMR studies of MIF will provide important biophysical insights that have been missing from the field of MIF biology.

The aims of this dissertation are to provide foundational insights into the allosteric regulation of MIF by identifying amino acids involved in its allosteric signaling, and how this

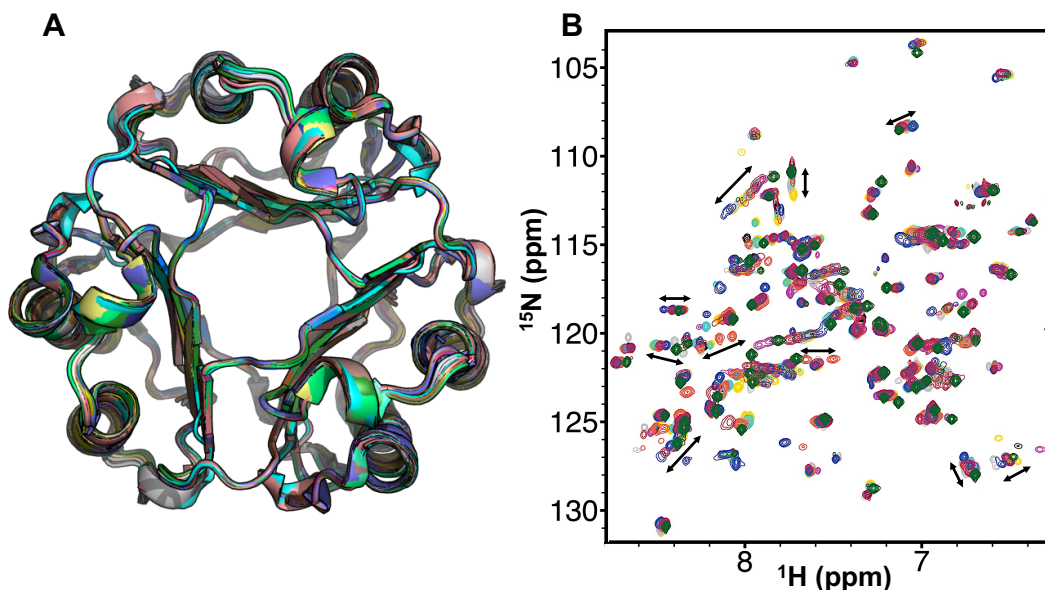


Figure 1. Solution NMR detects the conformational equilibrium of MIF that is not observed by X-ray crystallography. (A) An overlay of 30 X-ray crystal structures of MIF mutants and MIF-inhibitor complexes show few changes to its structure, while (B) NMR spectra of 10 MIF mutants suggest that each structure is different in solution, with arrows highlighting shifts in NMR peak (amino acid) positions, reflecting a clear conformational equilibrium.

network is altered under disease relevant conditions, to help resolve the mechanisms that drive its distinct functions. Small molecule inhibitors of MIF have primarily affected MIF active sites, thus the elucidation of novel points of functional control can be leveraged for the development of new allosteric inhibitors that selectively target MIF in disease.

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CHAPTER 2

Regulation of MIF Enzymatic Activity by an Allosteric Site at the Central Solvent Channel

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Regulation of MIF Enzymatic Activity by an Allosteric Site at the Central Solvent Channel

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Author contributions: **E.S.** expressed and purified protein, and conducted and analyzed the NMR and CD spectroscopy experiments. **G.P.** conceived the project, expressed and purified protein, and conducted and analyzed crystallography experiments. **L.K.** performed and analyzed the kinetic experiments. **A.M.** conducted the protein-water interaction network analyses. **K.R.** performed MD simulations. **V.S.B.** supervised the MD simulations. **G.P.L.** supervised the NMR and CD experiments, and analyzed data. **E.J.L.** conceived and supervised the project. The manuscript was written through contributions from all authors.

Abstract

Protein motions play a key role in their diverse functions. In proteins with multiple functions, such as macrophage migration inhibitory factor (MIF), the study of its intramolecular dynamic network can offer a unique opportunity to understand how a single polypeptide chain is able to carry out several non-overlapping functions. Toward this effort, a dynamic mechanism that controls the MIF-induced activation of CD74 was recently discovered. In the present study, the regulation of MIF tautomerase activity was explored. The MIF catalytic base Pro1 is found to form dynamic communications with the same allosteric node that regulates activation of CD74. Signal transmission between the allosteric and catalytic sites takes place through intramolecular aromatic interactions and a hydrogen bond network that is formed among residues and well-ordered water molecules of the MIF solvent channel. Once thought to be a consequence of trimerization, we now define a biological function for the solvent channel. Collectively, these results provide mechanistic insights into the regulation of MIF catalytic activity and the role of solvent channel water molecules in MIF catalysis.

Introduction

MIF has been identified to have multiple, unrelated functions similar to other “moonlighting proteins”.¹⁻³ From an evolutionary point of view, these proteins served an initial function and were exapted to serve additional roles. A well-known example are the crystallin proteins in the eye that evolved from various dehydrogenases to serve as structural proteins that increase the transparency and refractive index of light.⁴ These gene products assumed their alternate functions based on a cell that evolved a mechanism to accommodate this purpose. In the case of MIF, various functions are related to its cell biology with respect to its presence in the cytosol, nucleus, and the extracellular space,⁵ yet we lack a full understanding of the intramolecular

and intermolecular mechanisms that regulate its multiple and nonoverlapping functions.

MIF is a well-known master regulatory cytokine originally described in the 1960s^{6,7} and named after its ability to inhibit macrophage migration.⁸ In the cytoplasm, it modulates the activities of JAB-1,⁹ ERK-1/2,¹⁰ and p53.¹¹ In the nucleus, MIF is reported to associate with the apoptosis-inducing factor (AIF) to function as an endonuclease for genomic DNA degradation resulting in chromatinolysis.¹² Cytoplasmic MIF is exported to the extracellular space after cell stimulation, resulting in the activation of the type II receptor CD74 and the G-protein coupled receptors CXCR2 and CXCR4.^{13,14} Functional regions on MIF responsible for some of these activities were identified using modeling and experimental approaches.^{12,15-20}

While MIF plays a pivotal role in orchestrating aspects of the innate and adaptive immune response, high levels of this cytokine contribute to a wide range of pathologies including inflammatory, autoimmune, cardiovascular, and malignant diseases.²¹⁻²³ MIF levels are governed by 5-8 tandem DNA repeats of CATT at position -794 of the MIF gene promoter region and a G/C SNP at position -173.^{24,25} Although the majority of published studies suggest that MIF upregulation has a negative impact in disease, in some cases such as cardiac surgery,²⁶ MIF plays a protective role.

The crystal structure of human MIF²⁷ reveals a homotrimer with a solvent channel along the 3-fold axis. The human D-dopachrome tautomerase (D-DT/MIF-2) and the bacterial enzymes 4-oxalocrotonate tautomerase (4-OT) and 5-carboxymethyl-2-hydroxymuconate isomerase (5-CHMI)²⁸ share a similar structural topology, and together comprise the MIF superfamily. The members of this family share a tautomerase or isomerase activity with a catalytic base, Pro1²⁹ that has an unusually low pKa of 5.6 in human MIF.³⁰ Despite the distinct physicochemical properties of the active site for the human MIF and D-DT/MIF2, they both catalyze the tautomerization of

D-dopachrome (a synthetic molecule), 4-hydroxylphenyl pyruvate (4-HPP), and phenyl pyruvate (PP).^{29,31,32} These substrates and catalytic activities were used to identify various inhibitors for MIF^{17,33-35} and D-DT.^{31,32} However, somewhat paradoxically, the physiological substrate of the MIF tautomerase site is unknown, despite universal conservation of the Pro1 active site in MIF species.

Molecular dynamics (MD) simulations were recently utilized to obtain insights into the MIF-induced activation of CD74.³¹ An allosteric site located at one end of the solvent channel was identified to regulate CD74 activation. Here, we employ a functional study of MIF mutants, including MD simulations, water molecule analysis, crystallography, and nuclear magnetic resonance (NMR) to understand how MIF catalytic activity is regulated. We identify an intramolecular dynamic mechanism formed among MIF residues and water molecules in the solvent channel that regulates the catalytic activity of MIF.

Results

Mutation of the allosteric node and adjacent residues alters MIF catalytic activity

MD simulations of WT MIF identified that the allosteric site composed of Tyr99 is strongly correlated with the catalytic residue Pro1.³¹ The two sites communicate *via* the intermediate residues His62 and Met2, which are located on adjacent β -strands. This dynamic pathway that controls the MIF catalytic activity differs from the corresponding pathway that regulates activation of CD74, both in terms of direction of the signal and the residues involved.³¹ To experimentally probe the potential dynamic correlation between Tyr99 and the catalytic activity of MIF, we mutated the key nodes of the dynamic pathway, Tyr99 (Y99F, Y99A and Y99G) and His62 (H62Y, H62F, H62A and H62G), and examined the impact of these mutations on the HPP keto/enol tautomerase activity of the protein (**Figure 1** and **Table 1**).

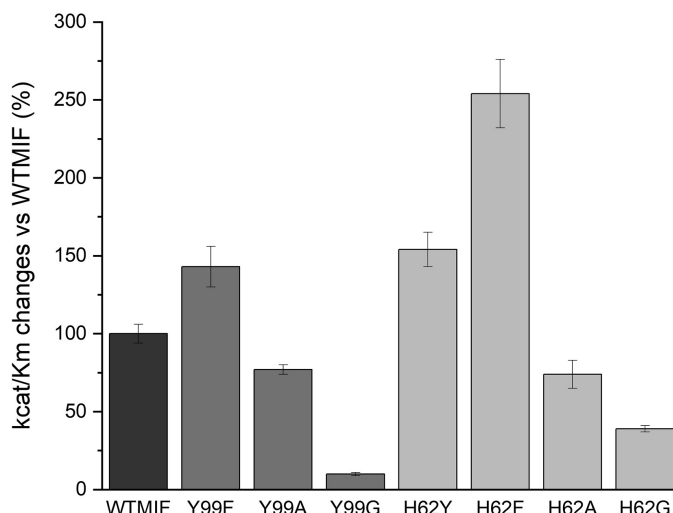


Figure 1. Impact of Tyr99 and His62 mutations on the catalytic activity of MIF. Kinetic analyses of WT MIF (black bar) and MIF mutants (Y99F, Y99A, Y99G - dark gray bars; H62Y, H62F, H62A, H62G – light gray bars) demonstrate the connection between the allosteric (Tyr99) and catalytic (Pro1) sites.

Based on the catalytic efficiencies ($k_{\text{cat}}/K_{\text{m}}$) of the MIF variants, we noticed three distinct outcomes. In the first group, we observed elevated catalytic activities due to substitution of Tyr99 or His62 by another aromatic amino acid (Tyr or Phe). Compared to wild-type (WT) MIF, the catalytic efficiencies of Y99F, H62F and H62Y were found to be increased to 143%, 254% and 154%, respectively (**Figure 1**). The kinetic parameters showed that the enhanced catalytic rates of Y99F and H62Y are related to k_{cat} values while for H62F, both k_{cat} and K_{m} are improved (**Table 1**). In the second group, Tyr99 and His62 substitutions to alanine (Y99A and H62A) reduced, to some extent, the catalytic efficiency of MIF to 77% (Y99A) and 74% (H62A) of the corresponding efficiency of WT MIF (**Figure 1**). Despite the similar activities, Y99A and H62A revealed distinguishable patterns in relation to their kinetic parameters (**Table 1**). For Y99A, the K_{m} value remained similar to that of WT MIF and only k_{cat} is reduced while for H62A the opposite effect is observed. The Gly mutations, which is the third distinct group, demonstrated decreased catalytic efficiencies resulting in 10% (Y99G) and 39% (H62G) of the corresponding efficiency of WT MIF (**Figure 1**). The K_{m} value of Y99G remain unaffected and only k_{cat} is significantly reduced, while

Table 1. Kinetic parameters for WT MIF and MIF mutants determined from tautomerase assays of 4-HPP

	$V_{\max}$ ($\mu\text{M s}^{-1}$)	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1}\text{s}^{-1}$)	$k_{\text{cat}}/K_{\text{m}}$ changes vs. WT MIF (%)
WT MIF	0.32 ± 0.01	6.47 ± 0.12	1.06 ± 0.05	6.1 ± 0.36	100 ± 6
Y99F	0.60 ± 0.03	11.9 ± 0.64	1.38 ± 0.13	8.7 ± 0.82	143 ± 13
Y99A	0.23 ± 0.02	4.67 ± 0.42	0.99 ± 0.10	4.7 ± 0.21	77 ± 3
Y99G	0.15 ± 0.01	0.59 ± 0.05	1.02 ± 0.20	0.6 ± 0.06	10 ± 1
H62Y	0.43 ± 0.02	8.67 ± 0.46	0.92 ± 0.02	9.4 ± 0.68	154 ± 11
H62F	0.49 ± 0.01	9.86 ± 0.12	0.64 ± 0.06	15.5 ± 1.35	254 ± 22
H62A	0.37 ± 0.10	7.40 ± 1.91	1.64 ± 0.42	4.5 ± 0.53	74 ± 9
H62G	0.52 ± 0.02	2.09 ± 0.08	0.88 ± 0.46	2.4 ± 0.12	39 ± 2

the H62G mutation alters both K_{m} and k_{cat} values (**Table 1**). Circular dichroism spectra indicate that all classes of mutants are folded (**Figure 2**), with small per-monomer energetic changes that likely only partially account for these functional observations.

Crystal structures of MIF mutants highlight subtle differences in molecular interactions within the allosteric pathway

Crystallographic analysis of the MIF mutants (Y99F, Y99A, Y99G, H62Y, H62F, H62A and H62G) revealed excellent superposition to WT MIF with root-mean-square deviation (RMSD) values that varied between 0.08 and 0.16 Å (**Appendix Table 1**). Overall, we did not observe any major conformational changes that could potentially explain the three distinct catalytic behaviors of the MIF mutants. Y99G, which is the mutant with the lowest catalytic activity (**Figure 1**), has a superposition agreement to WT MIF with an RMSD of 0.10 Å and does not show any significant conformational changes along the dynamic pathway under investigation (Tyr99-His62-Met2-Pro1). We therefore probed whether the solvent channel and residues located at the opening of the channel (Tyr99) or in close proximity to it (His62) were affected. Notably, we found that the size of the solvent channel opening correlates with the catalytic efficiency of the MIF variants (**Figure3**). Indeed, as the side chain volume decreases from Y99F to Y99G (Y99F-Y99A-Y99G)

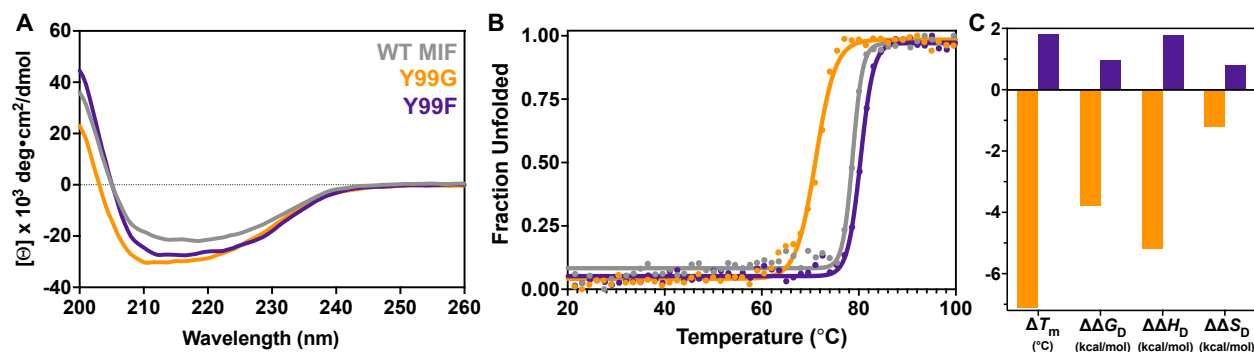


Figure 2. Secondary structure and stability of Tyr99 variants. (A) Circular dichroism spectra of WT (gray), Y99G (orange), and Y99F (purple) MIF. (B) Thermal unfolding experiments for WT MIF and the Y99 variants ($\lambda = 218 \text{ nm}$), plotted as a normalized fraction unfolded. (C) Summaries of apparent changes in T_m , free energy ($\Delta\Delta G_D$), enthalpy ($\Delta\Delta H_D$), and entropy ($\Delta\Delta S_D$) for Y99G and Y99F MIF, calculated relative to WT MIF.

and from H62F/H62Y to H62G (H62F/H62Y-H62A-H62G), the opening of the solvent channel increases and the catalytic activity of these MIF variants decreases. Interestingly, the two mutants H62Y and H62F, which demonstrate higher catalytic efficiency than WT MIF (254% and 154%, respectively), have similar solvent channel openings but their catalytic activities are different (Figure 1). These observations suggest that the channel opening plays a major role in regulating the catalytic activity from the allosteric node. We used crystal structures of these two variants to examine how the interatomic interactions promote these catalytic enhancements between the allosteric and catalytic sites. We observed movements of the Tyr99 side chain toward the Tyr62 (H62Y) and Phe62 (H62F) side chains by 0.9 Å and 1.2 Å, respectively (Figure 4A, top panel). The movement brings the center of Tyr99 and the two aromatic rings for H62F and H62Y to a distance of 4.1 Å and 4.3 Å, respectively, creating π - π stacking interactions more optimal than those of WT (Figure 4B, bottom panel) as confirmed by Arpeggio, a web server that calculates interatomic interactions in protein structures.³⁶ Using the crystal structure of WT MIF (PDB: 3DJH), we examined how the π - π stacking interactions can promote the transmission of a dynamic signal between the allosteric and catalytic sites. We found that His62 forms two backbone

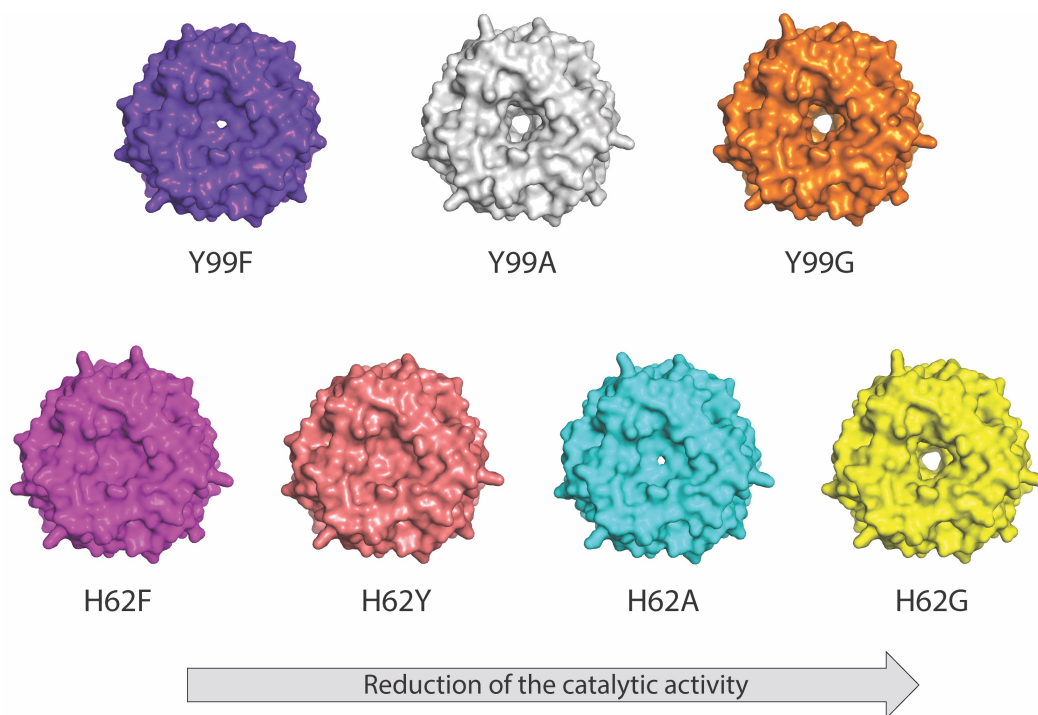


Figure 3. Effect of Tyr99 and His62 mutations at the opening of MIF solvent channel. Crystallographic analysis of the MIF variants showed that the opening of the solvent channel is clearly affected by the mutation of Tyr99 and His62. The increased opening of the solvent channel is oppositely associated with the catalytic activity of MIF.

hydrogen bond interactions with Met2, typical of two adjacent β -strands in a β -sheet (**Figure 4C**).

Through this network of interactions, a dynamic signal from Tyr99 is able to reach the catalytic site *via* the β -strand system of MIF. Of note, Tyr99 and the catalytic residue Pro1 are located 7.8 Å apart (PDB: 3DJH, **Figure 4D**). Therefore, the only way that the catalytic activity of MIF can be influenced by Tyr99 is through intramolecular dynamic signals that involve the β -strand system of the protein, something that has been previously described for other proteins.^{37,38}

Solvent channel water molecules mediate MIF catalysis

The increased catalytic efficiency of the H62Y, H62F, and Y99F mutants is unlikely due to the weak π - π interactions between residue 62 and 99 alone. To determine whether the solvent contributes to the observed differences in catalytic activity across MIF variants, we performed a

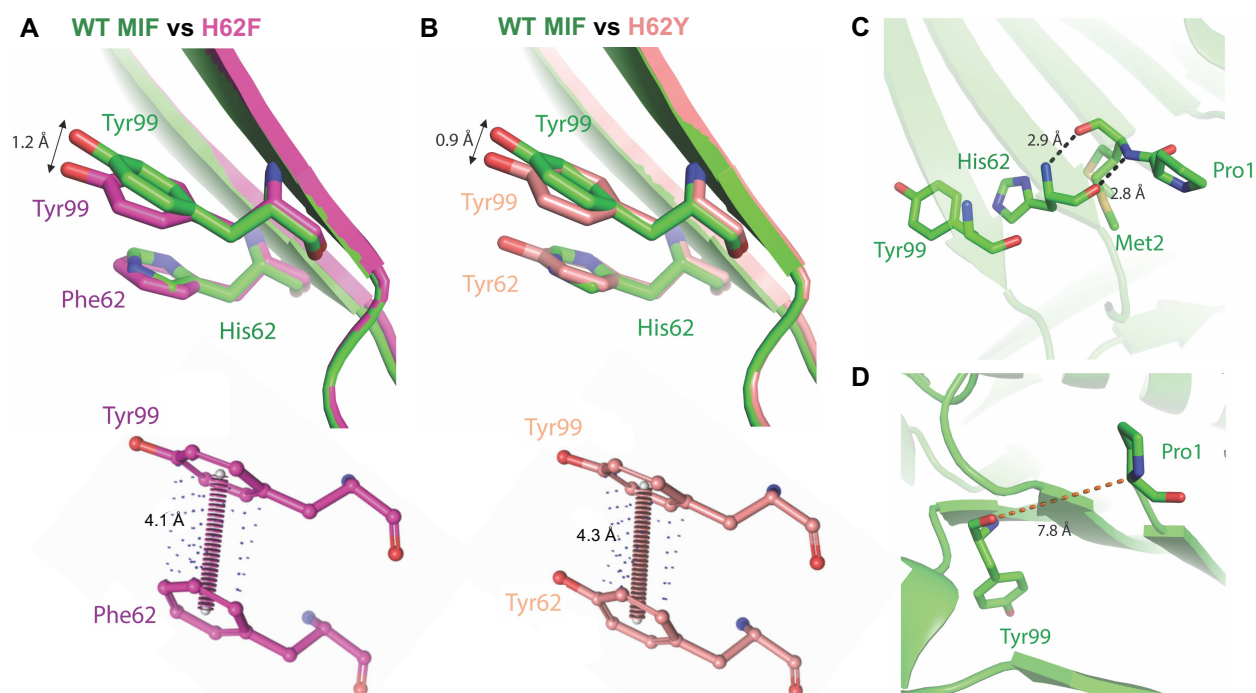


Figure 4. Crystallographic analysis of MIF variants. Comparison of the crystal structures of WT MIF with H62F in (A) and H62Y in (B) reveal that the larger aromatic residues at position 62 induce a distinct conformational change towards Tyr99, increasing the coupling between the two residues (top panel). Aromatic interactions between Phe62-Tyr99 and Tyr62-Tyr99 also become more favorable in the variants (bottom panel). (C) His62 forms two backbone hydrogen bond interactions with Met2 that promote communication along the dynamic pathway. (D) The nearest distance between Tyr99 and Pro1 is 7.8 Å. Tyr99 and Pro1 are shown as sticks.

contact network analysis involving the solvent channel water molecules. We computed the hydrogen bond network of water molecules near the allosteric site for the crystal structures of WT MIF and variants (Y99F, Y99A, Y99G, H62Y, H62F, H62A and H62G). For the MIF mutants with enhanced catalytic activities (Y99F, H62Y, and H62F), we observed an increased hydrogen bond network proximal to residue 99 relative to WT MIF (**Figure 5A**). In contrast, the mutants with reduced catalytic efficiency (Y99A, Y99G, H62A and H62G) exhibited few-to-no water mediated hydrogen bonds in the network proximal to the allosteric site. In WT MIF, water molecules are positioned to form hydrogen bond interactions with both Tyr99 and His62 and facilitate a bridge between the two residues (**Figure 5B**). The Y99A, Y99G, H62A and H62G mutations abolished any ability for bridging residues 99 and 62 by water molecules, thus

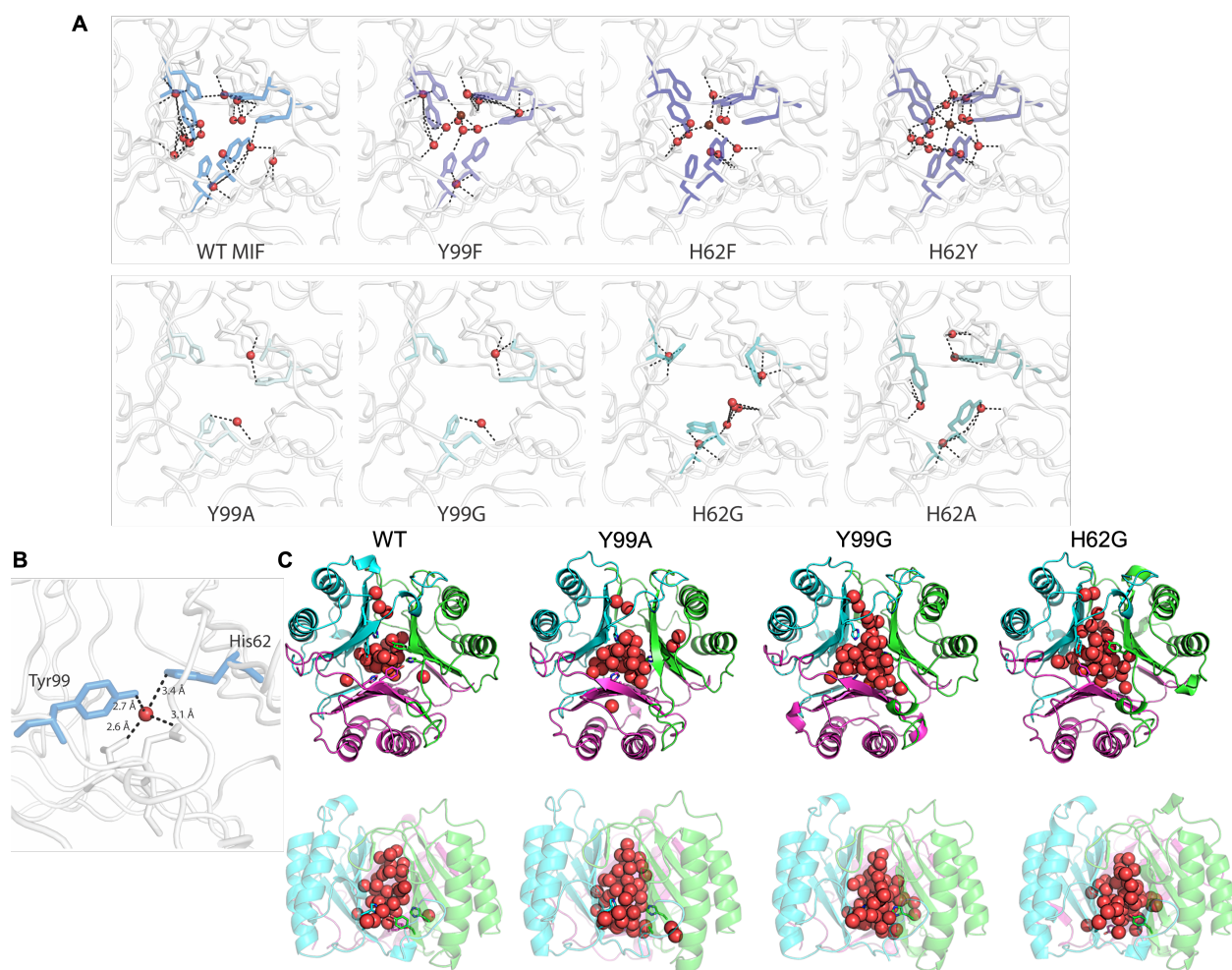


Figure 5. The role of water-mediated networks in MIF catalysis. (A) The degree of water network connectivity between residues 99 and 62 is strongly correlated with the MIF catalytic activity. Water molecules are shown as red spheres and the hydrogen bond as black dotted lines. The central water molecule found in the crystal structures of Y99F, H62F and H62Y is shown in brown. (B) In the crystal structure of WT MIF, water molecules serve as a bridge to facilitate communication between residues 99 and 62, via hydrogen bond interactions. (C) Models from 200 ns MD simulations examining hydrogen bond networks in WT, Y99A, Y99G, and H62G MIF. Water molecules within the solvent channel, as well as hydrogen bond donor-acceptor distances between gating residues Y99 and V42 (top and bottom of channel) were used to determine the number of central water molecules in each MIF model.

decoupling the two residues and leading to decreased catalytic activities.

To examine the influence of individual water molecules on MIF catalytic activity, we performed a protein-water network centrality analysis computed for WT MIF and all variants. From this analysis, we found that the MIF mutants Y99F, H62Y, and H62F have one common water molecule (shown in brown) that is positioned to facilitate water bridges between residues 99

and 62 (**Figure 5A**). For example, in the H62Y mutant, the central water molecule is stably positioned to form water bridges amongst any of the six neighboring tyrosine residues. The additional water molecules found around the central water help construct a network that connects residues 99 and 62. The denoted central water molecule found in the crystal structures of Y99F, H62Y, and H62F had the second highest degree centrality of any water molecule in the entire protein, thus confirming both its high stability and significance in transmitting dynamic signals between positions 99 and 62. With this analysis, we demonstrate that water network arrangements in the solvent channel can play a significant role in MIF catalysis.

Hydrogen bond analysis from 200 ns MD simulations revealed that mutations at His62 or Tyr99 reduced the hydrogen bond interactions connecting the active and Tyr99 sites. Of note, Tyr99 is connected to the active site through hydrogen bonds with Leu61 and Ser63 during the trajectories, which surround His62, and are in turn hydrogen bonded to Met2. In the Y99A mutant, the hydrogen bonding between the backbones of Ala99 and Ser63 was reduced by 16% based on hydrogen bond analysis of the latter 100 ns of the trajectory. Additionally, the interaction between Ala99 and Leu61 observed during WT trajectories was reduced by 8%. Predictably, mutating Tyr99 to glycine exacerbated this reduction. For Y99G, the hydrogen bonding between Gly99 and Ser63 was reduced by 25% and that between Gly99 and Leu61 by 6%. The H62G mutant was the most perturbed, as residue 62 sits at the center of the interaction path between Tyr99 and the active site. The hydrogen bonding between Tyr99 and Ser63 was reduced by 46%, between Gly62 and Met2 by 18%, and between Leu61 and Tyr99 by 9%, consistent with its drastic reduction in catalytic activity.

Examining the number of waters within the channel during MD simulations also shows the clear effect of Tyr99 and His62 mutants (**Figure 5C**). The WT model had, on average, 41.4 ± 3.5

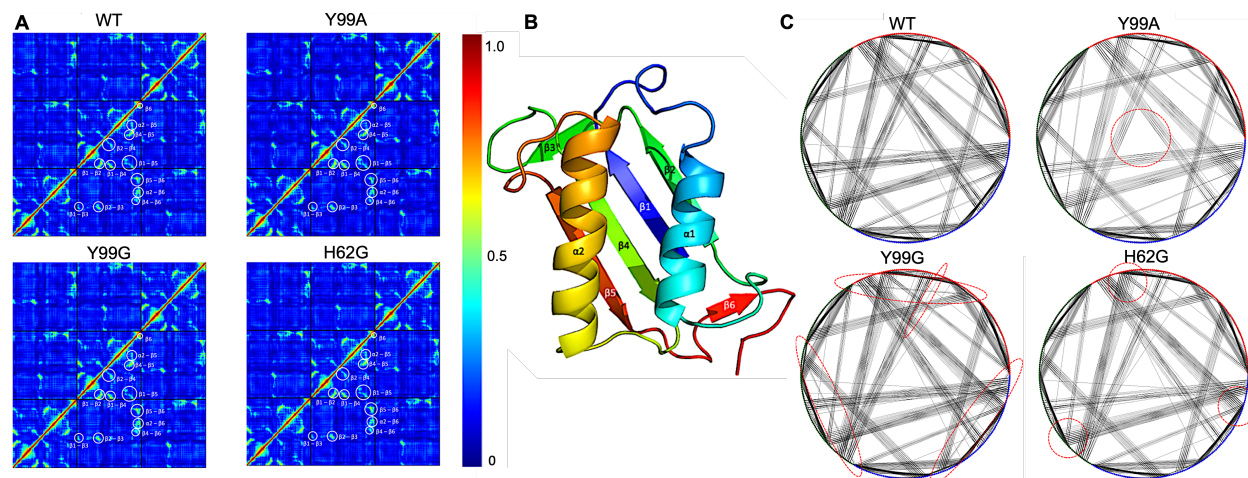


Figure 6. Impacts of allosteric mutations on intra-residue and inter-monomer communication in MIF. (A) Heat maps depicting MIF intra-residue correlation during 200 ns MD simulations quantify RMSF values from α -carbons of aligned trajectories for WT MIF, Y99A, Y99G, and H62G. Each square within the map represents a MIF monomer (i.e. A, B, or C; bottom-to-top, and left-to-right). The diagonals show the A-A, B-B, and C-C correlations. Red indicates strong correlation between structural elements, while blue indicates little-to-no correlation. (B) Critical secondary structure elements are shown on the MIF monomer and circled on the correlation maps. (C) Community networks in MIF, with black lines depicting intra- and inter-monomer crosstalk, and red circles illustrating significant differences in variants relative to WT. For example, correlations at the central solvent channel of Y99A (middle of circle) are weakened relative to WT, while inter-monomer correlations are fragmented in Y99G and H62G. The MIF trimer is represented as a circle, with red, blue, and green perimeters defining each of the MIF monomers.

water molecules in the central channel. The Y99A and Y99G mutants had 46.6 ± 3.8 (+13%) and 52.0 ± 3.7 (+26%), respectively. The H62G mutant showed no significant change in the amount of waters in the channel (41.5 ± 3.8), likely due to its placement away from the channel's opening. However, the H62G mutant increases the RMSD of Tyr99 from 0.6 Å in WT to 1.6 Å, likely contributing the mutant's loss of hydrogen bonding noted above. There is no similar increase in the RMSD of His62 with the Y99A/G mutants and all simulations had an overall C_{α} RMSD of 0.6 – 0.7 Å. Disruptions to intra-monomer correlations are apparent in all MIF mutants studied, and residue networks are altered from those of WT (**Figure 6**). Based on these analyses, the reduced activity in the studied mutants can be attributed to either a disruption of the hydrogen bonds between the residues themselves (as in H62G) or positional alterations within the central water channel (as in Y99A and Y99G).

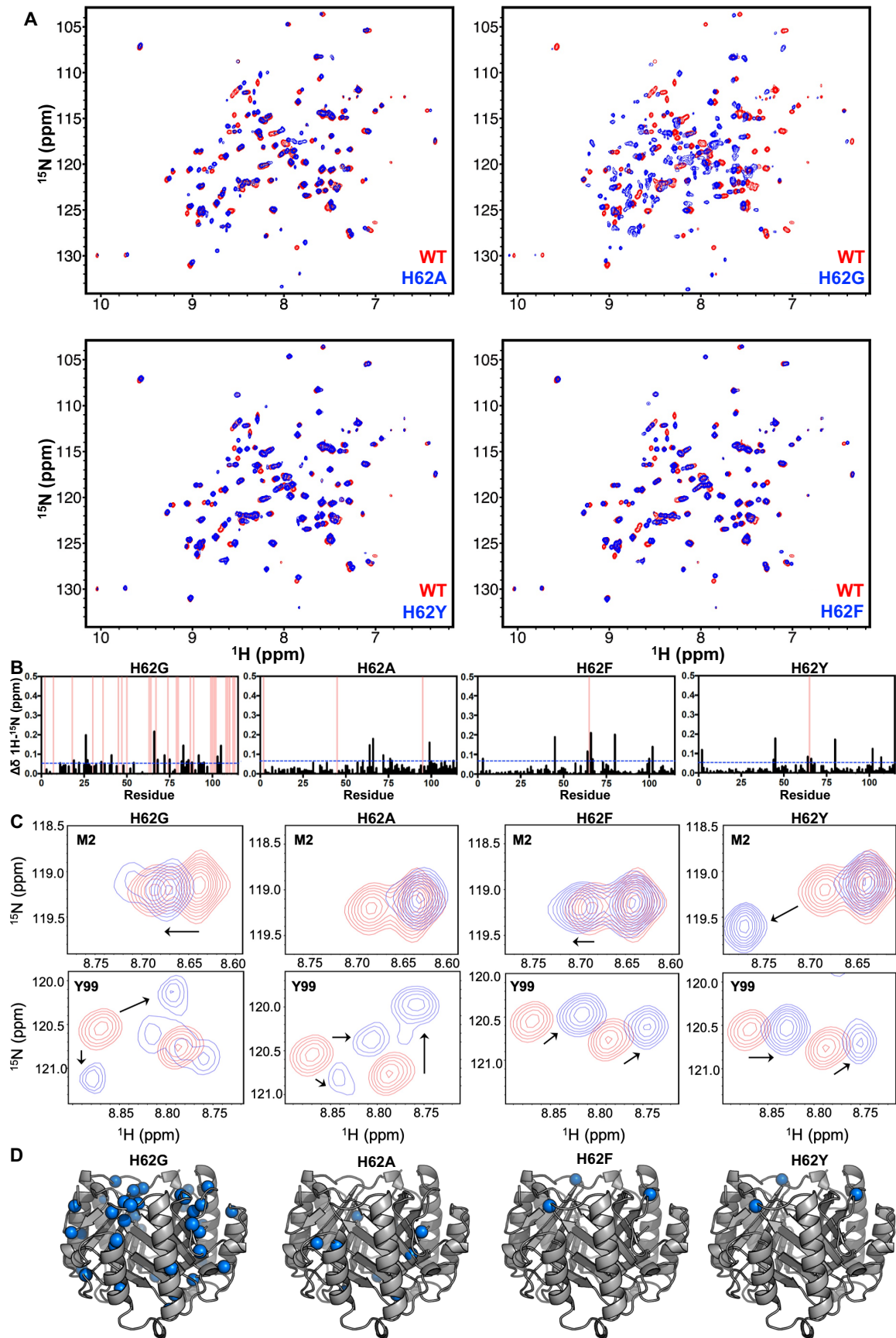


Figure 7. NMR probe of perturbations to the MIF allosteric pathway induced by His62 mutations. (A) ^1H - ^{15}N TROSY HSQC NMR spectral overlays of His62 variants (blue) with WT MIF (red). (B) Combined ^1H - ^{15}N chemical shift perturbations (black lines) caused by mutations at His62. Blue dashed lines represent 1.5σ above the 10% trimmed mean of all shifts and pink bars denote sites of exchange broadening in each NMR spectrum. (C) Close-up views of ^1H - ^{15}N HSQC spectral overlays of WT MIF (red) and His62 variants (blue), highlighting perturbations to resonances corresponding to allosteric nodes Tyr99 and Met2. (D) Summary of amino acids broadened beyond detection (blue spheres) in ^1H - ^{15}N NMR spectra of MIF mutants. The degree of line broadening increases from F/Y $\rightarrow$ A $\rightarrow$ G mutations, and qualitatively denotes sites within MIF with heightened flexibility on the *ms* timescale.

Solution NMR highlights the dynamic character of the allosteric pathway

The ^1H - ^{15}N TROSY-HSQC NMR spectra of MIF mutants revealed an increase in the flexibility of the entire protein along the series H62Y-H62F-H62A-H62G and Y99F-Y99A-Y99G (**Figures 7-8**). The residue-residue and residue-water interactions we observed for Y99F, H62Y, and H62F are absent from the remaining mutants and consistent with the NMR findings. While the chemical shift perturbation profiles of MIF variants are rather modest (**Figures 7B and 8B**), a high degree of line broadening due to motion of the protein on the millisecond timescale is observed in variants with low catalytic activity (**Figures 7D and 8D**). We monitored dynamic signatures of critical relay points along the Tyr99-His62-Met2-Pro1 pathway and found that mutation of Tyr99 or His62 to Gly broadens the correlated node residues, a classic signature of heightened dynamics at those sites,³⁹ suggesting coupling of the N-terminus of MIF to Tyr99 in a manner consistent with MD simulations.³¹ A reciprocal effect is observed at Tyr99 in the presence of Pro1/Met2 mutations (**Figure 9A**). Interestingly, Tyr99 correlations from MD simulations, as well as mutations at Tyr99, propagate to the CD74 and tautomerase sites, however, mutations at Pro1 affect Tyr99 but not the CD74 site in NMR experiments (**Figure 9B**). We also observe a significant reordering of the solvent channel network along a continuum of mutations that may facilitate the enhanced (diminished) tautomerase function. In cases where MIF catalytic efficiency is only marginally attenuated (Y99A, H62A), fewer flexible residues are observed, occurring only at sites outside of the allosteric relay. Mutations that enhance MIF activity show very small, if any,

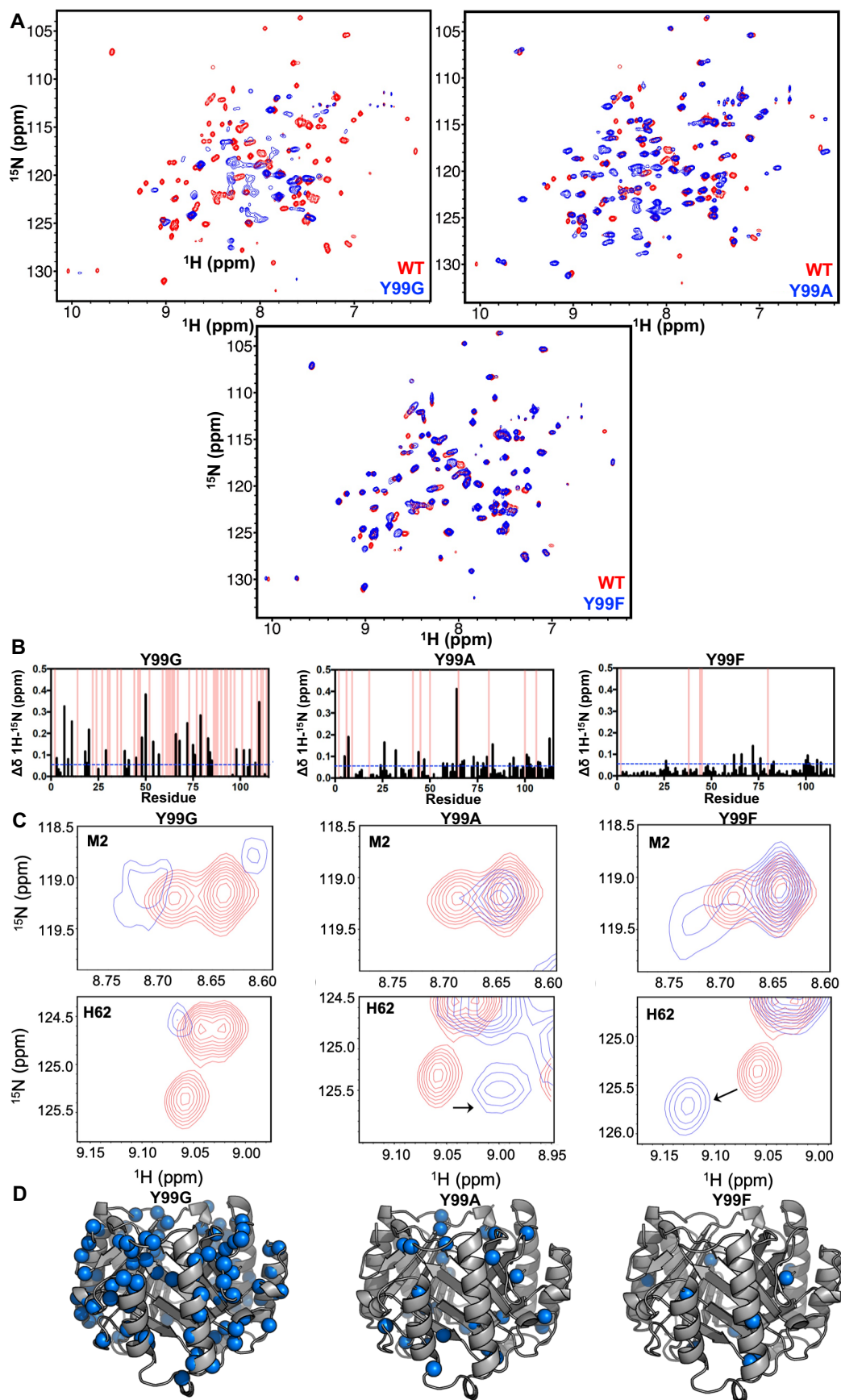


Figure 8. NMR probe of perturbations to the MIF allosteric pathway induced by Tyr99 mutations. (A) ^1H - ^{15}N TROSY HSQC NMR spectral overlays of Tyr99 variants (blue) with WT MIF (red). (B) Combined ^1H - ^{15}N chemical shift perturbations (black lines) caused by mutations at Tyr99. Blue dashed lines represent 1.5σ above the 10% trimmed mean of all shifts and pink bars denote sites of exchange broadening in each NMR spectrum. (C) Close-up views of ^1H - ^{15}N HSQC spectral overlays of WT MIF (red) and Tyr99 variants (blue), highlighting perturbations to resonances corresponding to allosteric nodes Tyr99 and Met2. (D) Summary of amino acids broadened beyond detection (blue spheres) in ^1H - ^{15}N NMR spectra of MIF mutants. The degree of line broadening increases from F $\rightarrow$ A $\rightarrow$ G mutations, and qualitatively denotes sites within MIF with heightened flexibility on the *ms* timescale.

changes to the structure or dynamics of the entire protein. The NMR results are also consistent with His62 playing a role in propagating a dynamic signal between the active and regulatory sites, as shown by substantial exchange broadening in resonances corresponding to His62 (**Figure 8C and 9A**). Additional signatures of enhanced flexibility in Y99G and H62G MIF are localized at the monomer-monomer interfaces near the catalytic N-terminus, indicating that changes at the active site are largely dynamic, rather than structural, in nature. It should be noted that despite relatively small changes in raw CD spectra indicating folded structures of MIF mutants in solution, Y99G is ~ 1.3 kcal/mol, on a per monomer basis, less thermostable than WT MIF while Y99F is ~ 0.32 kcal/mol more thermostable (**Figure 2**). These energetic differences track with the dynamic character of the MIF mutants, particularly the NMR exchange broadening that is typically ascribed to heightened intra-residue dynamic signaling and in allosteric proteins. Further, the WT MIF structure itself displays significant fast timescale flexibility by NMR (**Figure 10**), suggesting that even though X-ray crystallography, CD, and NMR indicate stable folded structures, MIF is intrinsically flexible. Based on these data, it is clear that mutations introducing aromatic interactions to these sites strengthen hydrogen bonding networks essential for catalysis, while those that promote structural flexibility disrupt the residue-residue and residue-solvent interactions.

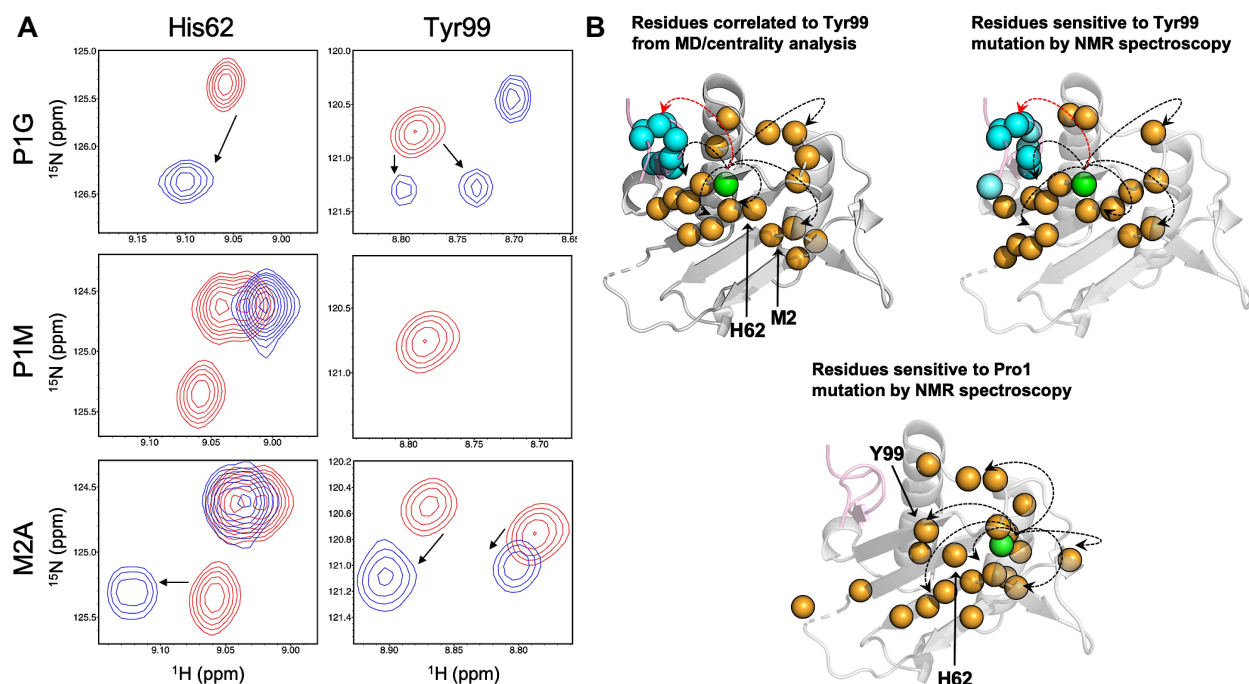


Figure 9. Allosteric reciprocity and sensitivity of the tautomerase and solvent channel sites. (A) NMR studies of P1G, P1M, or M2A mutants shows significant chemical shift perturbations or line broadening at the Tyr99 allosteric site and His62 node. **(B)** Summary of correlated residues in MIF by MD and NMR. Residues identified to be correlated with Tyr99 by MD³¹ are mapped onto the MIF monomer (top left). Tyr99 is shown in green, and residues at the C-terminus of the adjacent monomer are shown in cyan. Most of these correlations are also observed in NMR studies of Y99A MIF (top right), where spheres represent residues showing line broadening or chemical shifts 1.5σ above the 10% trimmed mean of all shifts. Residues sensitive to Pro1 mutations by NMR, however, show correlations at the Tyr99 allosteric site (bottom center), but not the C-terminal CD74 activation region. These data suggest Try99 is a critical node in both pathways, but may not enable direct concerted crosstalk between these sites. This is consistent with the different levels of attenuation of tautomerase and neutrophil recruitment³¹ activity by the Y99A mutant.

Ligand-induced dynamic signals are propagated from the catalytic site to Tyr99

Since our data show a dynamic association between the active site Pro1 and the allosteric Tyr99 residue, we explored whether substrates or inhibitors at the active site have any effect on Tyr99. Nineteen high-resolution human MIF structures from the Protein Data Bank (PDB) with all three active sites of the homotrimer occupied by a ligand were examined (**Figure 11**). The ligand library consisted of a substrate (hydroxyphenylpyruvate solved at 2.5 Å), four competitive inhibitors (resolutions between 1.8-2.0 Å), and fourteen covalent inhibitors bound to Pro1 (resolutions between 1.53-2.07 Å). The PDB entries, RMSD values to WT MIF, and resolutions

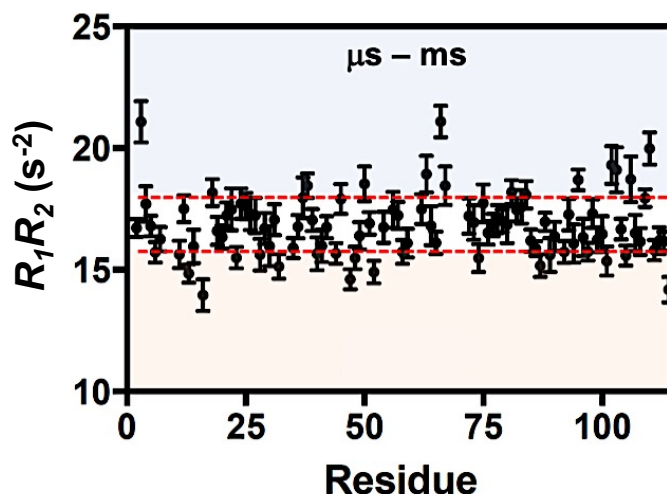


Figure 10. NMR spin relaxation rates for WT MIF. The longitudinal (R_1) and transverse (R_2) relaxation rates are plotted as the R_1R_2 product, showing clear sites of micro-millisecond (1.5σ above the 10% trimmed mean, red dashed line). These data indicate the WT MIF structure is intrinsically flexible on multiple timescales.

of the 19 structures used in this study are provided (**Appendix Table 2**). Depending on the ligand, the side chain hydroxyl group of Tyr99 can vary relative to other ligand-dependent positions of Tyr99 by up to 1.4 Å (**Figure 11A**). Larger fluctuations are limited due to the presence of the side chain of His62. To understand the correlation between Tyr99 fluctuation and ligand binding, we explored the amino acid interaction network around this area. In addition to the two backbone hydrogen bond interactions between His62 and Met2 and the π - π interactions between Tyr99 and His62, we found a cross-subunit hydrogen bond interaction formed between the side chains of His62 and Tyr99 (**Figure 11B**). Thus, binding of a ligand to the catalytic site causes movements of Pro1, which in turn affects the Met2 backbone atoms. These movements are transmitted to His62, and then, Tyr99 (**Figure 11C**). The ligand-dependent conformational changes of Tyr99 are also consistent with the dynamic connection between the two sites (**Figures 7-9**).

Discussion

We previously performed MD simulations to provide insight into the MIF-induced activation of CD74.³¹ This method identified an allosteric site, which we experimentally tested,

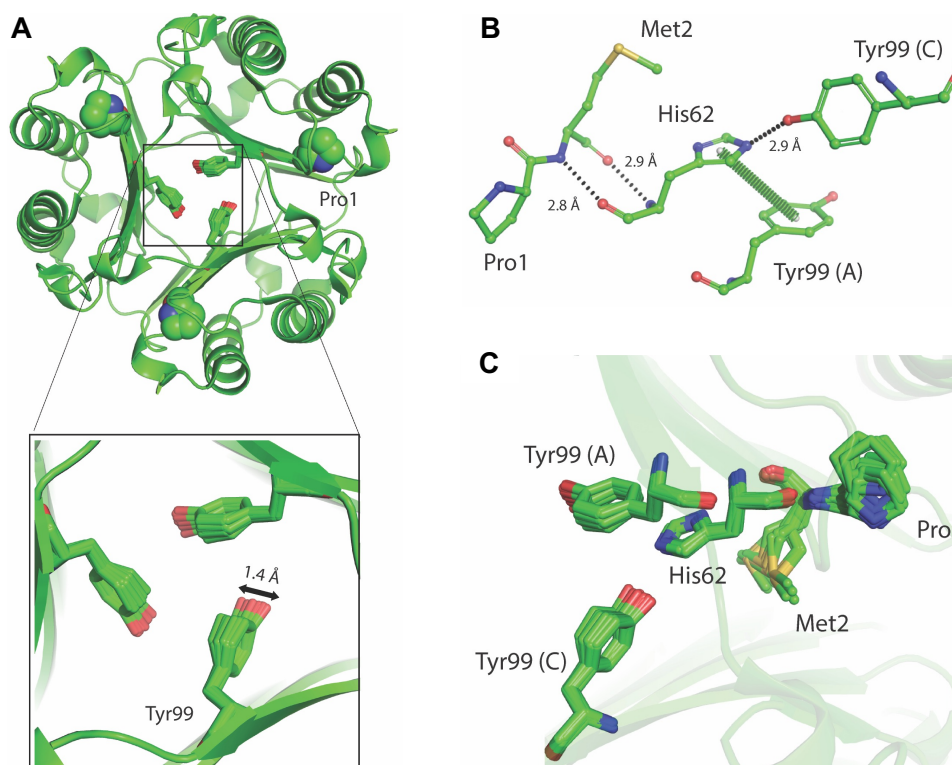


Figure 11. Active site ligands and the allosteric site Tyr99. (A) Ligands that bind to the active site pocket of MIF induce dynamic motions of Tyr99 side chain. We used 19 high-resolution crystal structures in which the ligands occupy all three active sites. Pro1 is shown as spheres and Tyr99 as sticks for each monomer. (B) His62 forms two backbone hydrogen bond interactions with Met2 (β strand) and a side chain hydrogen bond interaction with Tyr99 ($\beta 5$ strand) from the adjacent monomer. The hydrogen bond interactions are shown as black dotted lines. His62 also forms aromatic interactions (shown in red dotted line) with Tyr99 from the same monomer. (C) The ligand-induced dynamic effect are shown on Pro1, Met2, His62, and Tyr99 by superposition the 19 crystal structures.

verified, and determined its intramolecular pathway to regulate CD74 activity. In this new study, we experimentally demonstrate that the MIF allosteric site, which is located at one opening of the solvent channel also regulates the protein's catalytic activity. Crystallographic analysis show that Tyr99 and Pro1 are dynamically connected *via* aromatic interactions and a hydrogen bond network that involves His62 and Met2. The residues Tyr99, His62 and Met2 located on $\beta 5$, $\beta 4$, and $\beta 1$ strands, respectively, implicate the β -strand system of MIF in the regulatory mechanism. The dynamical connection between the allosteric and catalytic sites is supported by mutagenesis, protein-water network analyses, crystallographic, and NMR studies. Nineteen MIF-ligand crystal

structures obtained from PDB independently support this conclusion.

High resolution crystallography and MD simulations have revealed the importance of water molecules in mediating protein folding, function, and signaling.^{40,41} Crystallographic and kinetic analyses of the MIF mutants led us to identify a functional role for the solvent channel. In the crystal structures of Y99F, H62Y, and H62F, the dense hydrogen bonding network created proximal to the allosteric site and around a stable central water molecule greatly strengthens the allosteric signal from Tyr99 to Pro1 and increases the catalytic efficiency of these MIF mutants. These mutants display enhanced aromatic interactions between the positions 99 and 62, and well-ordered water molecules in the solvent channel compared to the WT protein. In contrast, the absence of the aromatic interactions in the Y99G and H62G mutants result is a poorly connected hydrogen bonding network in the solvent channel and a dramatic increase in the dynamic properties of Y99G and H62G.

Collectively, our findings demonstrate that at least two of the MIF activities, CD74 activation and enzymatic activity, are regulated by the same allosteric node *via* separate intramolecular dynamic signals that are transmittable to distal functional regions (**Figure 7**). For the MIF enzymatic activity, we found a Tyr99-His62-Met2-Pro1 pathway connecting the allosteric and catalytic sites. Experimental evidence from NMR suggests the relationship between these two sites/pathways is quite complex. The tautomerase site is sensitive to Tyr99 mutations, and reciprocity of the allosteric signal (at Tyr99) is observed in tautomerase mutants (Pro1, Met2) in the form of chemical shifts and line broadening at His62 and Tyr99 (**Figures 8-9**). MD simulations show connectivity from Tyr99 to both the CD74 and tautomerase sites,³¹ and mutations at Tyr99 (this work) also perturb chemical shifts at both functional sites. Mutations at Pro1 only affect the Tyr99 site, while no chemical shift perturbations are observed along the CD74 pathway (**Figure**

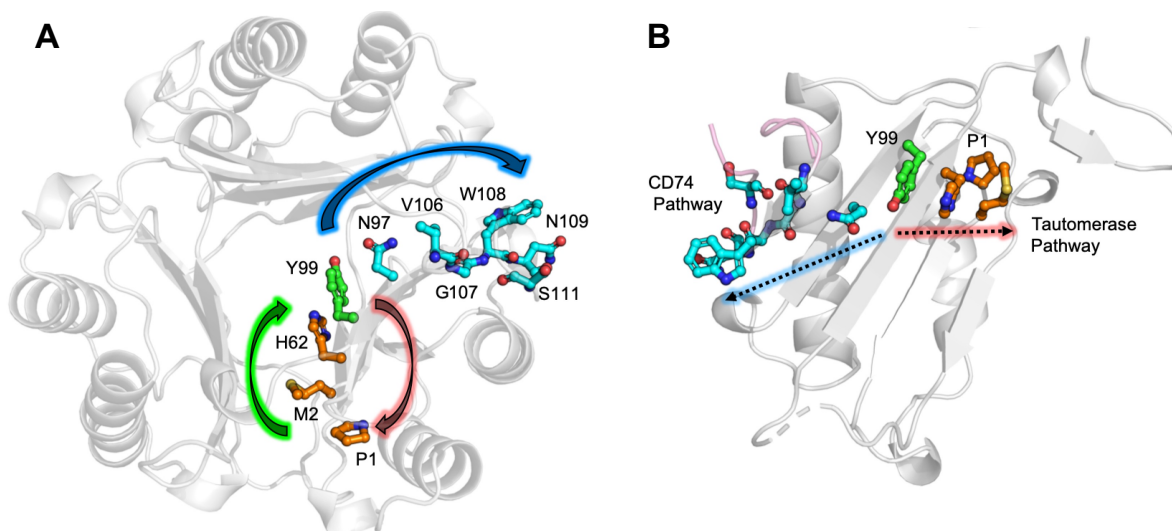


Figure 12. Allosteric pathway(s) influencing MIF activity. (A) Communication from the solvent channel (red arrow) to the N-terminal tautomerase site (orange sticks) was established in this work. Reciprocity of this effect is shown by NMR studies of Pro1 mutants (green arrow). Tyr99 (green sticks) is also a central node in the CD74 activation network (blue arrow, sticks) shown by MD simulations.³¹ (B) Allosteric pathways mapped onto a MIF monomer highlight the majority of the CD74 network residing on the C-terminus of an adjacent subunit (pink cartoon).

9B). One explanation for this experimental result could be that the MIF C-terminal binding site is not directly coupled to Pro1, and that while they share the Tyr99 node, MIF utilizes two distinct, pathways (**Figure 12**). Additionally, many other distal residues in MIF alter CD74 activation, suggesting this activity may encompass a broader network than was previously known. Although it is clear that Tyr99 is a critical node in both processes, the fact that neutrophil recruitment (a reporter of CD74 activation) is completely abolished in Y99A MIF³¹ but tautomerase is attenuated only 33%, suggests that the influence of Tyr99 differs in these processes.

We also found mutants with increased catalytic activity to have decreased dynamics as observed by NMR, slightly more favorable energetics, and a new solvent network around the allosteric site. Other mutants of Tyr99 and His62 with decreased enzymatic activity have substantially increased dynamics, poorer structural energetics, and lacked the solvent network. The intrinsic flexibility of the MIF structure is modulated along a continuum with mutations at or near the allosteric node. Quantitating rates of conformational exchange and dynamic equilibrium

populations of MIF residues in future studies may enable us to definitively identify sites that share similar motional parameters indicative of a higher degree of coupling in allosteric pathways.⁴² This would, potentially, allow us to address the question of whether or not Tyr99 is a node in two distinct, or one contiguous pathway(s). Our study demonstrates that the MIF solvent channel is not merely a result of protein folding but contains a critical allosteric node that also serves an important role for at least the MIF enzymatic activity.

Macrophage migration inhibitory factor (MIF) is a critical immunoregulatory cytokine and enzyme that is strongly implicated in inflammatory diseases such as asthma, arthritis, acute respiratory distress syndrome, and sepsis, as well as several cancers. Attenuation of these disease phenotypes is well documented in MIF deficient and knockout animals, however the molecular mechanisms of the pathophysiology of MIF are unclear. Further, efforts to target MIF with inhibitors or therapeutics are complicated by its nearly ubiquitous cellular localization and protective role in innate immunity. Additional mechanistic studies are therefore required to leverage control over the pathological effects of MIF. We recently identified Tyr99 of the MIF solvent channel, formed along the 3-fold axis of its trimeric structure, to be a critical mediator of MIF-induced activation of its CD74 receptor. Here, we confirm Tyr99 as an allosteric site in MIF, and reveal the structural, dynamic, and biochemical determinants of an allosteric pathway that couples Tyr99 to the MIF catalytic residue, Pro1. In addition to residue-residue interactions, we identified a functional role for solvent channel water molecules in MIF allostery, suggesting that this previously unexplored channel is not simply a consequence of MIF trimerization. The atomistic characterization of this novel allosteric network may provide alternate routes of therapeutic inhibition of MIF.

Materials and Methods

Mutagenesis, Expression, and Protein Purification

All the MIF mutants, encoded in the pET-11b vector, were produced by the QuikChange II site-directed mutagenesis kit (Agilent Technologies) using the cDNA of wild type MIF as a template.^{15,43} Cloning of Y99A was described elsewhere.³¹ WT and mutant MIF proteins were expressed in *Escherichia coli* BL21-Gold (DE3) cells and purified as described.^{44,45} Briefly, bacteria cells expressing the MIF proteins were grown in a shaking incubator at 37°C to an O.D.₆₀₀ of 0.6 and induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. After a 4-hour incubation at the same temperature, the cells were centrifuged and stored in -80°C. For protein purification, the cells were resuspended in the lysis buffer (20 mM Tris, pH 7.4 with 20 mM NaCl) containing a mini EDTA-free protease inhibitor cocktail tablet (Sigma-Aldrich) and lysed by sonication. The cell debris was removed by centrifugation at 4°C and the supernatant was filtered using 0.22 μ m filter units (Millipore) and loaded onto Q-Sepharose and SP ion exchange chromatography columns connected in series. WT MIF and MIF mutants were eluted in the flow-through with ~95% purity. After concentrating the samples using 10 kDa molecular weight cut-off centrifugal filters (Millipore), the proteins were loaded onto size-exclusion column (16/60 Superdex 75) to remove the remaining ~5% of contaminants. Purification was carried out using 20 mM Tris, pH 7.4 with 20 mM NaCl as the running buffer. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Enzyme Kinetics

A 100 mM stock solution of 4-hydroxyphenylpyruvate (4-HPP) in 0.5 M ammonium acetate, pH 6.2 was freshly prepared and equilibrated for 24 hours at room temperature to produce

the predominating keto-form under this condition. Kinetic assays were carried out using keto form of 4-HPP at 0 - 2 mM. For each concentration, 10 μ L of 4-HPP was mixed with 130 μ L borate at a final concentration of 0.420 M in a UV transparent flat bottom 96-well plate. The reaction was initiated by addition of 10 μ L of protein at a final concentration of 50 nM (for WT MIF and Y99F, Y99A, H62Y, H62F, H62A) or 250 nM (for H62G and Y99G). Absorbance measurements for the conversion to the enol form of HPP (the enol-borate complex has an extinction coefficient of $\epsilon_{306} = 11,400 \text{ M}^{-1} \text{ cm}^{-1}$) were recorded at 10 second intervals for a total of 360 seconds using a Tecan Infinite M200. All experiments were performed at 25°C. Initial velocities were plotted as a function of 4-HPP concentration and the Michaelis–Menten parameters (V_{max} , K_m , k_{cat}) were determined by nonlinear regression using ORIGIN 9.0. Each assay was carried out in triplicate.

Crystallization and Structure Determination

Crystallization of Y99A was described elsewhere.³¹ The MIF mutant proteins (Y99F, Y99G, H62Y, H62F, H62A and H62G) were crystallized in 24-well hanging drop trays. Crystallization was accomplished by mixing the stock protein solution concentrated to ~18 mg/ml with 20 mM Tris, pH 7.5, 2 M ammonium sulfate and 3% 2-propanol at various volume ratios. In all cases the drop size was 4 μ L. Trays were stored at 20°C and crystals were formed overnight. Individual crystals were transferred to a new drop containing the mother liquor enriched with 28% glycerol for cryoprotection, mounted on a loop, and flash frozen in liquid nitrogen. Crystal screening and complete data set collection occurred at the Macromolecular X-ray Facility at the Yale School of Medicine using a Rigaku Pilatus 200K Detector with a Rigaku 007 rotating copper anode X-ray generator (wavelength=1.5418 Å). All the data sets were collected at a temperature of 100K. The HKL2000 program suite⁴⁶ was used for integration and scaling. The CCP4 supported program PHASER⁴⁷ was used to obtain solutions for the MIF mutants. For molecular replacement,

the crystal structure of WT MIF (PDB: 3DJH) was used as the starting model. Refinement of the structures were carried out using Refmac⁴⁸ and COOT.⁴⁹ Ramachandran analyses of the MIF mutants showed 0% outliers and 98.8%, 99.1%, 99.1%, 98.5%, 98.5%, or 98.8% residues in the preferred regions, for Y99F, Y99G, H62F, H62Y, H62A and H62G, respectively. The crystal structures of WT MIF, the MIF mutants, and the nineteen MIF-ligand complexes obtained from PDB were superimposed using the CCP4-supported program SUPERPOSE,⁵⁰ and visualized and analyzed in PyMOL.⁵¹ The interatomic interactions around the allosteric site were examined using Arpeggio.³⁶ The detailed statistics of the Tyr99 and His62 mutants are presented in **Appendix Table 1**. The RMSD values obtained for the MIF mutants and nineteen MIF-ligand complexes vary between 0.08-0.16 Å and 0.20-0.36 Å, correspondingly. Coordinates and structural factors have been deposited in PDB under the accession codes 6OYE (Y99F), 6OY8 (Y99G), 5UMK (H62Y), 6OYG (H62F), 5UMJ (H62A) and 6OYB (H62G).

NMR Spectroscopy

Samples for NMR spectroscopy were expressed under identical conditions to those utilized for X-ray or kinetic studies. Isotopically enriched ¹⁵N-MIF was grown in deuterated M9 minimal medium containing CaCl₂, MgSO₄, and MEM vitamins and utilizing glucose (¹²C₆H₁₂O₆, natural abundance) and ammonium chloride (¹⁵NH₄Cl, Cambridge Isotope Labs) as the sole carbon and nitrogen sources, respectively. Cells were adapted to be grown in D₂O as follows. A small culture of MIF was grown for 8 – 10 hours in LB medium. The resulting cloudy suspensions were then used to inoculate 25 mL of LB containing 50% D₂O, which was grown for 8 – 10 hours and then used to inoculate a final LB culture (~50 mL) made with 95% D₂O. After 7 hours of incubation at 37°C, these cells were collected by centrifugation and resuspended in 1 L of M9 minimal medium. MIF was grown at 37°C to an OD₆₀₀ ~ 0.8 – 1.0 and induced with 1 mM IPTG. After an additional

4 – 6 hours of incubation, cells were harvested by centrifugation and stored at -80 °C. MIF was purified identically to the manner described earlier. $^1\text{H}^{15}\text{N}$ -TROSY HSQC NMR spectra were collected at 30°C on a Bruker Avance NEO 600 MHz spectrometer. The ^1H and ^{15}N carriers were set to the water resonances and 120 ppm, respectively. All NMR spectra were processed with NMRPipe⁵² and analyzed in SPARKY.⁵³

Circular Dichroism (CD) Spectroscopy

CD spectra and thermal unfolding experiments were collected on a JASCO J-815 spectropolarimeter equipped with a variable temperature Peltier device. Denaturation curves of 15 – 20 μM MIF were recorded at 218 nm in a 1 mm quartz cuvette over a temperature range of 20 – 90 °C (293 K – 363K). *Thermodynamic parameters were extracted via nonlinear curve fitting of CD data as:*

$$\text{Ellipticity}(T) = \frac{[(m_f T + b_f) + (m_u T + b_u)] \exp\left[\left(-\frac{\Delta H_{D,vH}}{R}\right)\left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}{1 + \exp\left[\left(-\frac{\Delta H_{D,vH}}{R}\right)\left(\frac{1}{T} - \frac{1}{T_m}\right)\right]} \quad (1)$$

where m_f , b_f , and m_u , b_u are the slopes and y-intercepts of the folded (low temperature) and unfolded (high temperature) regions of the melting curve; R is the gas constant; T_m and $\Delta H_{D,vH}$ are the unfolding midpoint and van't Hoff enthalpy of denaturation at T_m , respectively. Free energy analysis was performed with calculated values of ΔC_p , the unfolding heat capacity, estimated from the reports of Privalov and Makhatadze^{54,55} as $\Delta C_p = 4.8$ kcal/molK for the MIF trimer. The apparent differences in unfolding enthalpy, entropy, and free energy between these MIF variants was calculated as using a reference temperature (T_{REF}) of 351.9 K, the T_m of WT MIF, and using.

$$\Delta H_{D,app} = \Delta H_{D,vH} + \Delta C_p (T_{REF} - T_m) \quad (2)$$

$$\Delta S_{D,app} = \frac{\Delta H_{D,vH}}{T_m} + \Delta C_p \ln \left(\frac{T_{REF}}{T_m} \right) \quad (3)$$

$$\Delta G(T) = \Delta H_{D,vH} \left(1 - \frac{T_{REF}}{T_m} \right) + \Delta C_p \left[(T_{REF} - T_m) - T_{REF} \ln \left(\frac{T_{REF}}{T_m} \right) \right] \quad (4)$$

Computing water-mediated interactions

Hydrogen bonds were computed in crystal structures with GetContacts.⁵⁶ We applied a simple distance cutoff of < 3.5 Å between donor and acceptor atoms. No angle criteria were imposed since crystal structures generally lack hydrogen atoms. A water-mediated interaction is defined to occur between a pair of residues if they form hydrogen bonds to a common water molecule or a pair of water molecules held together by a hydrogen bond. All water-mediated interactions were computed throughout the WT MIF, H62A, and H62Y crystal structures to compare protein-water interaction networks.

Protein-water graph network analysis

Previous studies have shown that residues critical for protein function tend to have high centrality measures within the non-covalent residue interaction network.⁵⁷⁻⁵⁹ Here, we perform a similar network centrality analysis that involves both the protein and solvent to assess the significance of individual water molecules in assisting protein function. The protein-water interaction graph network is represented by $G(V, E)$ where V is the set of nodes including all residues and water molecules in the system, and E is the set of hydrogen bonds forming edges between nodes. Degree centrality was computed using the NetworkX Python library.⁶⁰ The degree centrality of each node v in G is defined as the fraction of nodes in G that shares an edge with v . We rank each node by degree centrality to identify the water molecules with greatest functional significance.

Molecular Dynamics Simulations

Four models of MIF (WT, Y99A, Y99G, and H62G) were made using the PDB structure 3DJH.⁶¹ In the case of Y99A, Y99G, and H62G, the mutations were made uniformly on all three MIF monomers. The models were each placed in a periodic water box and neutralized with NaCl. Simulations were run for 200 ns at 300 K using NAMD⁶² and the CHARMM36m force field,⁶³ and the last 100 ns were analyzed. Hydrogen bond and RMSD analyses were conducted using the respective VMD plugins.⁶⁴ Hydrogen bonds were defined as an acceptor-donor distance of no more than 3.0 Å and an acceptor-donor-hydrogen angle no more than 30°. The average number of hydrogen bonds in the solvent channel per MD frame was averaged across the three MIF monomers, and the percentage change in hydrogen bonding was determined as (Mutant-WT)/WT. Overall RMSD values were calculated from the alpha carbons of aligned trajectories and the RMSDs of His62 and Tyr99 were calculated using the entire residue of interest, excluding hydrogens. In order to calculate the number of waters in the central channel of each mutant, the mouth at either end of the channel was defined using the alpha carbons of Val42 and Tyr99. The number of waters within these limits was averaged for the final 100 ns of the trajectory using 100 frames/ns for a total of 10,000 frames. The percent change in water molecules was determined similarly to the method described for hydrogen bonds (*vide supra*).

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Appendix

Table 1: Data collection and refinement statistics for the Tyr99 and H62 variant crystal structures. Coordinates and structural factors have been deposited in PDB under the accession codes 6OYE (Y99F), 6OY8 (Y99G), 5UMK (H62Y), 6OYG (H62F), 5UMJ (H62A) and 6OYB (H62G).

	Y99F	Y99G	H62Y	H62F	H62A	H62G
Data collection						
Space group	P 21 21 21	P 21 21 21	P 21 21 21	P 21 21 21	P 21 21 21	P 21 21 21
Cell dimensions						
a, b, c (Å)	68.09, 68.11, 86.74	67.95, 68.08, 86.92	68.11, 68.31, 86.63	68.08, 68.26, 86.97	67.99, 68.22, 86.41	67.99, 68.21, 87.00
α, β, γ (°)	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00	90.00, 90.00, 90.00
Resolution (Å)	50.00-1.53 (1.56-1.53) *	50.00-1.53 (1.56-1.53) *	50.00-1.73 (1.76-1.73) *	50.00-1.55 (1.58-1.55) *	50.00-1.61 (1.64-1.61) *	50.00-1.53 (1.56-1.53) *
R_{sym} or R_{merge}	0.035 (0.300)	0.048 (0.241)	0.041 (0.067)	0.047 (0.225)	0.054 (0.112)	0.052 (0.331)
$I / \sigma I$	39.7 (4.1)	39.5 (5.5)	30.2 (10.2)	39 (6.4)	30.8 (7.7)	41.4 (4.7)
Completeness (%)	98.8 (95.0)	99.9 (99.2)	99.2 (99.5)	99.7 (98.7)	99.6 (96.9)	100 (99.8)
Redundancy	6.7 (4.3)	6.6 (4.2)	4.1 (2.2)	6.9 (4.5)	4.9 (2.5)	8.6 (5.4)
Refinement						
Resolution (Å)	48.16-1.53	48.09-1.53	48.23-1.73	48.21-1.55	48.16-1.61	48.15-1.53
No. reflections	57876	58529	40663	56183	49953	58470
$R_{\text{work}} / R_{\text{free}}$	0.15/0.17	0.15/0.18	0.15/0.18	0.12/0.15	0.15/0.18	0.11/0.16
No. atoms						
Protein	2649	2600	2585	2634	2585	2629
Ligand/ion	55	47	38	37	35	58
Water	345	316	321	327	384	349
B -factors						
Protein	13	13	18	15	12	14
Ligand/ion	25	24	31	74	22	29
Water	29	29	29	33	26	33
R.m.s. deviations						
Bond lengths (Å)	0.016	0.015	0.026	0.017	0.025	0.018
Bond angles (°)	1.760	1.832	2.047	1.751	2.141	1.759

Table 2: The PDB entries, RMSD values to WT MIF, and resolution of the 19 MIF structures with all three active sites occupied.

PDB entry	RMSD (Å)	Resolution (Å)	Ligand Type	Ligands/MIF	Citation
3DJH	-	1.25	-	-	(Crichlow et al., 2009)
1CA7	0.20	2.5	Substrate (p-hydroxyphenyl pyruvate)	3	(Lubetsky et al., 1999)
1GCZ	0.20	1.9	Competitive inhibitor	3	(Orita et al., 2001)
1LJT	0.22	2.0	Competitive inhibitor	3	(Lubetsky et al., 2002)
1MFI	0.36	1.8	Competitive inhibitor	3	(Taylor et al., 1999)
3L5U	0.27	1.9	Competitive inhibitor	3	(McLean et al., 2010)
4F2K	0.34	1.53	Covalent inhibitor	3	(Tyndall et al., 2012)
3B9S	0.36	1.8	Covalent inhibitor	3	(Winner et al., 2008)
3WNT	0.32	2.07	Covalent inhibitor	3	(Spencer et al., 2015)
3CE4	0.20	1.55	Covalent inhibitor	3	(Crichlow et al., 2009)
3SMB	0.28	1.6	Covalent inhibitor	3	(Crichlow et al., 2012)
3SMC	0.29	1.8	Covalent inhibitor	3	(Crichlow et al., 2012)
4OYQ	0.20	1.7	Covalent inhibitor	3	(Spencer et al., 2015)
3JSF	0.26	1.93	Covalent inhibitor	3	(McLean et al., 2009)
3JSG	0.25	1.58	Covalent inhibitor	3	(McLean et al., 2009)
3JTU	0.26	1.86	Covalent inhibitor	3	(McLean et al., 2009)
4P01	0.20	2.07	Covalent inhibitor	3	(Pantouris et al., 2015)
4TRF	0.29	1.63	Covalent inhibitor	3	(Pantouris et al., 2015)
4P0H	0.27	1.93	Covalent inhibitor	3	(Pantouris et al., 2015)
4PLU	0.22	1.63	Covalent inhibitor	3	(Pantouris et al., 2015)

CHAPTER 3

A Cysteine Variant at an Allosteric Site Alters MIF Dynamics and Biological Function in Homo- and Heterotrimeric Assemblies

This chapter is adapted from a published manuscript in Frontiers in Molecular Biosciences (2022) titled:

A Cysteine Variant at an Allosteric Site Alters MIF Dynamics and Biological Function in Homo- and Heterotrimeric Assemblies

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Author Contributions: **E.S.** expressed and purified protein, validated heterotrimeric assemblies, and conducted and analyzed NMR and CD spectroscopy experiments. **G.P.** conducted crystallographic experiments and analyses. **D.S.** conducted and analyzed neutrophil recruitment experiments with **V.B.** supervision. **R.M.** performed and analyzed steady state kinetics. **N.K.M.** collected and analyzed analytical ultracentrifugation experiments. **M.O.**, **G.P.L.** and **E.L.** conceived and supervised the project. The manuscript was written through contributions from all authors.

Abstract

Macrophage migration inhibitory factor (MIF) is an inflammatory protein with various non-overlapping functions. It is not only conserved in mammals, but it is found in parasites, fish, and plants. Human MIF is a homotrimer with an enzymatic cavity between two subunits with Pro1 as a catalytic base, activates the receptors CD74, CXCR2, and CXCR4, has functional interactions in the cytosol, and is reported to be a nuclease. There is a solvent channel down its 3-fold axis with a recently identified gating residue as an allosteric site important for regulating, to different extents, the enzymatic activity and CD74 binding and signaling. In this study we explore the consequence of converting the allosteric residue Tyr99 to cysteine (Y99C) and characterize its crystallographic structure, NMR dynamics, stability, CD74 function, and enzymatic activity. In addition to the homotrimeric variant, we develop strategies for expressing and purifying a heterotrimeric variant consisting of mixed wild-type and Y99C for characterization of the allosteric site to provide more insight.

Introduction

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine involved in multiple non-overlapping physiological pathways.¹ It is expressed in cells from a variety of tissues with pronounced expression in the cells of the immune system. MIF forms a homotrimeric structure with a solvent channel coincident with its 3-fold axis and possesses three putative catalytic cavities formed at the interface between each of two adjacent subunits.^{2,3} These were first identified by the similarity of the MIF structure to two microbial enzymes that catalyzed isomerase or tautomerase reactions with a conserved and unusual N-terminal proline functioning as a catalytic base.⁴⁻⁶ These enzymes and MIF have 5-20% sequence homology but share an N-terminal proline that is evolutionarily invariant for MIF.⁷ The *bona fide* substrate for MIF has yet to be

identified, but “pseudosubstrates” D-dopachrome and hydroxyphenylpyruvate have been useful for designing inhibitors or optimizing high throughput screens.⁸⁻¹¹ These inhibitors were used as tools to probe MIF biological activities and signaling, including functional interactions with p53, ERK-1/2, PI3K/Akt in the cytosol,¹²⁻¹⁴ and the extracellular receptors CD74, CXCR2, and CXCR4.^{1,15-18} More recently, a nuclease function was ascribed to MIF in a process known as parthanatos.^{19,20}

The mechanisms by which MIF promotes these activities are unclear, though signaling studies, biochemistry, and structures have provided some molecular details of possible mechanisms. A hypothesis that trimeric MIF functions as an enzyme at higher concentrations in the cytosol and subsequently dissociates into monomers when it is diluted in the extracellular milieu to activate receptors was disproven. Sedimentation velocity and equilibrium ultracentrifugation experiments found MIF has an unusual partial volume for a protein and forms an extremely tight trimer.²¹ Up to four structure-based mutations at the subunit interface also failed to disrupt the homotrimer; MIF with a fifth mutation did not express or was extremely unstable.²² Despite the tight homotrimeric association of MIF, it displays remarkable dynamics by NMR.²³⁻²⁶ Recently, we discovered an allosteric regulatory site at the center of the symmetric MIF homotrimer that serves as a hub for two of its biochemical activities: the enzymatic enol-keto tautomerase and CD74 receptor activation (**Figure 1**).^{25,26} The allosteric residues, Tyr99 from all three subunits, modulate enzymatic and CD74 receptor activation to different extents when mutated, while concurrently altering the structural and dynamic properties of the protein, as well as hydrogen bonding with solvent molecules in the central solvent channel. Notably, the size of the solvent channel opening and dynamics at the allosteric site were found to be critical diagnostics of functional outcomes, though it is still unclear exactly how the chemical properties of the

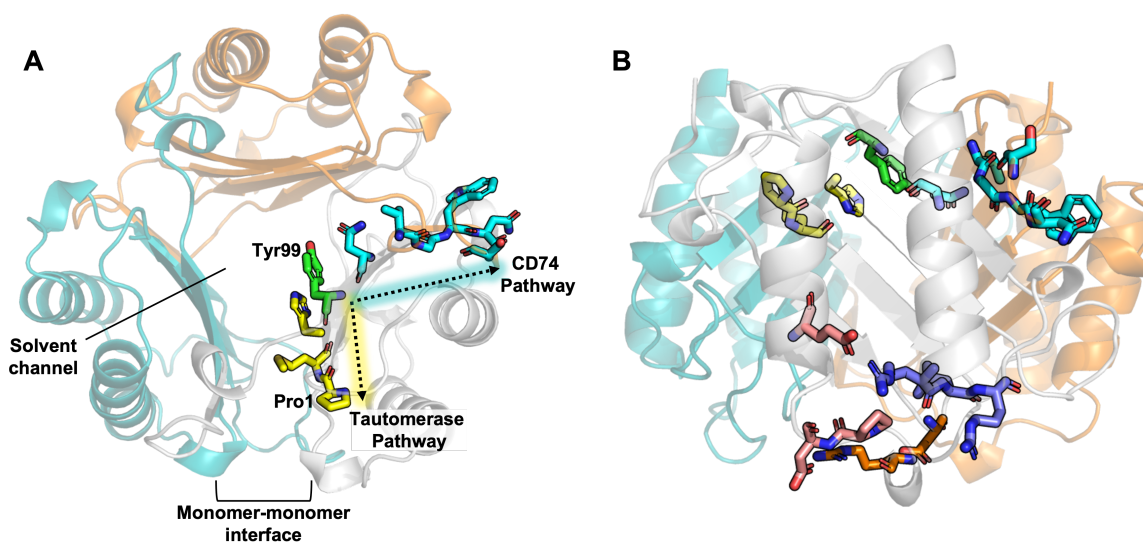


Figure 1. Schematic of important structural features in MIF. (A) A top view of the MIF trimer shows the monomeric subunits in gray, teal, and orange (PDB: 1MIF). Previously characterized allosteric pathways controlling tautomerase activity and pro-inflammatory CD74 activation are denoted in yellow and teal, respectively, including the common allosteric node, Tyr99, highlighted in green. Pro-1, the catalytic base for tautomerization, and the monomer-monomer interface, essential for catalysis, are also indicated. (B) A side profile of MIF highlights the allosteric residues and sites shown above, as well as regions of the protein involved in binding to the receptors CXCR4 (purple) and CXCR2 (orange), and residues implicated in nuclease activity (pink).

allosteric site at Tyr99 or the solvent channel influence the biophysical signatures of MIF. Further insights would facilitate the rational design of site-specifically modified allosteric pathways to regulate the information flow to-and-from Tyr99, creating enhanced spatial and temporal resolution of MIF function.

To investigate this relationship further, we introduced a cysteine residue at position 99 (Y99C). The sulfhydryl group of Cys99 is the same molecular distance (~ 8 Å) from the enzymatic active site as the C γ of Tyr99. We hypothesized that this mutation would enable disulfide stabilized dimers within the MIF trimers, providing a more comprehensive understanding of the structural, dynamic, and functional impact of Cys99 substitution. This method was used in a previous MIF study, when a cysteine mutation was introduced at N110 and formed an inter subunit disulfide with a Cys80 and induced changes in the oligomeric and functional properties.²² We generated a unique Y99C MIF cysteine variant, as well as a method to generate homo- and hetero- trimeric

Cys proteins, and characterized their molecular structure, dynamics, and effect on enzymatic and CD74 activities using X-ray crystallography, NMR spectroscopy, biochemical assays, and *in vivo* experiments.

Results

Biophysical Characterization of Y99C and Y99C/WT MIF trimers

Since Tyr99 is located the end of a β -strand of the MIF structure, we speculated it would take local unfolding to move the Cys residues in proximity to form disulfide linkages. We also set out to generate the first mixed trimer species of MIF, with the rationale of using the steric bulk of a Tyr99 side chain to constrain other Cys99 residues into forming disulfide bonds. This approach is also a proof-of-principle demonstration for the engineering of novel MIF variants with structural scaffolds and activities distinct from wild-type (WT) MIF and homotrimeric variants. While the Y99C MIF homotrimer displays a very similar size exclusion chromatographic profile to that of WT MIF, purification of the Y99C/MIF mixed trimers, facilitated by a SNAP-tag and a His₆-tag on Y99C monomeric subunits, shows distinct MIF species with differing molecular weights that can be followed by SDS-PAGE (**Figure 2A-B**). The MIF monomer band (12.4 kDa) increases in intensity, relative to the MIF monomer/SNAP-tag band (31.8 kDa), from peak 1 (P1) through peak 3 (P3) on the size exclusion chromatogram, representative of MIF species with increasing numbers of WT MIF monomers within the MIF trimer (**Figure 2C**). Thus, P1 is likely the Y99C homotrimer (96.1 kDa with SNAP-tags), P2 is the Y99C/WT heterotrimer with two Y99C monomers and one WT monomer (76.7 kDa with SNAP-tags), and P3 is the Y99C/WT heterotrimer with one Y99C monomer and two WT monomers (57.3 kDa with SNAP-tag). When P1-P3 are pooled and cleaved of the SNAP-tags and His₆-tags, the Y99C/WT mixed trimers elute at the same volume as WT MIF at 37.9 kDa.

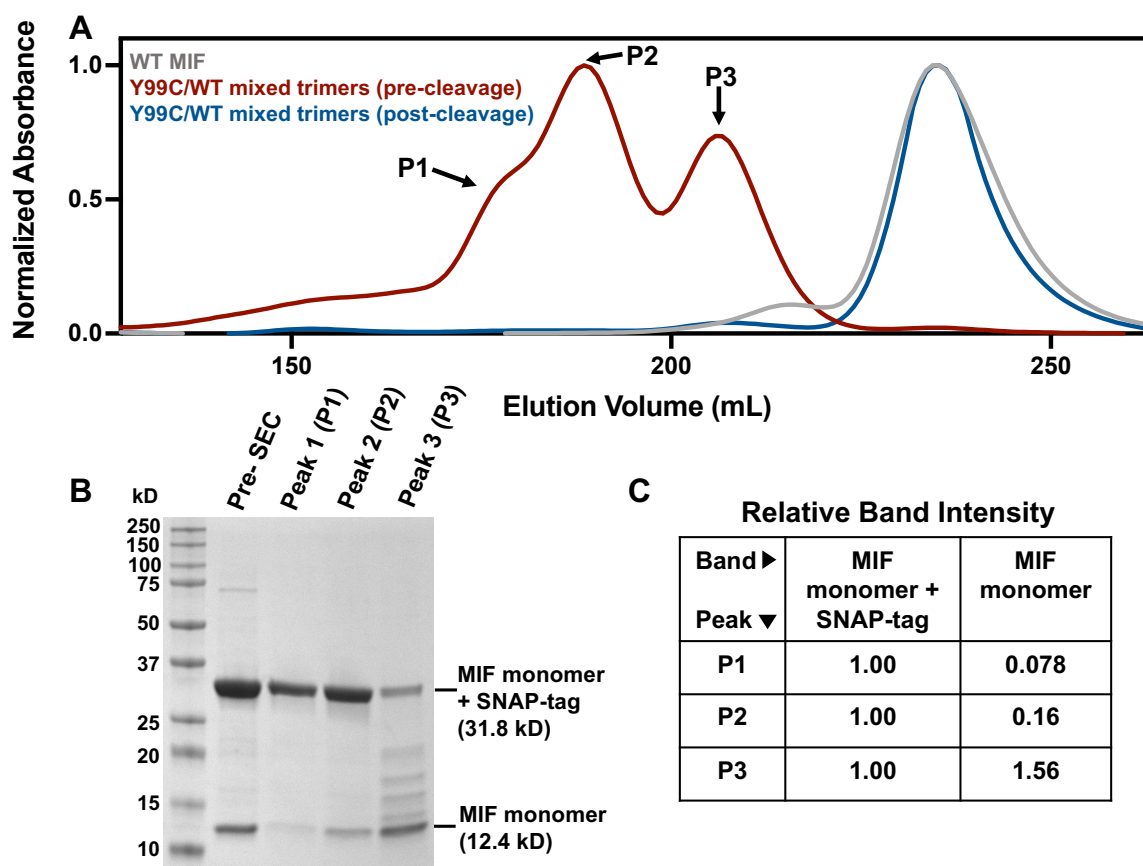


Figure 2. Verification of novel Y99C/WT mixed trimers. (A) Chromatographic overlay of WT MIF and the Y99C/WT mixed trimers pre- and post-tag cleavage as purified by size exclusion chromatography (SEC) (B) SDS-PAGE analysis of the Y99C/WT mixed trimer species showing the presence of the MIF monomer (12.3 kDa) and the MIF monomer + SNAP-tag (31.7 kD) in peaks 1-3. (C) Relative SDS-PAGE band intensities of the MIF monomer and the MIF monomer + SNAP-tag bands from (B) via ImageJ software (NIH). Each lane was analyzed separately and normalized such that the MIF monomer + SNAP-tag band is equal to 1.00.

The Y99C MIF homotrimer was probed by analytical ultracentrifugation at three loading concentrations (**Figure 3**). The sedimentation coefficient of the Y99C homotrimer is in good agreement with published values for WT MIF.²¹ The molar mass of 37.8 ± 0.8 kDa calculated directly from the primary data agrees very well that of WT MIF (37.9 ± 2.8 g/mol per trimer). Thus, the Y99C variant is a trimer and also has the same elevated partial specific volume seen for WT MIF (within error). With these data, we see no differences between WT MIF and the Y99C MIF homotrimer in terms of oligomeric state, shape, or partial specific volume.

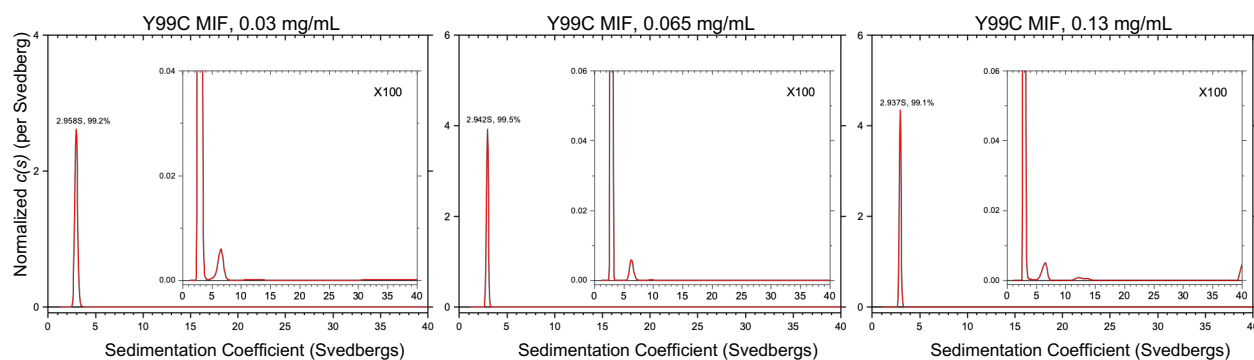


Figure 3. Sedimentation coefficient distribution for the Y99C MIF homotrimer at different concentrations. The inset has the vertical scale expanded 100-fold so that the minor components can be observed. The distribution is normalized so the total area under the curve is 1.0, and thus the area of each peak gives the fraction of that concentration.

X-ray crystallography of the Y99C MIF homotrimer.

The structure of homotrimeric Y99C MIF was solved to 1.60 Å to probe whether there were sufficient protein dynamics for the Cys99 side chain to form a disulfide and to detect any unexpected effects (**Appendix Table 1**). The structural alignment of WT MIF, Y99A, and Y99C were calculated and viewed in PyMol (**Figure 4A**). From this alignment, the residue 99 sidechains indicate the C β carbons overlap very well (**Figure 4A**, right panel). Interestingly, the thiol group in all three monomers has two conformations in the channel as shown in **Figure 4A** where Y99 and Y99A are also shown. The 2Fo-Fc and omit map of Y99C is also shown (**Figure 4B**). The closest distances among the thiol groups of adjacent subunits are ~6.5 Å (**Figure 4B**). The two conformations of the thiol groups in this study do not interact with other protein atoms nor do they induce large conformational changes (**Figure 4C**). The RMSD of Y99C to WT MIF (3DJH) and the Y99A (5EIZ) structures are 0.134 Å and 0.077 Å, respectively, and support a lack of sufficient movement of Y99C to induce conformational changes. Though the shared C β atom for WT, Y99A, and Y99C MIF align very well, the radius of the solvent channel opening where the three Y99C residues appear is 3.4 Å and similar to Y99A (3.6 Å), in contrast to WT MIF (1.4 Å) as calculated by MOLEonline.²⁷ In a previous study involving three Tyr99 variants and four His62 mutations,

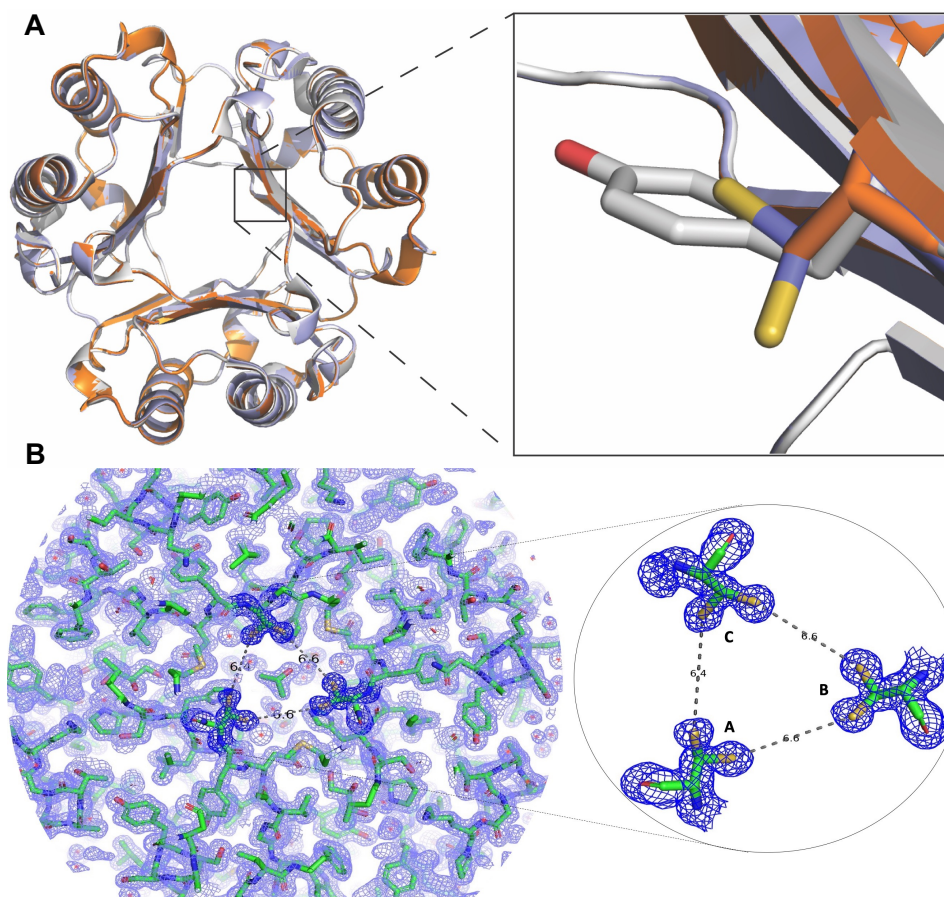


Figure 4. Changes in the MIF structure with mutations at Tyr99. (A) The crystal structure of Y99C MIF has two thiol group conformations per monomer in the solvent channel (PDB: 7KQX). The inset shows overlapping side chains for residue 99 in WT MIF (3DJH; gray) and the Y99C (7KQX; blue) and Y99A (5EIZ; orange) MIF variants. (B) Electron density map for MIF Y99C homotrimer. On the left, the 2Fo-Fc electron density map for the Y99C MIF homotrimer is shown. On the right, the 2Fo-Fc map and the two conformations for the thiol groups of Y99C are enlarged. The distances among the closest sulfur atoms from each subunit are over 6 Å apart and too far from each other to form disulfides.

there was an inverse correlation between the size of the opening and catalytic efficiency.²⁶

The MIF structure is destabilized by Cys99 residues

To assess the impact of the Cys99 mutation on the secondary structure and stability of MIF, far-UV circular dichroism (CD) experiments were conducted. CD spectra of the Y99C homotrimer and Y99C/WT mixed trimers display α - β secondary structure consistent with that of WT MIF, with only subtle differences observed (**Figure 5A**), which is in good agreement with the crystallographic analysis of the Y99C homotrimer. Similarly, thermal unfolding experiments

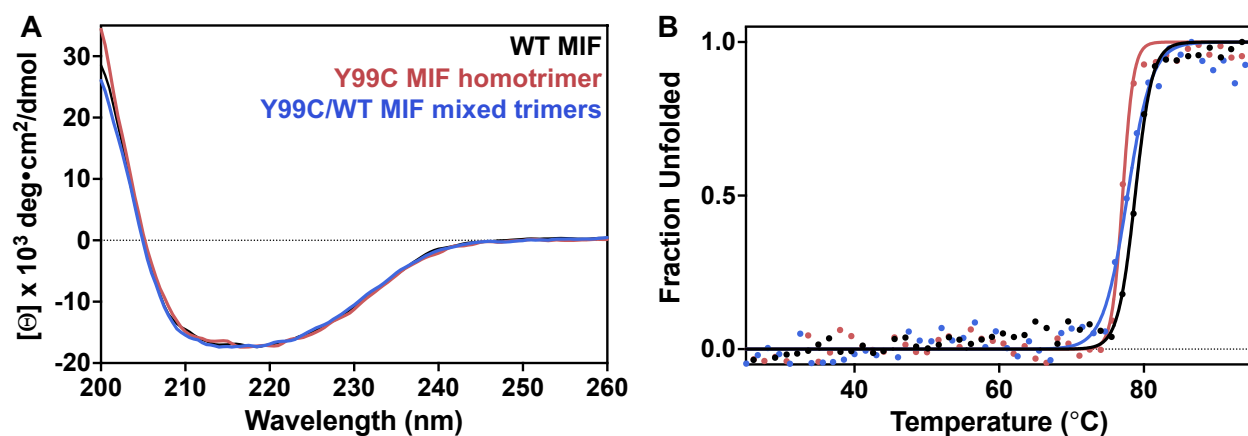


Figure 5. Secondary structure and stability of Y99C MIF variants. (A) Far-UV circular dichroism spectra of WT MIF (black), the Y99C MIF homotrimer (red), and the Y99C/WT MIF mixed trimers (blue) at 25°C. (B) Thermal stabilities of WT MIF (black; $T_m = 78.5^\circ\text{C}$), the Y99C MIF homotrimer (red; $T_m = 76.9^\circ\text{C}$), and the Y99C/WT MIF mixed trimers (blue; $T_m = 77.5^\circ\text{C}$), normalized and plotted as a fraction of unfolded protein.

reveal that the Y99C mutation has a destabilizing effect on the MIF structure, with T_m values for the Y99C homotrimer and Y99C/MIF mixed trimers of 76.9°C ($\Delta T_m = -1.6^\circ\text{C}$) and 77.5°C ($\Delta T_m = -1.0^\circ\text{C}$), respectively, relative to WT MIF ($T_m = 78.5^\circ\text{C}$) (**Figure 5B**). Together, these data suggest that MIF becomes increasingly destabilized as the number of Cys99 residues increases per trimer.

NMR spectroscopy highlights altered solution structures and dynamics of Y99C variants

We recently employed NMR spectroscopy to characterize significant solution structural and dynamic changes associated with mutations at the Tyr99 allosteric site that are not obvious through crystallographic analysis.²⁶ Here, we find that the NMR spectrum of the Y99C homotrimer is distinct from that of WT MIF, with significant chemical shift perturbations observed in residues at or near sites previously implicated in the allosteric signaling that controls MIF function (**Figure 6**). Chemical shift perturbations of residues at the N-terminus and proximal to His62 are consistent with prior studies of Tyr99 variants that confirmed the allosteric pathway (Tyr99-His62-Met2-Pro1) that modulates MIF enzymatic activity.²⁶ We also observed significant perturbations at the

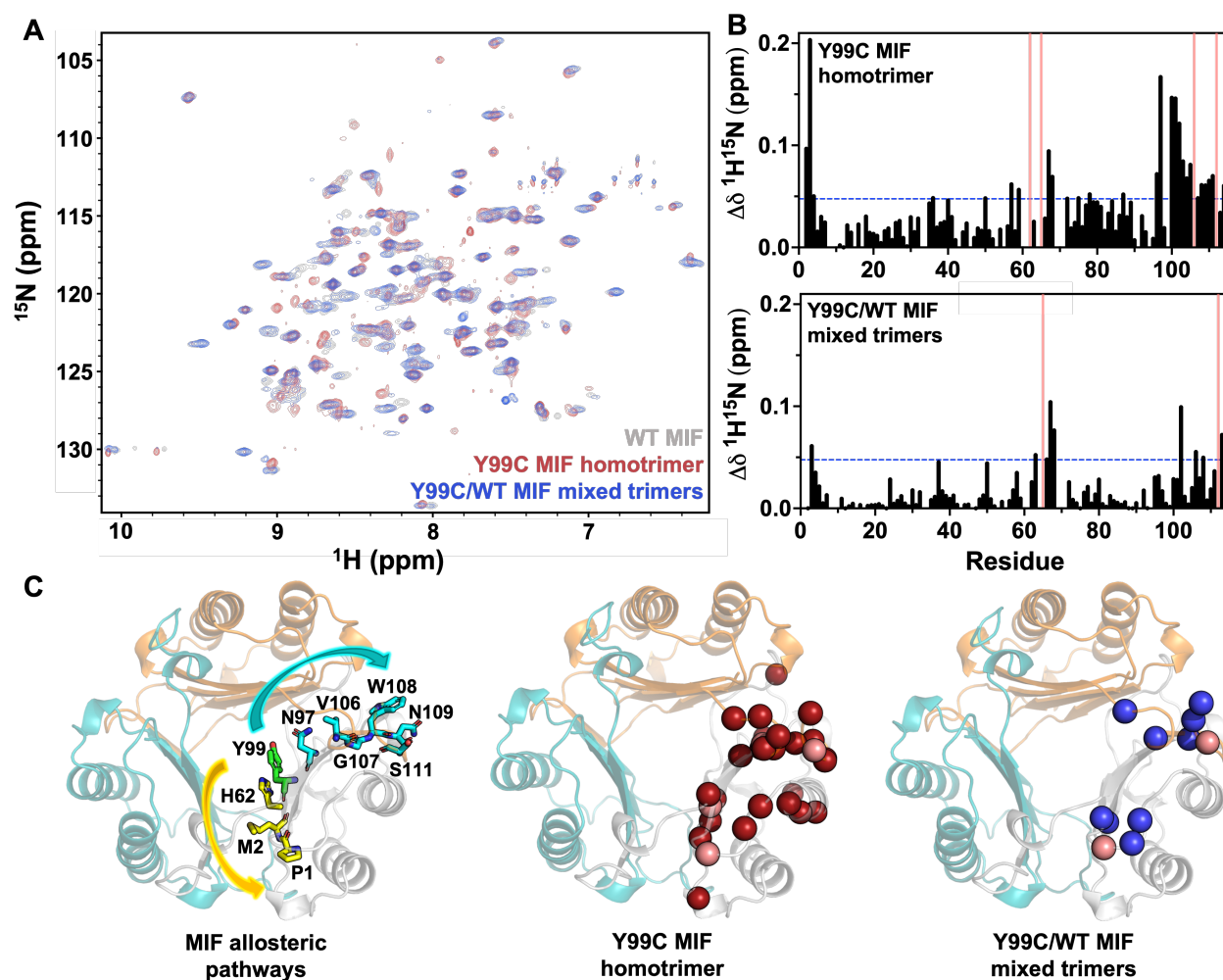


Figure 6. Structural effects of the Y99C variants by NMR. (A) ^1H - ^{15}N TROSY HSQC spectral overlay of WT MIF (gray), the Y99C homotrimer (red), and the Y99C/WT mixed trimers (blue). (B) ^1H - ^{15}N combined chemical shift perturbations ($\Delta\delta$) for the Y99C homotrimer (top) and the Y99C/WT mixed trimers (bottom). Pink bars denote residues that are broadened beyond detection, and blue lines represent 1.5 σ above the 10% trimmed mean of all shifts. (C) Significant chemical shift perturbations observed for the Y99C homotrimer (middle, red spheres) and Y99C/WT mixed trimers (right, blue spheres) are mapped onto the MIF structure for comparison to the previously characterized MIF allosteric pathways (left) affecting tautomerase activity (yellow) and CD74 activation (teal). Pink spheres denote residues broadened beyond detection. Residues after position 99 are mapped onto the adjacent monomer, as reported for the CD74 activation site.

C-terminus, where the CD74 receptor activation site has been mapped (Figure 6).^{25,28}

Comparatively, the NMR spectrum of the Y99C/WT mixed trimers reveals chemical shift perturbations in the same regions of the MIF structure as the Y99C homotrimer, though to a lesser extent. The highly symmetric WT MIF appears as a 114-residue without the cleaved initiating

methionine with a molecular weight of 12,388 Da protein by NMR, although it is actually a 37,164 Da trimer. While the heterozygous incorporation of mutations into MIF alters its local chemical environment, it does not perturb the global symmetry of MIF to an extent that renders its NMR spectrum more difficult to interpret (**Figure 6A**). Although the similarity of the NMR spectra indicate that mixed trimers also do not facilitate disulfide formation, this is the first evidence that heterozygous MIF samples can be studied with molecular detail in solution.

In order to assess the impact of the Y99C mutation on dynamically-driven signaling in MIF, we quantified longitudinal (R_1) and transverse (R_2) relaxation rates and the heteronuclear ^1H - ^{15}N NOE to report fluctuations in the protein backbone (**Figure 7A**). We postulated that the dynamic profile of the Y99C/WT mixed trimers would be “in between” that of WT MIF and the Y99C homotrimer, as was observed structurally by chemical shift perturbations of the Y99C variants (**Figure 6B**). Interestingly, we observe a greater overall deviation from WT-like dynamics in the Y99C/WT mixed trimers. In fact, there are limited differences in flexibility between WT MIF and the Y99C homotrimer. Only a few sites of significantly altered R_1 and R_2 relaxation rates for the Y99C homotrimer are observed, including Ile67, proximal to the His62 allosteric site, and Ile96 and Ala104, proximal to the Tyr99 allosteric site and the CD74 binding site (**Figure 7A**). Further, the ^1H - ^{15}N NOE for the Y99C homotrimer reveals a slight global elevation relative to WT MIF, suggesting that the Y99C homotrimer is actually less flexible overall than WT MIF. In contrast, there is a large variance in the dynamic profile of the Y99C/WT mixed trimers. Regions with altered R_1 and R_2 relaxation rates include residues at the monomer-monomer interface (3, 5, 32, 36, 49), residues proximal to the His62 allosteric site (63, 67), and residues near Tyr99 and the CD74 allosteric pathway (95-98, 100 102, 107, 113). The increased flexibility of the Y99C/WT mixed trimers is most apparent in the ^1H - ^{15}N NOE, where a global depression of NOE values is

observed, including significant deviations from WT MIF in functionally relevant regions of the protein similar to those observed by R_1 and R_2 relaxation rates.

We compared the dynamics of the Y99C homotrimer and Y99C/WT mixed trimers to those of WT MIF, and to each other, using correlation plots of the R_1/R_2 relaxation rates (suggestive of μs – ms molecular motions), analyzed as a product to minimize anisotropic molecular tumbling artefacts and ^1H - ^{15}N NOEs, reporting on ps – ns dynamics (**Figure 7B-C**). Deviations from linearity in these plots denote substantial differences between the samples, allowing us to pinpoint specific residues affected by disruption of the allosteric site. There is a much lower correlation between the dynamic profiles of WT MIF and the Y99C/WT mixed trimers, indicating that the Y99C/WT mixed trimers exhibit altered flexibility to a much greater extent than the Y99C homotrimers, consistent with our analysis of the raw data. Further, the dynamics of the Y99C variants are distinct from each other, even more so than either variant relative to WT MIF. Residues outside of the 1.5σ correlation boundaries, mapped onto the MIF monomer in **Figure 7**, highlight regions of altered flexibility suggestive of motion on multiple timescales. Most notably, we observe dynamic changes at the C-terminus and the solvent channel β -sheet, regions that allosterically mediate MIF function (**Figure 6C**), suggesting that the Y99C/WT mixed trimers may be functionally distinct from WT MIF and the Y99C homotrimers. The heightened flexibility observed for the Y99C/WT mixed trimers may be, in part, due to the heterogeneity of the Y99C/WT mixed trimers, as the subtle loss of symmetry of the heterotrimers may disrupt stabilizing interactions, despite maintaining a relatively similar overall MIF structure by NMR.

The Y99C mutation modulates MIF enzymatic activity and ability to activate CD74 in vivo

The stimulation of neutrophil recruitment in murine lungs is tied to CD74 receptor activation *in vivo*.²⁹ Our previous work with a Y99A variant showed significant abrogation of

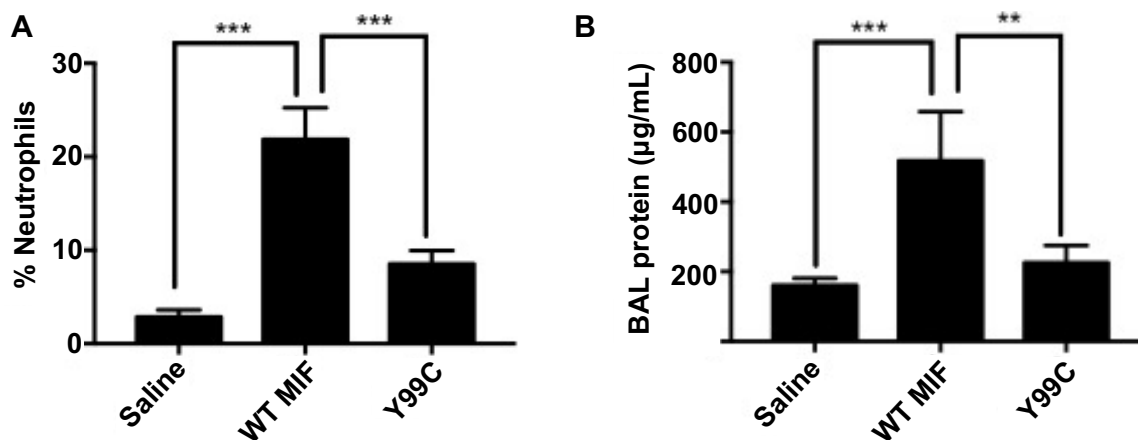


Figure 8. *In vivo* CD74 activation in the lung by WT MIF and the Y99C MIF homotrimer. (A) Neutrophil recruitment demonstrates a statistically significant decrease in cells recruited to the lung by the Y99C MIF homotrimer compared to that of WT MIF. **(B)** The Y99C MIF homotrimer leads to significantly decreased total BAL protein levels, a surrogate marker for alveolar–capillary leak/pulmonary edema *in vivo*. ** $p < 0.01$, *** $p \leq 0.001$.

neutrophil recruiting activity, suggesting a coupling of this allosteric residue to the C-terminal CD74 binding site.²⁵ We measured the percentage of neutrophils and total protein content as biomarkers for CD74 activity and alveolar-capillary leak/pulmonary edema, respectively, in bronchoalveolar lavage (BAL) fluid obtained from mice sacrificed after 6 hours of administration of a vehicle (saline) only or vehicle with Y99C MIF (**Figure 8**). The neutrophil influx was significantly increased in the WT MIF group (compared to controls), but significantly decreased in groups administered the Y99C homotrimeric variant, similar to Y99A.²⁵ The decrease in lung inflammation is likely due to the same factor as the Y99A variant: the absence of Tyr99 and the inability of the variant to undergo correlated motions with a number of proximal and distal residues originally shown by molecular simulations.²⁵ NMR studies of Y99C (**Figures 6A-B and 7**) show perturbation in residues Ile64, Lys66, Trp108, and Asn109, which are involved in CD74 receptor binding and activation. We also cannot eliminate the two sidechain orientations of Cys99, the local changes it causes at receptor binding residues, and other residues in its proximity.

We previously noted that increasing the radius of the solvent channel opening by

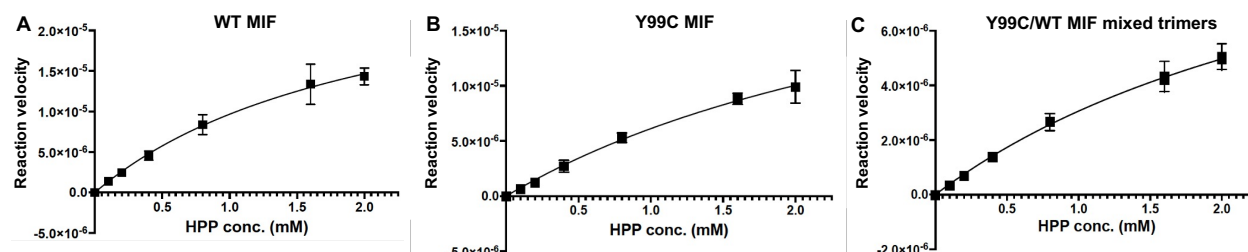


Figure 9. Altered catalytic activity of the Y99C MIF variants. Tautomerase activity of (A) WT, (B) Y99C MIF homotrimer, (C) and Y99C/WT MIF mixed trimers was monitored by the formation of the hydroxyphenylpyruvate (HPP) enol-borate complex at $\lambda_{\text{max}} = 306$ nm with 50 nM MIF and 0-2 mM of HPP. Reactions were repeated in triplicate per protein sample, with black box representing the mean and error bars denoting the standard deviation.

substituting Tyr99 with other residues is correlated to reduced catalytic activity.²⁶ Though we observed a solvent channel opening for Y99C similar to that of Y99A, the catalytic activity of the Y99C homotrimers has reduced (55%) catalytic efficiency relative to WT MIF and compared to Y99A (77%)³⁰ (Figure 9 and Table 1). Our previous work evaluated a range of amino acid substitutions at position 99, including glycine, alanine, and phenylalanine.³⁰ While the catalytic efficiencies of Y99G and Y99F were found to be 10% and 143%, respectively, compared to WT MIF, Y99A only exhibited a small decrease in catalytic activity to 77%, suggesting that amino acids with side chain sizes between the extremes of glycine and phenylalanine are less functionally predictable, and there are likely additional factors that influence activity. The catalytic efficiency of the Y99C/WT mixed trimers was predicted to be between that of the Y99C homotrimer and WT MIF, as there are fewer changes (mutations) compared to the Y99C homotrimer. Interestingly, the catalytic efficiency of the Y99C/WT mixed trimers is reduced to ~28% of WT MIF. This result may be attributed to the significant increase in flexibility observed by NMR for the Y99C/WT mixed trimers, particularly at the C-terminus near the monomer-monomer interface and in proximity to the allosteric sites on the solvent channel β -sheet, where signaling, ligand binding, and/or active site chemistry may be altered (Figure 7). We observed limited differences in flexibility of WT MIF and the Y99C homotrimer, which may be a contributing factor in the higher

Table 1. Kinetic parameters of the WT MIF and Y99C MIF variants tautomerase activity

	$V_{\max}$ ($\mu\text{M s}^{-1}$)	k_{cat} (s^{-1})	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1}\text{s}^{-1}$)
WT MIF	0.30 ± 0.03	6.05 ± 0.67	2.13 ± 0.35	2.84 ± 0.44
Y99C MIF	0.28 ± 0.04	5.6 ± 0.81	3.57 ± 0.33	1.57 ± 0.17
Y99C/WT MIF mixed trimers	0.13 ± 0.03	2.65 ± 0.61	3.33 ± 0.84	0.80 ± 0.47

activity of the Y99C homotrimer compared to the heterotrimeric variants. In addition to the altered dynamic profile of the Y99C/WT mixed trimers, the reduced enzymatic efficiency could be due to do the importance of symmetry at the allosteric site and its effects in catalytic activity. The use of heterotrimers was thus important to suggest symmetry is also significant at the allosteric site.

Discussion

The unexpected discovery of an allosteric site at the center of the MIF trimer has spawned protein engineering and small molecule studies aimed at modulating two non-overlapping functions of MIF from a single location. This approach highlights a strategy to make the catalytic activity, CD74 agonism and antagonism, and conformational ensemble of MIF more intuitively controllable. Mutagenesis studies employed here attempted to probe dynamics at the gating residue of the solvent channel. Engineering of an intersubunit disulfide via mutagenesis at a different residue (N110C) was used to study MIF aggregation by the compound ebselen via interaction with a natural MIF cysteine (Cys80) and the effect of a locked trimer on the catalytic and CD74 activity of MIF.^{22,31} The intersubunit disulfide induced global conformational changes, a higher-order oligomer, and decreased catalytic and CD74 activation.²² The X-ray structure of the Y99C MIF homotrimer did not reveal the presence of a disulfide between any two of three subunits. Interestingly, it did reveal two conformations for each thiol group (per monomer). The Y99C homotrimer abolished CD74 activation *in vivo* but possessed reduced enzymatic activity,

supporting the role of Tyr99 as a common allosteric node within two distinct allosteric pathways. We also demonstrate in this study a proof-of-principle that MIF can be engineered to form Y99C/WT MIF heterotrimers with different structural and biophysical properties than WT MIF or the corresponding Y99C homotrimeric variant. Surprisingly, the catalytic efficiency of the mixed heterotrimeric variants was lower than Y99C. While NMR studies reveal only slight differences in the dynamics of the Y99C homotrimer and WT MIF, the Y99C/WT mixed trimers studied here exhibit heightened flexibility at critical regions of the protein, as well as the absence of symmetry compared to the Y99C homotrimer, suggesting that the slight loss in symmetry of the heterotrimers may play an important role in the altered dynamics and functional effects of the Y99C/WT mixed trimers. Future studies will attempt to isolate the heterotrimers to study their structures and dynamics individually.

The advance in engineering heterotrimeric MIF species will allow us to characterize the biological activities and biophysical properties of this and other heterotrimeric mutants to provide insight on how this protein carries out its multi-functional activities. It is noteworthy that two other examples of heterotrimeric proteins exist for use as an anti-inflammatory, and in the other case, as mutations that cause inflammatory disease. A strategy of structure-based design was used to convert the inflammatory homotrimeric tumor necrosis factor (TNF) protein into a heterotrimeric mutant that inactivates the native TNF via its sequestration from its two receptors.³² A disease-causing example involves more than 100 autosomal dominant mutations in the extracellular binding domain of the heterotrimeric tumor necrosis factor receptor 1 (TNFR1) responsible for the TNF Receptor-Associated Periodic Syndrome (TRAPS). The underlying symptoms are recurrent and prolonged episodes of fever and inflammation.³³ The TNFR2-Fc therapeutic, etanercept, has limited efficacy toward TRAPS, demonstrating that the interactions between TNF and its receptors

is not the basis of the disease. This supports the reduced binding of TNF to the TNFR1 heterotrimeric T50M variant and absence of almost any binding to the C33Y and C52F mutants, even though these receptor mutants are present on the cell surface.³⁴ Further studies indicate the variant heterotrimeric receptors are mostly retained within the ER, resulting in ER stress, and activate inflammatory signaling and secretion of pro-inflammatory cytokines.³⁵ Our studies of Y99 MIF variants serve as another example of a homotrimeric and heterotrimeric protein with unique biological effects. Heterotrimeric MIF species also provide an additional avenue for characterizing the allosteric network proposed to control the distinct functions of MIF, including the role of inter- and intra-subunit signaling.

Materials and Methods

MIF mutagenesis and protein purification

The Y99C MIF cDNA was produced by site-directed mutagenesis using the PfuTurbo DNA Polymerase (Agilent). DpnI was utilized to digest the parental pET-11b plasmid with the WT MIF cDNA that contained methylated and hemimethylated DNA.²⁵ BL21-Gold (DE3) cells (Agilent) were transformed with the sequenced Y99C MIF plasmid and selected for on LB agar containing 100 µg/ml Amp (LB/Amp). WT MIF or Y99C MIF-containing cells were grown in 1L LB/Amp at 37°C, and induced by 1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.6. Levels of expression were assessed using a NuPAGE 4-12% Bis-Tris gels 4 hours after addition of IPTG. The cells were centrifuged and stored in -80°C until further use. Frozen cells were thawed on ice and dissolved in 20 mM Tris, 20 mM NaCl, pH 7.4 containing a mini EDTA-free protease inhibitor cocktail tablet (Sigma-Aldrich) and lysed by sonication. After centrifugation at 4°C, the filtered supernatant was loaded onto Q-Sepharose and SP ion-exchange chromatography columns connected in series. MIF mutant was in the flow-through with ~95%

purity. After concentrating the sample using 10 kDa MWCO Amicon centrifugal filters (Millipore), the protein was loaded onto a 16/60 Superdex 75 size-exclusion column to remove the remaining ~5% of contaminants with 20 mM Tris, pH 7.4, with 20 mM NaCl as the running buffer. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Y99C/WT MIF mixed trimer: plasmid design, expression, and purification

The Y99C MIF sequence was synthesized and cloned into the pET-24b(+) vector (Kan-resistance) with a C-terminal TEV protease cleavage site, followed by a SNAP-tag (19.4 kDa) and a His₆-tag (GenScript USA, Inc.). The Y99C MIF/SNAP-tag/His₆-tag plasmid was co-transformed with the WT MIF plasmid (pET-11b; Amp-resistance) into BL21-Gold (DE3) competent cells (Agilent) and selected for on LB agar plates containing 1.0 mg/mL Amp and 0.75 mg/mL Kan. 1 L LB with 1.0 mg/mL ampicillin and 0.75 mg/mL kanamycin was inoculated with a colony containing the WT MIF and the Y99C MIF/SNAP/His₆ plasmids, and grown at 37 °C to an OD₆₀₀ of 0.6 to 0.8. Cells were cooled on ice for 30 minutes, then induced with 0.2 mM IPTG and incubated for 16 hours at 18°C. The cells were harvested by centrifugation and stored at -80 °C.

Cells were resuspended in 25 mL lysis buffer (20 mM sodium phosphate, 300 mM NaCl, and 5 mM imidazole at pH 7.4) with a mini EDTA-free protease inhibitor cocktail tablet, lysed by sonication, and centrifuged to remove cell debris. Y99C/WT MIF mixed trimers were purified by Ni-NTA affinity chromatography, such that WT MIF homotrimers, containing no His₆-tags, elute from the column in the flow through, while Y99C/WT MIF mixed trimers containing 1-3 monomers of the Y99C MIF/SNAP-tag/His₆-tag construct are bound to the Ni-NTA resin (GoldBio) and subsequently washed with 50 mL of 20 mM sodium phosphate, 300 mM NaCl, and 25 mM imidazole buffer at pH 7.4 and eluted with 15 mL of 20 mM sodium phosphate, 300 mM

NaCl, and 500 mM imidazole buffer at pH 7.4. To confirm the presence of Y99C/WT MIF mixed trimers, the protein was purified over a HiLoad 26/600 Superdex 200 size exclusion column (GE Healthcare) in 20 mM sodium phosphate, 150 mM sodium chloride, and 1 mM EDTA at pH 7.4. The SNAP-tag and His₆-tag were then cleaved with TEV protease and subsequently removed via Ni-NTA chromatography.

SDS-PAGE band intensities were analysed using densitometry via ImageJ software (NIH).³⁶ Briefly, the optical density of each band was determined by the software, with band intensities calculated as the area of the resulting optical density peaks. Each lane was analysed separately due to differing protein concentration per sample. The MIF monomer and MIF monomer + SNAP-tag bands were compared relative to each other and normalized such that the MIF monomer + SNAP-tag band was equal to 1.00.

Analytical Ultracentrifugation (AUC) of the Y99C MIF homotrimer using sedimentation velocity

Y99C MIF homotrimer samples were loaded into cells with 2-channel charcoal-epon centerpieces with 12 mm optical pathlength. Samples were analyzed in PBS (from Corning). The reference buffer (PBS) was loaded into the reference channel of each cell to serve as an optical reference blank. The cells were then loaded into an AN-60Ti analytical rotor, which was then placed into a Beckman-Coulter ProteomeLab XL-A analytical ultracentrifuge. After thermal equilibrium was established at 20°C, the samples were scanned at 3,000 RPM at 230 nm to confirm proper cell loading. The rotor was stopped, and then accelerated to the final run speed of 50,000 rpm. Scans were recorded at this rotor speed every ~4 min for 110 total scans for each sample (~7.3 hours total sedimentation time).

The raw data were analyzed using the SEDFIT software, which implements the $c(s)$ method developed by Peter Schuck at the N.I.H.³⁷ In this approach the raw data scans are analyzed by non-

linear least squares to determine the sedimentation coefficient distribution associated with the sample. This method models the influence of diffusion on the data in order to enhance the resolution of the corresponding size distribution. A maximum entropy regularization probability of 0.683 ($1\ \sigma$) was used, and time-invariant noise was removed. The resultant size distributions were graphed, and the peaks were integrated using OriginLab Origin[®] version 7.

CD Spectroscopy

CD spectra and thermal unfolding experiments were acquired on a JASCO J-815 spectropolarimeter equipped with a variable temperature Peltier device and using a 2 mm quartz cuvette. CD spectra were collected at 25 °C and denaturation curves were recorded at 218 nm over a temperature range of 20 – 100 °C (293K – 373K). T_m for WT MIF and MIF variants were determined via nonlinear curve fitting using GraphPad Prism.

Crystallization of Y99C MIF, structure determination, and characterization of solvent channel

Using the Formulatrix NT8 robot, 400 nl of 18 mg/ml protein was mixed with 200 nl reservoir solution (2 M ammonium sulfate, 3% 2-propanol, 0.1 M Tris-HCl, pH 7.5) in one drop and set to crystallization at 20°C using a hanging drop vapor diffusion method with a reservoir volume of 100 μ L. Crystallization was observed with a RI-1000 Formulatrix Imager, and crystals grew to their maximum size in 30 days. Crystals were then flash-frozen in liquid N₂ with 15% glycerol which acts as a cryoprotectant during the data collection. X-ray crystallographic data sets were collected at 100 K using a Rigaku-007 Micromax Generator with a PILATUS Dectris 200K Pixel Array Detector and AFC 4-axis goniometer. Diffraction data were integrated and scaled using HKL3000³⁸. The crystal structure was solved by molecular replacement method using the MIF monomer structure (PDB ID:2OOW) and PHASER.³⁹ The structure solution yielded three

monomers of MIF in the asymmetric unit. The refinement of the structure was performed using the module *Phenix.refine* of the *PHENIX* package.⁴⁰ Cycles of refinement and model building were done using REFMAC⁴¹ and Coot.⁴² The stereochemistry of these crystal structures was assessed with *MOLPROBITY*.⁴³ The X-ray data collection, scaling, and refinement statistics are summarized in **Appendix Table 1**. Figures of the solvent channels and the minimal radius for WT, Y99C, and Y99A were calculated with MOLE 2.0⁴⁴ and MoleOnline,²⁷ respectively.

NMR spectroscopy

Isotopically labeled MIF samples for NMR studies were grown in 1 L M9 minimal media supplemented with glucose (¹²C₆H₁₂O₆, natural abundance) and ammonium chloride (¹⁵NH₄Cl, Cambridge Isotope Labs) as the sole carbon and nitrogen sources, respectively. The ¹⁵N-MIF samples were expressed and purified as described above, then dialyzed into 20 mM sodium phosphate, 1 mM EDTA, and 7.5% D₂O at pH 7.0 and concentrated to 0.5 – 1.0 mM with a 10 kDa MWCO Amicon centrifugal filter (Millipore).

NMR experiments were carried out on a Bruker Avance NEO 600 MHz spectrometer at 30°C. ¹H-¹⁵N TROSY HSQC and relaxation data were collected with the ¹H and ¹⁵N carriers set to the water resonance and 120 ppm, respectively. NMR spin relaxation experiments were performed using TROSY-based pulse sequences adapted from Palmer and coworkers.⁴⁵ Longitudinal relaxation rates (*R*₁) were measured with *T*₁ delays of 20, 60, 100, 200, 600, 800, and 1200 ms. Transverse relaxation rates (*R*₂) were collected with 1.0 ms spacing between 180° CPMG pulses at total relaxation delays of 1, 2, 4, 8, 10, and 12 ms. The recycle delay in these experiments was 2.5 s. Longitudinal and transverse relaxation rates were extracted by non-linear least squares fitting of the peak heights (major peaks in cases of slow exchange) to a single exponential decay using in-house software. Uncertainties in these rates were determined from replicate spectra. The

heteronuclear cross-relaxation rate (^1H -(^{15}N) NOE) was obtained by interleaving pulse sequences with and without proton saturation and calculated from the ratio of peak heights from these experiments. NMR data were processed using NMRPipe⁴⁶ and analysed in SPARKY⁴⁷ along with in-house scripts.

In vivo neutrophil recruitment assays

Wild-type mice of genetic background strain (C57BL6/J) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free animal facility at Cooper University Healthcare. All experiments were done in 10-12 weeks old male mice. Mice were administered a one-time intratracheal instillation of 100 μl of normal saline alone (vehicle) or 10 $\mu\text{g}/\text{ml}$ of WT-MIF or Y99C MIF in a normal saline solution. Mice were sacrificed after 6h of vehicle only or vehicle + experimental agent administration and bronchoalveolar lavage (BAL) fluid was collected. BAL collection was performed by cannulating the trachea with a blunt 22-gauge needle and lavaging the lungs with 800 μl of pre-cooled sterile PBS solution. Total protein concentration in the BAL fluid was measured using the PierceTM BCA assay kit (Thermo Scientific, Rockford, IL), as previously described.⁴⁸ The animal study protocol was approved by the Institutional Animal Care and Use Committee of Cooper University Healthcare, Camden, NJ.

Enzymatic Assays

A 100 mM stock of 4-hydroxyphenyl pyruvate (4-HPP) in 0.5 M ammonium acetate, pH 6.2, was prepared by overnight equilibration at room temperature to generate the predominant keto form. The absorbance of a reaction mixture containing final concentrations of 0.425M boric acid (pH 6.2), 100 nM MIF, and 0-2 mM HPP (saturating) was measured at 306 nm in a 96-well plate using a Tecan Infinite M200, where an increase in absorbance is due to the formation of the enol-

borate complex between HPP and boric acid as MIF catalyzes the tautomerization of HPP to the enol form. Absorbance was measured every 10 seconds for 1.5 minutes, and each reaction was conducted in triplicate per protein sample.

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Appendix

Table 1. Crystallographic Statistics for the Y99C MIF homotrimer (PDB: 7KQX).

	Y99C MIF (7KQX)
Wavelength (Å)	1.5428
Resolution range (Å)*	48.1 - 1.6 (1.657 - 1.6)
Space group	P 21 21 21
Unit cell dimensions (Å)	67.984, 68.062, 86.737
Total reflections	2201046
Unique reflections*	53570 (5219)
Multiplicity*	5.7 (3.4)
Completeness (%)*	99.55 (98.32)
Mean I/sigma (I)*	31.40 (3.29)
Wilson B-factor (Å ²)	14.29
R-meas*	0.050 (0.391)
R-pim*	0.018 (0.204)
CC1/2*	0.997 (0.898)
Reflections used in refinement*	53547 (5219)
Reflections used for R-free*	2579 (248)
R-work*	0.1529 (0.1873)
R-free*	0.1798 (0.2408)
Number of non-hydrogen atoms	3113
macromolecules	2622
ligands	125
solvent	366
Protein residues	342
RMS(bonds) (Å)	0.016
RMS(angles) (°)	1.35
Ramachandran favored (%)	98.81
Ramachandran allowed (%)	1.19
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	1.01
Clashscore	5.57
Average B-factor (Å ²)	17.78
Macromolecules (Å ²)	15.02
Ligands (Å ²)	32.63
Solvent (Å ²)	32.52

CHAPTER 4

Redox-dependent Structure and Dynamics of Macrophage Migration Inhibitory Factor Reveal Sites of Latent Allostery

This chapter is adapted from a published manuscript in Structure (2022) titled:

Redox-dependent Structure and Dynamics of Macrophage Migration Inhibitory Factor Reveal Sites of Latent Allostery

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Abstract

Macrophage migration inhibitory factor (MIF) is a multifunctional immunoregulatory protein that is a key player in the innate immune response. Given its overexpression at sites of inflammation and in diseases marked by increasingly oxidative environments, a comprehensive understanding of how cellular redox conditions impact the structure and function of MIF is necessary. We used NMR spectroscopy and mass spectrometry to investigate biophysical signatures of MIF under varied solution redox conditions. Our results indicate that the MIF structure is modified and becomes increasingly dynamic in an oxidative environment, which may be a means to alter the MIF conformation and functional response in a redox-dependent manner. We identified latent allosteric sites within MIF through mutational analysis of redox-sensitive residues, revealing that a loss of redox-responsive residues attenuates CD74 receptor activation. Leveraging sites of redox-sensitivity as targets for structure-based drug design therefore reveals an avenue to modulate MIF function in its “disease state.”

Introduction

Macrophage migration inhibitory factor (MIF) is a ubiquitous human protein that plays a crucial role in the pathophysiology of inflammation.¹ MIF is expressed by nearly every cell in the body,¹ and many of its effects are elicited by its binding to the chemokine receptors CXCR2 and CXCR4^{2,3} or its cognate receptor CD74.⁴⁻⁶ Circulating levels are reported in the low nM range in healthy humans,⁷⁻⁹ but MIF is overexpressed under inflammatory conditions and has been linked to glucocorticoid overriding activity,¹⁰ asthma,^{7,11} rheumatoid arthritis,¹² colitis,¹³ pancreatitis,¹⁴ atherosclerosis,¹⁵⁻¹⁷ systemic lupus erythematosus (SLE),^{18,19} acute respiratory distress syndrome (ARDS),²⁰ and cancer,²¹⁻²⁵ as well as complications from COVID-19.²⁶ Efforts to elucidate the pathogenic mechanism of MIF have attempted to connect its disease state to cellular redox

properties,^{11,27} most recently by investigating its cysteine residues as conformational switches.²⁸ Mutation of either C56 or C59 in a canonical ⁵⁶CXXC⁵⁹ (XX = AL in MIF) motif modulates the enzymatic and macrophage activation functions of MIF.^{29,30}

An oxidative cellular environment is one of the hallmarks of inflammatory disease, characterized by excess reactive oxygen species (ROS) at sites of inflammation.³¹ Despite the acknowledged contribution of redox imbalance to asthma,^{32,33} ARDS,³⁴ pulmonary fibrosis,³⁵ atherosclerosis,^{36,37} SLE,^{38,39} and cancer,⁴⁰ as well as the link between these pathologies and MIF, our understanding of the redox behavior of MIF is limited, particularly at the structural level. A redox-dependent conformation of MIF, termed “oxMIF,” was previously proposed to account for selective binding to anti-MIF antibodies under oxidizing conditions. Using antibody binding as a marker for oxMIF, this altered conformation has been implicated in human-derived samples and animal models of inflammatory disease.²⁷ Little work, however, has directly addressed how the global MIF structure is modulated by cellular redox conditions and the functional implications of these structural changes.

We recently characterized an allosteric pathway that links two distinct functions of MIF, effectively establishing a regulatory network that mediates functional output.⁴¹ In addition, studies of the conformational dynamics of MIF, a 37.9 kD symmetric homotrimer, have implicated its conformational plasticity in allosteric signal transmission and function.^{6,42} We wondered whether MIF could utilize pro-inflammatory redox imbalances to act as an allosteric sensor to toggle its structure based on the chemical environment of the cell. Altered conformations of MIF at local sites of inflammation could then modulate its function or create different modes of interaction with accessory proteins, thereby controlling downstream biological responses. In a first step toward exploring such a mechanism, we investigated the impact of solution redox conditions on the MIF

structure and identified regions of the protein that are sensitive to oxidative environments. We used solution nuclear magnetic resonance (NMR) spectroscopy to assess the redox-dependent dynamics of MIF with spin relaxation experiments and used mass spectrometry to identify redox-modified cysteine residues. We then leveraged these findings, using further NMR structural studies and *in vitro* and *in vivo* assays, to confirm redox-sensitive amino acids as latent points of functional control in MIF.

Results

Oxidizing solution alters MIF structural stability

To assess the effect of solution redox potentials on the MIF structure, we performed far-UV circular dichroism experiments on redox-neutral MIF samples as well as MIF samples that had been oxidized or reduced. CD spectra of redox-altered MIF show characteristic α - β structure, with only minor differences (**Figure 1A**). These data are consistent with numerous MIF structures in the Protein Data Bank (PDB) that have no obvious alterations in the presence of substrates, inhibitors, or mutations. Despite similarly folded structures, temperature-dependent CD experiments (**Figure 1B**) show that an oxidizing environment destabilizes MIF to unfolding

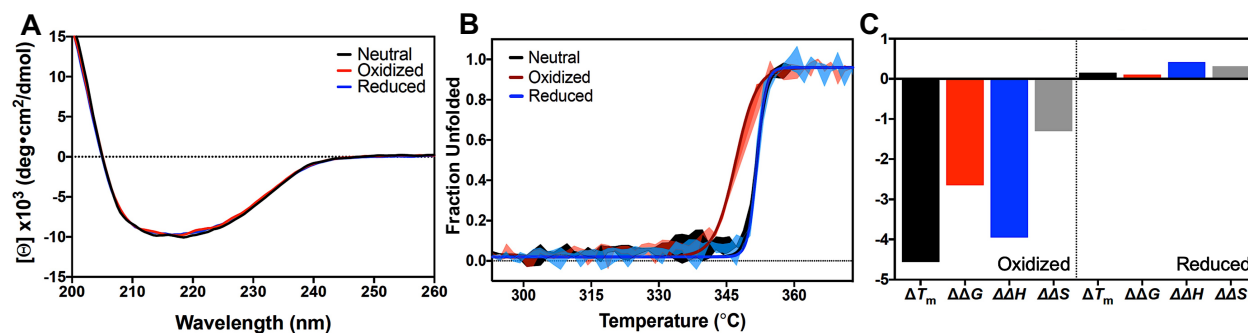


Figure 1. Redox-dependent secondary structure and stability of MIF. (A) Far-UV circular dichroism spectra of redox-neutral (black), oxidized (red) and reduced (blue) MIF. (B) Redox-dependent thermal denaturation profiles of MIF ($\lambda = 218 \text{ nm}$) normalized as a fraction of unfolded protein. Solid lines represent fits of the data to Equation 1, and shaded areas represent the error in the data points over multiple unfolding experiments. (C) Summaries of apparent redox-dependent changes in T_m (black), free energy (red), enthalpy (blue), and entropy (gray) of MIF, calculated relative to redox-neutral MIF.

relative to reduced and redox-neutral MIF ($\Delta T_m = -4.56^\circ\text{C}$, $\Delta\Delta G = -2.65$ kcal/mol, **Figure 1C**).

This energetic difference (expressed per trimer) is modest, but suggests a loosening of the MIF structure that may affect its conformational ensemble.

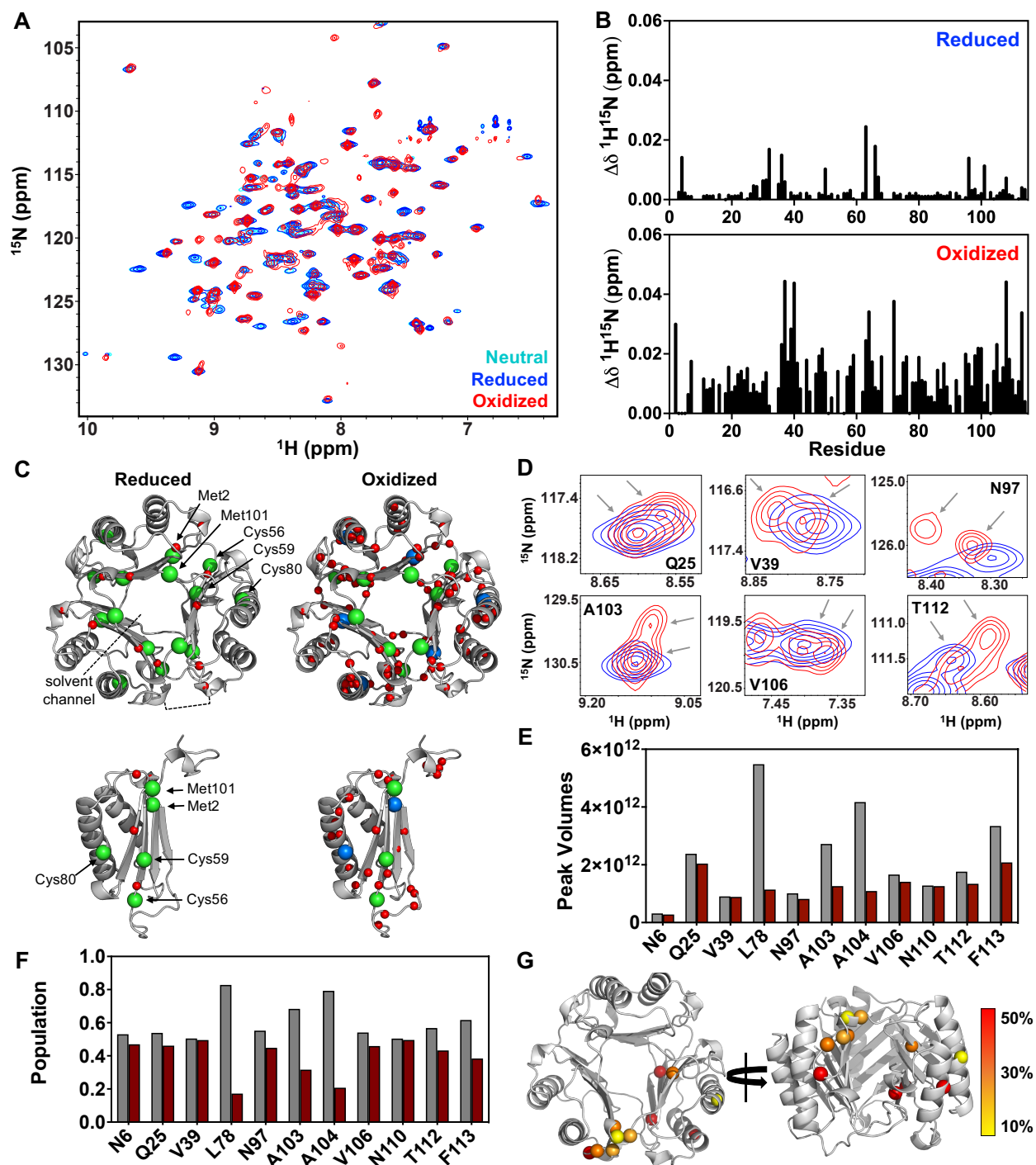


Figure 2. Redox-dependent changes in MIF detected by NMR. (A) $^1\text{H}^{15}\text{N}$ TROSY HSQC spectral overlay of MIF under redox-neutral (teal), reduced (blue), and oxidized (red) conditions. Contour levels for the reduced and oxidized spectra are lowered for enhanced visibility of the neutral spectra. Buffer conditions include 20 mM sodium phosphate and 1 mM EDTA at pH 7.0, with 6 mM DTT, 6 mM H_2O_2 , or no oxidant/reductant depending on the redox condition tested. (B) $^1\text{H}^{15}\text{N}$ combined chemical shift perturbations ($\Delta\delta$) for reduced and oxidized MIF, relative to a redox-neutral sample. (C) Sites of redox-sensitive chemical shift perturbations caused by reducing (left) and oxidizing (right) conditions are mapped onto the MIF structure (PDB: 1MIF⁴³). In both cases, red spheres represent spectral perturbations relative to redox-neutral MIF. Traditionally redox-sensitive Cys and Met residues are shown in larger green spheres and sites where chemical shift perturbations occur at Cys/Met residues are shown in larger blue spheres. Important structural features, including the solvent channel and monomer-monomer interface, are also indicated for reference. (D) Selected resonances from the $^1\text{H}^{15}\text{N}$ HSQC spectral overlay of reduced (blue) and oxidized (red) MIF collected during equilibration (< 6 hours) show sites of slow exchange observed under oxidizing conditions. The major and minor peaks are indicated by arrows. Peak volumes (E) and populations (F) of the major (gray) and minor (red) peaks at sites of slow exchange in oxidized MIF are plotted. The peak intensity and volume for each pair of slow exchanging peaks were calculated in SPARKY,⁴⁴ where peak volume was calculated by integrating each peak as a Gaussian. The minor state populations were estimated by taking the volume of the minor peak and dividing by the total volume of the slow exchanging pairs. (G) Populations of MIF conformers are mapped onto a monomeric subunit of the MIF trimer, where the percentage indicated in the legend denotes the minor species (*i.e.* satellite peak).

Redox-dependent NMR spectra of MIF highlight multiple conformations and altered dynamics

We used solution NMR to pinpoint regions of the MIF structure that are sensitive to the redox environment. $^1\text{H}^{15}\text{N}$ TROSY-HSQC spectral overlays of reduced, oxidized, and redox-neutral MIF (**Figure 2**) show that while reduced and redox-neutral samples are spectrally similar, more significant spectral changes are observed for MIF under oxidizing conditions. Redox-sensitive chemical shift perturbations (relative to redox-neutral MIF) are observed throughout the protein, including for residues at the solvent channel and monomer-monomer interface, where the N-terminal enzymatic active site⁴⁵ is formed (**Figure 2B-C**). Perturbations to MIF are not exclusive to redox-active Cys residues, at times occurring 10 - 20 Å from the ⁵⁶CALC⁵⁹ motif or C80. However, Cys and Met residues serve as nucleation points for our observations, with a majority of redox-dependent changes to the MIF structure occurring proximal to these traditionally sensitive sites (**Figure 2C**). Additionally, for oxidized MIF samples, duplicate resonances for 11 residues are observed, suggesting that an oxidizing environment modulates an equilibrium between two MIF conformations on the NMR timescale (**Figure 2D-G**). Seven sites of slow

exchange map to the C-terminus at the monomer-monomer interface (**Figure 2G**), indicating that the structure of this region of the protein is strongly affected by oxidizing solution, consistent with the lower thermal stability observed by CD. This region of the MIF structure is also implicated in CD74 activation,⁶ suggesting that oxidizing conditions may alter its pro-inflammatory response. These data are consistent with the alternate oxMIF conformation most recently probed by its selective binding to anti-MIF antibodies.²⁸

Analysis of longitudinal (R_1) and transverse (R_2) NMR relaxation rates⁴⁶ highlights local differences caused by redox conditions. Consistent with the altered thermodynamic parameters of the oxidized MIF samples, individual relaxation rates, as well as the R_1R_2 product, qualitatively imply that MIF exhibits heightened flexibility under oxidizing conditions (**Figure 3A**). Analysis of R_1R_2 values⁴⁶ highlights 21 sites suggestive of μs – ms flexibility in oxMIF ($R_1R_2 > 1.5\sigma$ above the 10% trimmed mean of the data), 7 of which do not appear flexible in both redox-neutral and reduced MIF. Despite patches of distinct flexibility, the dynamic profiles of reduced and redox-neutral MIF are similar to each other. ^1H - ^{15}N NOE values and order parameters also hint at local changes to flexibility in oxMIF, though fast timescale dynamics do not differ substantially between samples. Residues with relaxation parameters suggestive of μs – ms flexibility are mapped onto the MIF trimer and monomer structures in **Figure 3B-C**. Correlation plots of R_1R_2 and S^2 parameters for reduced and oxidized MIF reveal a weaker correlation for R_1R_2 , suggesting that MIF experiences greater dynamic changes on the μs – ms timescale over a range of redox conditions (**Figure 3D**). Contributions to the variations in R_1R_2 parameters are most strongly affected by oxidizing solution conditions (**Figure 3E**, left panel), while reducing conditions contribute more significantly to the ps – ns dynamics of MIF via S^2 (**Figure 3E**, right panel). Redox-sensitive residues with apparent μs – ms flexibility include T30, G50, L58, S63, I64*, K66,

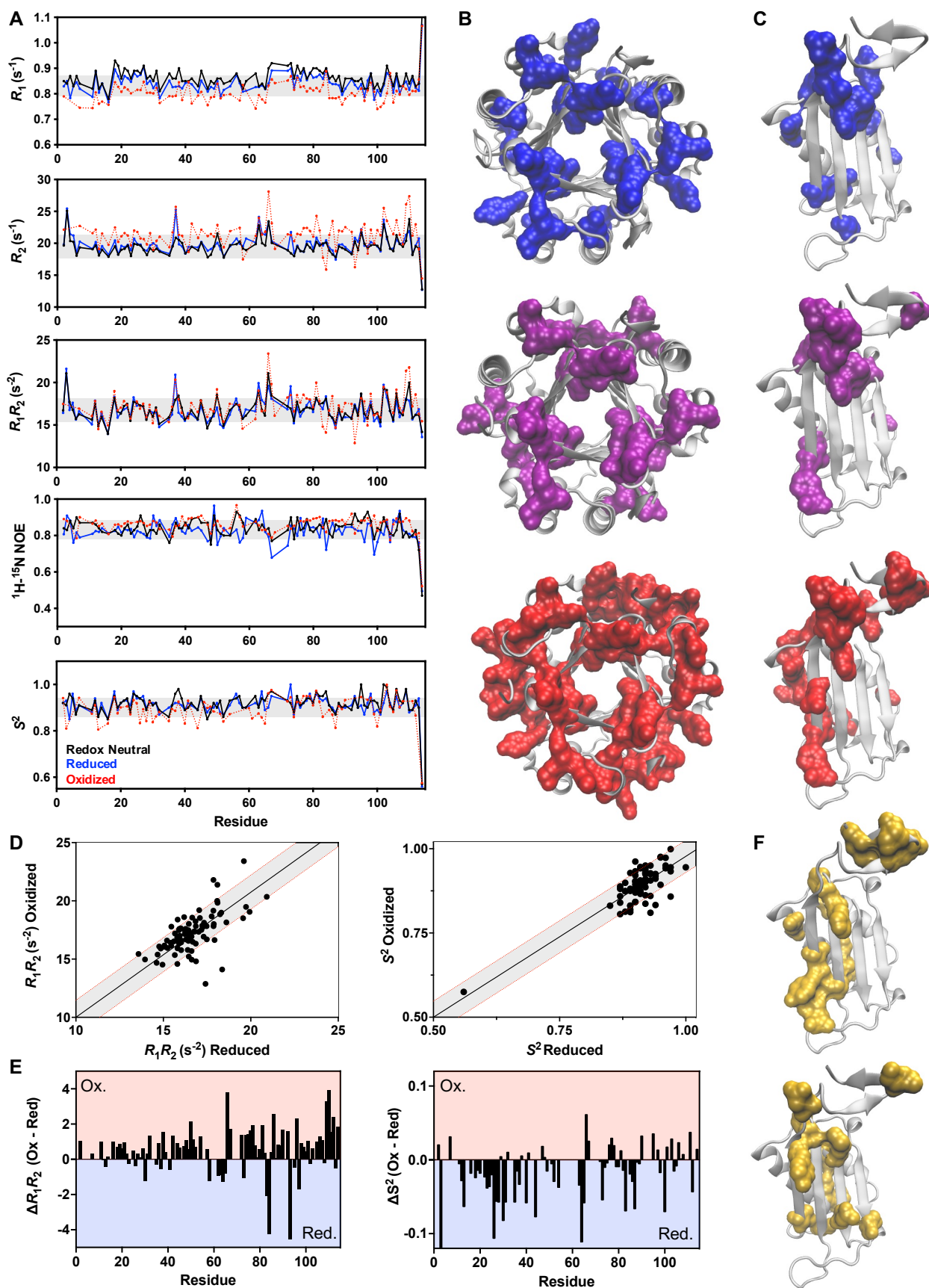


Figure 3. The dynamic profile of MIF is modulated by solution redox conditions. (A) Plots of R_1 , R_2 , R_1R_2 , and the ^1H - ^{15}N NOE and order parameters (S^2) for reduced (blue), redox neutral (black), and oxidized (red dash) MIF. Gray shaded bars denoted $\pm 1.5\sigma$ of the 10% trimmed mean of all data collected for each parameter. Sites suggestive of μs – ms chemical exchange in MIF determined from R_1R_2 values 1.5σ above the 10% trimmed mean of the data are mapped onto the MIF trimer (B) and monomer (C) structures as a function of solution redox potential. Surfaces correspond to reduced (blue), redox-neutral (purple), and oxidized (red) MIF. (D) Correlation plots of the R_1R_2 (left) and S^2 (right) parameters for oxidized and reduced MIF. Gray shaded areas represent $\pm 1.5\sigma$ from the linear least-squares fits of the data (black lines). (E) Per-residue differences in R_1R_2 (left) and S^2 (right) plotted as oxidized – reduced. Bars in the shaded red area denote a greater value in oxMIF, while those in the blue area denote a greater value in reduced MIF. (F) Residues outside of the 1.5σ correlation boundaries in (D) are mapped onto the MIF monomer, highlighting regions of the protein with redox-dependent differences in μs – ms (top) and ps – ns (bottom) dynamics.

R73, L83, A84, R86, R93, I96, N97*, N109*, N110, T112, and A114, and those with apparent ps – ns fluctuations are F3, T7, L26, T30, D44, I64*, K66, K77, L83, L87, S90, Y95*, D100, A103, and S111* (Figure 3E). Asterisks indicate residues previously identified as controlling MIF enzymatic activity and CD74 activation via direct interaction or through allosteric signaling,^{6,45,47} indicating redox-dependent perturbations in MIF may have functional consequences.

To confirm the influence of solution redox potential on μs – ms dynamics in MIF, we quantified the exchange contribution to transverse relaxation, R_{ex} , via CPMG relaxation dispersion experiments (Figure 4). The average global R_{ex} value ($\langle R_{\text{ex}} \rangle$) for MIF is fairly similar across the redox regimes tested (Figure 4A), but site-specific differences are revealed on careful analysis of ΔR_{ex} under reducing and oxidizing conditions (Figure 4B). There is a high degree of similarity in the dynamics of redox-neutral and reduced MIF, evidenced by very small ΔR_{ex} values across the protein, with the exception of a region encompassing Y99-D100-M101-N102. Aside from housing M101 that is known to be redox-modified under almost all experimental conditions (Table 1), an allosteric site that regulates enzymatic catalysis and CD74 activation was recently reported at Y99. The dynamic perturbations observed here are consistent with the high sensitivity of this site in NMR experiments of MIF mutants and ligand-bound complexes. Likewise, strong ΔR_{ex} perturbations are observed in this region of oxMIF, however a much greater overall degree of

fluctuation relative to redox-neutral MIF is also observed across the protein sequence. Several sites of flexibility in redox-neutral MIF are lost in oxMIF (negative ΔR_{ex}) while a large number of newly flexible sites in oxMIF appear around redox sensitive Cys and Met residues (positive ΔR_{ex}).

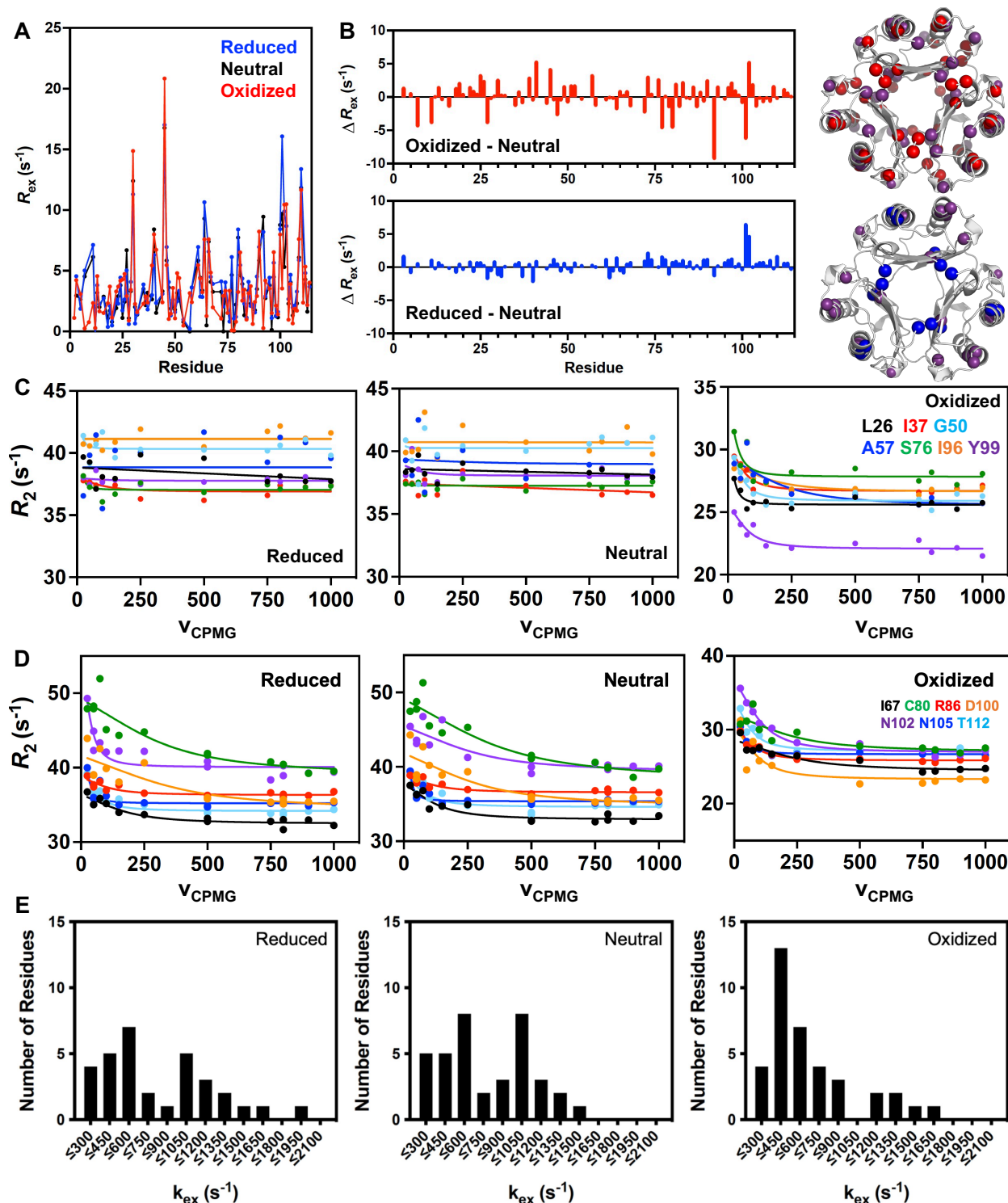


Figure 4. Sites of μ s – ms flexibility are revealed under oxidizing conditions. (A) Per-residue exchange contribution to relaxation (R_{ex}) for reduced (blue), redox-neutral (black), and oxidized MIF (red). (B) Site-specific differences in the exchange contribution to relaxation (ΔR_{ex}) for oxidized (red) and reduced (blue) MIF relative to redox-neutral MIF. Residues with heightened flexibility (positive ΔR_{ex}) in oxidized (red spheres) and reduced MIF (blue spheres) are plotted on the trimer structure, while residues with diminished flexibility relative to redox-neutral MIF (negative ΔR_{ex}) are indicated by purple spheres. (C) CPMG relaxation dispersion profiles for selected MIF residues collected in reducing (left), redox-neutral (center), or oxidizing (right) solution, demonstrating sites that exhibit μ s – ms dynamics only under oxidizing conditions. (D) CPMG relaxation dispersion profiles for residues in MIF, highlighting selected examples of conserved ms timescale motions in MIF across redox regimes. (E) Distribution of k_{ex} values determined from CPMG relaxation dispersion experiments on reduced, redox-neutral, and oxidized MIF, with bin sizing determined from a protocol described by Scott in *Biometrika*.⁴⁸

newly flexible sites in oxMIF appear around redox sensitive Cys and Met residues (positive ΔR_{ex}).

Numerous residues with μ s – ms dynamics are observed in all redox-modulated MIF samples (Figure 4D), however, oxidizing solution stimulates the most widespread relaxation dispersion, affecting 39 residues (~34%) in the MIF structure (Figure 4C). These data are consistent with R_1/R_2 values that estimated a stronger contribution from oxidizing solution to μ s – ms motions (Figure 3). Regions of MIF with known functional importance become flexible, including the monomer interface (L26, I37, G50) and solvent channel allosteric site (I96, Y99).

Despite similar dynamics in large portions of MIF, Figure 4 highlights selected sites that only appear flexible under oxidizing conditions. With the exception of the solvent channel, these flexible sites have not been well characterized for functional impact, suggesting that studies of MIF redox dependence can reveal new target residues for structure-function correlations. Rates of conformational exchange (k_{ex}) determined from analysis of CPMG data range from ~ 300 s⁻¹ to 1,500 s⁻¹ in redox-neutral MIF (Figure 4E). Reducing conditions broaden the distribution of k_{ex} values slightly, where a greater number of residues sample $k_{ex} \geq 1,000$ s⁻¹. In contrast, oxMIF skews the distribution toward $k_{ex} \leq 750$ s⁻¹. However, these differences are subtle and the high degree of dynamic overlap in these redox-modulated structures makes it difficult to relate these changes to a mechanistic driving force at present.

Cysteine 80 is a selectively modified redox switch

Prior work suggests that C80 functions as a redox switch to the oxMIF conformation when modified by thiol reactive compounds.²⁸ As a next step in elucidating the structural role of C80 in the redox behavior of MIF, we performed quantitative mass spectrometry to explore the possibility of the selective modification of C80 by redox reagents (**Figure 5** and **Appendix Figure 1**). Thiol (SH) groups present in the MIF structure under oxidizing or redox-neutral conditions were irreversibly alkylated with iodoacetamide (IAA). Then, natively oxidized cysteines were reduced with DTT and alkylated by *N*-ethylmaleimide (NEM; **Figure 5A**). As an experimental control, reduced MIF was modified with only IAA or NEM (**Figure 5B**). Mass spectra confirmed the presence of 1) C80 modified exclusively by IAA or NEM in reduced MIF samples modified by a

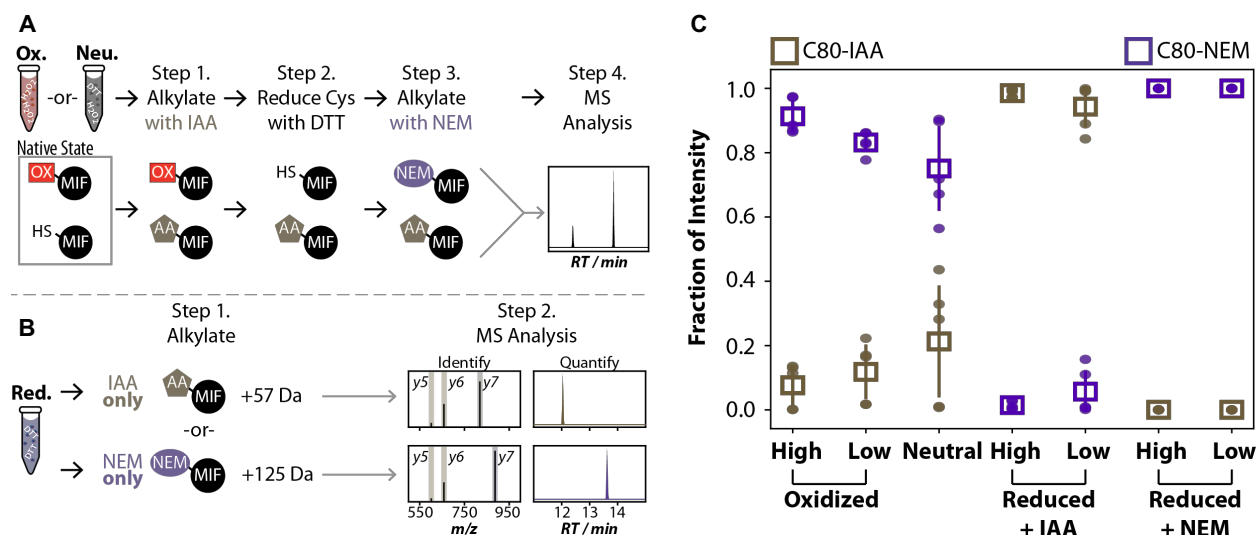


Figure 5. C80 is selectively modified under oxidizing conditions. (A) Stepwise alkylation of MIF cysteine residues to determine the relative abundance of oxidized residues via mass spectrometry. The alkylation workflow involved three reaction steps: Alkylation by iodoacetamide (IAA), reduction by dithiothreitol (DTT), and alkylation with *N*-ethylmaleimide (NEM), followed by LC-MS analysis. **(B)** MIF equilibrated under reducing conditions was treated with only one alkylating agent and served as a quantitative control. C80 modification was confirmed through mass spectrometry and relative quantitation was achieved through integration of the chromatographic peaks with respect to the base peak chromatogram intensity. **(C)** Relative quantitation of C80 alkylation revealed conformational dependence and sensitivity to the surrounding redox environment. Squares represent mean values from five sample injections, vertical bars correspond to standard deviation, and circle markers are observed values per replicate. Data were collected at low (10x) or high (20x) concentrations of oxidant or reductant.

single alkylating agent and 2) a mixture of IAA- and NEM-bound C80 from oxidizing and redox-neutral conditions (**Figure 5C** and **Appendix Figure 1A-C**). The abundance of each modification, and therefore relative cystine (S=S) abundance, was determined from the contribution of the respective chromatographic peaks to the total base peak intensity of the chromatogram.

Cystine abundance is reflected by NEM-bound C80, as this reaction is only possible after an existing cystine is reduced (**Figure 5A**). Indeed, using NEM-bound C80 as a proxy for the abundance of cystine residues reveals an increase from 75.1% to 91.3% cystine abundance between redox-neutral and oxidizing conditions (**Figure 5C**). Under redox-neutral conditions, C80 exhibits the greatest variance in cystine abundance, further highlighting the reactivity and sensitivity of this residue to environmental conditions. The relative abundance of cystine is also sensitive to the amount of oxidative stress. Increasing the concentration of oxidant in solution from 10:1 to 20:1 shifts the C80 cystine relative abundance from 83.1% to 91.3%. In addition to C80, MIF also contains two cysteine residues in its ⁵⁶CALC⁵⁹ motif; however, the peptide fragment containing C56 and C59 is a large 34-mer, precluding quantitation by the MS methods utilized here. Although we focus on C80, we cannot rule out a significant role for other cysteines in MIF redox-dependent structural changes.

Oxidation of much more labile methionine residues was also observed in the mass spectra. Modified Met2 was detected independent of redox conditions. Additionally, oxidation at Met49 was observed under 20:1 oxidizing conditions and 20:1 reducing conditions treated with IAA. Finally, Met101 was found to be oxidized under 10:1 and 20:1 oxidizing conditions, redox neutral conditions, and both reducing conditions treated with NEM. The noted methionine modifications are detailed in **Table 1** and the corresponding MS2 spectra for each identification are shown in and **Appendix Figure 1D**. Interestingly, Met can also cause double NMR resonance lines of

Table 1. Peptides with oxidized methionine residues detected by mass spectrometry.

Peptide	Position	Oxidation Site	Conditions detected
1. PMFIVNTNVPR	1-12	M2	ALL
2. PPQYIAVHVVPDQLMAFGGSSEPCALCSLHSIGK	34-67	M49	OH, RHI OL, OH, N, RLN, RHN
3. VYINYDDMNAANVGWNNSTFA	95-115	M101	

protons and nitrogens close in structural space. However, we only observe slow exchange behavior in MIF under oxidizing conditions, suggesting that Met oxidation alone does not drive the observed NMR spectral changes.

Mutation of redox-sensitive residues alters MIF structure and function

To test the possibility that biologically relevant conditions, such as changes in cellular redox, could reveal sites of allosteric regulation in MIF that are not otherwise obvious, we selected representative test residues that fit two criteria: a residue that can be redox modified (C80) and a residue that is redox sensitive (though not covalently modified) as revealed through structural and dynamic studies of MIF by NMR (K66). C80 was shown by mass spectrometry to be selectively modified under oxidizing conditions (**Figure 5**) and has been implicated as a ‘switch’ to adopt the oxMIF conformation.²⁸ K66 was found to have a large variation in the R_1R_2 parameter in oxMIF, as well as a low ^1H - ^{15}N NOE in reduced MIF (**Figure 3**), indicating that it undergoes significant changes in flexibility between the oxidized and reduced states. Interestingly, Thiele and coworkers postulated that an opening and/or disruption of the protein occurs during the conformational switch to oxMIF.²⁸ Similarly, the location of K66 within the MIF structure plays a stabilizing role in the relative orientation of the two helices and beta strands. Increased dynamics of K66 under oxidizing conditions may destabilize the structure, consistent with the lower stability observed for oxidized MIF by CD (**Figure 1**), and thus allow for a redox-dependent conversion to oxMIF. In addition,

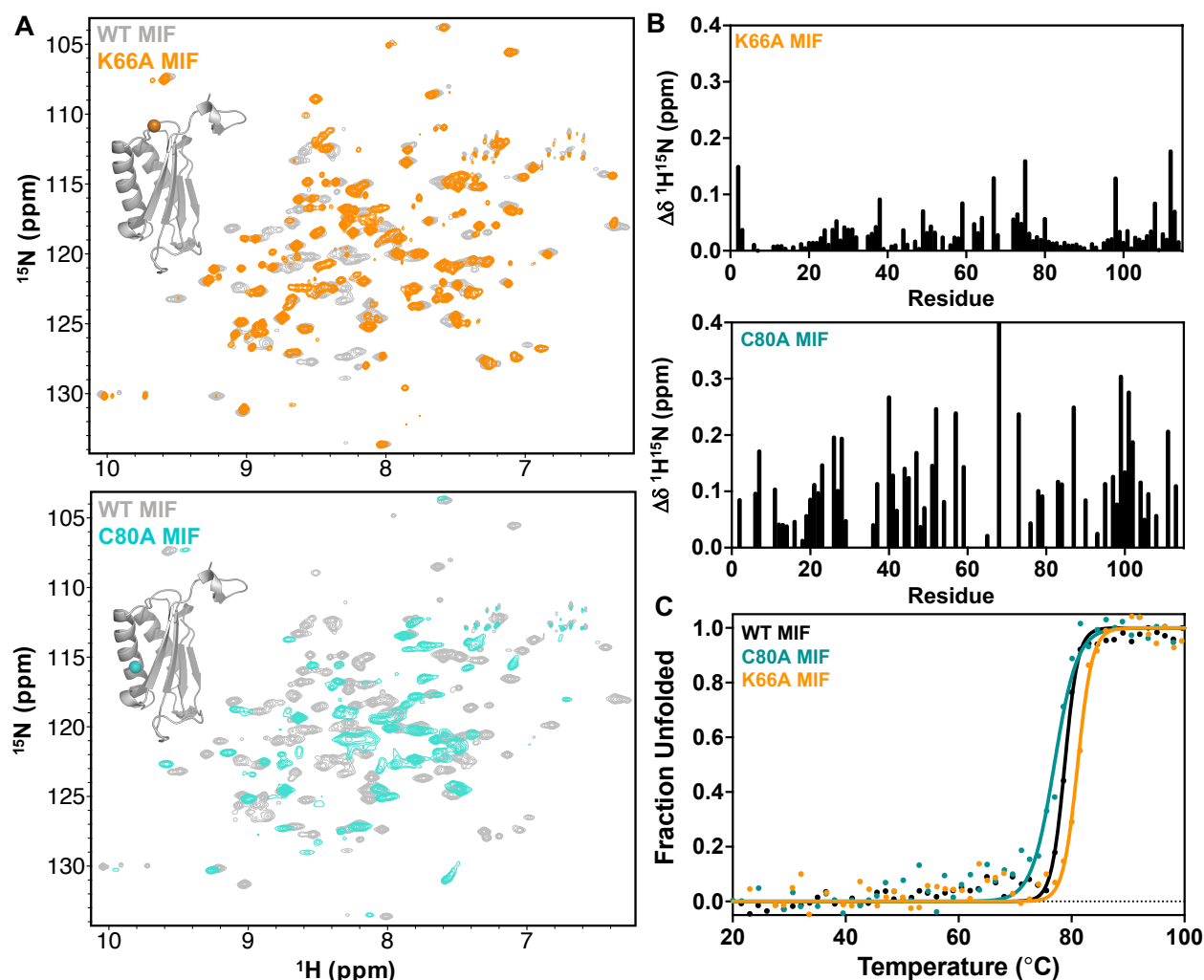


Figure 6. Mutations at K66 and C80 cause biophysical changes to the MIF structure. (A) ^1H - ^{15}N TROSY HSQC spectra of K66A (orange) and C80A (teal) overlaid with the WT MIF spectrum (gray). The inset structures indicate the location of residues 66 (orange sphere) and 80 (teal sphere) within the MIF monomer. (B) ^1H - ^{15}N combined chemical shift perturbations ($\Delta\delta$) for K66A (top) and C80A (bottom), relative to WT MIF. (C) Structural stability of K66A and C80A MIF variants. Thermal denaturation profiles ($\lambda = 218$ nm) of K66A MIF (orange), C80A MIF (teal), and WT MIF (black) are normalized as a fraction of unfolded protein. Despite minor stability changes resulting from single point mutations in the MIF variants, as compared to WT MIF, K66A and C80A MIF are fully folded under NMR experimental conditions (30 $^{\circ}\text{C}$), which is consistent with observed stability changes of other MIF variants as previously reported.⁴¹

K66 and C80 are spatially distinct from known MIF functional sites, establishing them as interesting targets for the study of MIF allostery at long ranges. It was also recently discovered that a cysteine residue can covalently crosslink with a lysine for form an NOS bridge, which was demonstrated to regulate the enzymatic function of a transaldolase ortholog by a redox-driven

mechanism.⁴⁹ Though K66 and C80 do not form crosslinks to each other or to other lysine/cysteine residues in MIF due to spatial restraints, it expands the possibility that these surface exposed residues may form covalent interactions with lysine residues on CD74 that are in proximity to the predicted site of interaction,⁵⁰ or with surface amenable lysine/cysteine residues on CXCR2 and CXCR4, in a redox-dependent manner.

To interrogate their role in MIF structure and function, we created single point mutations at K66 (K66A) and C80 (C80A) and collected ¹H¹⁵N TROSY-HSQC NMR spectra of the K66A and C80A variants in redox-neutral, reducing, and oxidizing solutions. Baseline structural changes caused by the K66A and C80A mutations are significant under redox-neutral conditions, particularly for the C80A variant (**Figure 6A-B**) when compared to wild-type (WT) MIF, though CD spectroscopy indicates the variants are fully folded at NMR sample temperatures (**Figure 6C**). Interestingly, chemical shift perturbations observed for K66A MIF under oxidizing and reducing conditions are modest when compared to a redox-neutral K66A sample (**Figure 7**). Most notably, oxidized K66A exhibits a chemical shift profile much smaller in magnitude than that observed in the corresponding WT MIF experiments. In addition, reduced K66A exhibits larger chemical shift perturbations than oxidized in both number and magnitude, the inverse of what was found for WT MIF. These data alone suggest that K66 may play a role in modulating the redox-dependent structural changes of WT MIF, which are substantially diminished upon mutation. Likewise, the C80A variant strongly attenuates redox-sensitive NMR chemical shift changes in both reduced and oxMIF (**Figure 7**). These data, along with mass spectrometry, indicate that C80 modulates the conformational landscape of MIF under oxidative conditions.

To understand whether this altered redox behavior foreshadowed changes in MIF function, we conducted enzymatic studies of WT MIF and the K66A and C80A variants under redox

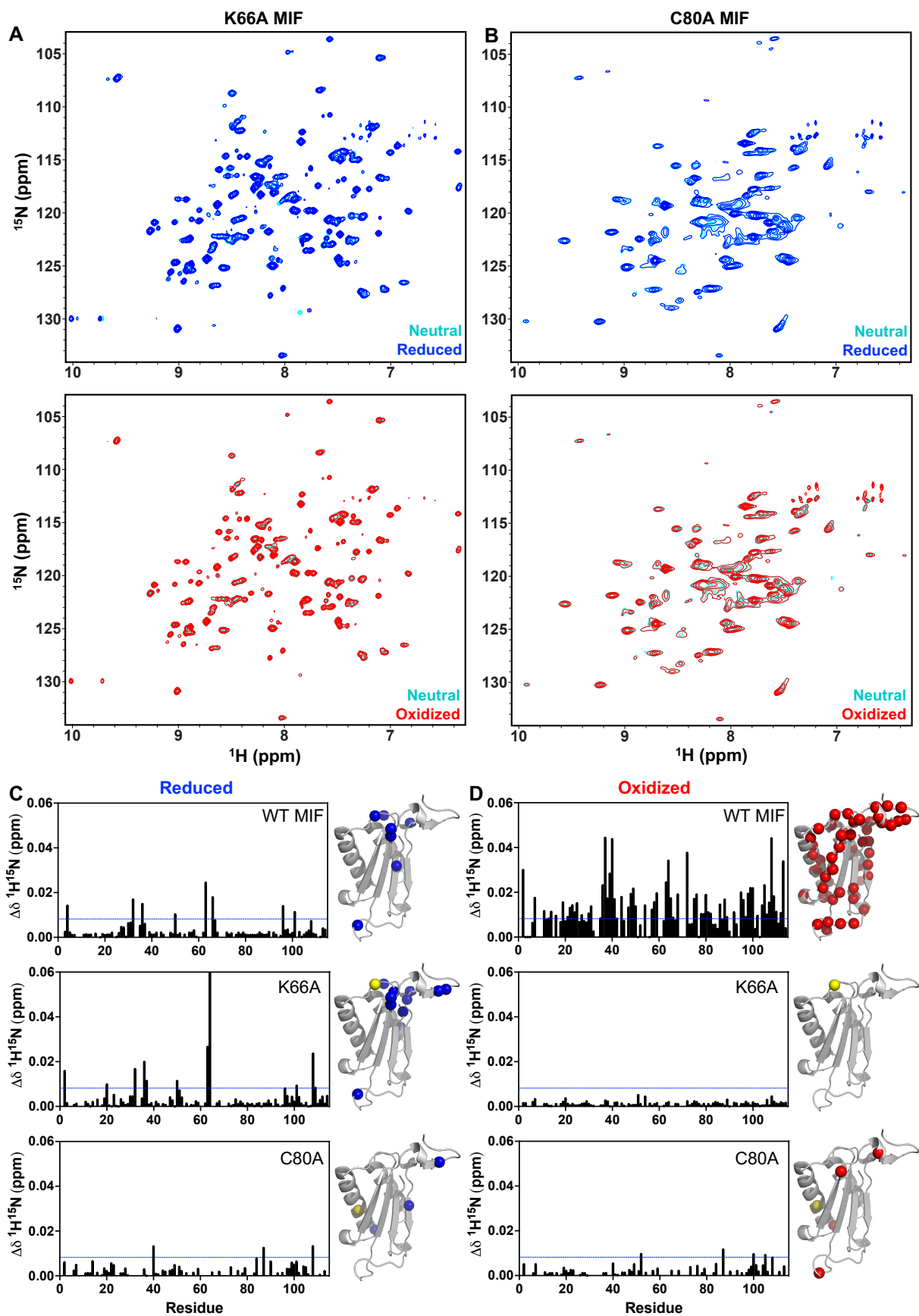


Figure 7. Mutation of the redox-sensitive K66 and C80 residues alters the redox sensitivity of the MIF structure. ^1H - ^{15}N TROSY HSQC spectra of K66A and C80A MIF collected under reduced (blue) and oxidized (red) solution conditions are shown in (A) and (B), respectively. Spectra are overlaid with that of redox-neutral MIF variants (teal). Contour levels for the reduced and oxidized spectra are lowered for enhanced visibility of the neutral spectra. The high degree of line broadening in C80A spectra occurs upon mutation but does not change as the solution redox potential is altered. ^1H - ^{15}N chemical shift perturbation profiles of reduced and oxidized MIF, relative to redox-neutral K66A and C80A samples used as references, are shown in (C) and (D), respectively. Blue lines denote 1.5σ above the 10% trimmed mean of all shifts. Combined ^1H - ^{15}N chemical shift plots for reduced and oxidized WT MIF are repeated here from **Figure 2** for comparison. Chemical shifts above 1.5σ are mapped onto the MIF monomer, with the sites of mutation indicated in yellow.

conditions (**Figure 8A**). K66A MIF exhibited reduced tautomerase activity at ~48% of WT MIF, while the tautomerase activity of C80A was abolished, implicating K66 and C80 as allosteric sites that regulate MIF enzymatic activity. Further, a significant body of literature supports a role for the catalytic Pro1 in MIF signaling,⁵¹⁻⁵⁶ suggesting that the K66A and C80A mutations, which exhibit altered enzymatic activity, may also impact its pro-inflammatory function. We did not observe redox-dependent changes to the tautomerase activity for WT MIF, consistent with previously reported findings for hydrogen peroxide-modulated MIF.⁵⁷ We cannot exclude, however, the effect that other reactive oxidants may have on modulating the enzymatic activity of MIF, as hypochlorous acid was shown to modify the catalytic Pro1 to a proline imine and significantly reduce its activity.⁵⁷ K66A and C80A also did not exhibit redox-dependence to the enzymatic activity, which is consistent with the minimal NMR structural changes observed for the variants under redox conditions (**Figure 7**).

We then evaluated the K66A and C80A variants in neutrophil recruitment assays in murine lungs, where we measured total immune cells, percentage of neutrophils in BAL fluid, and protein content (as a marker of alveolar capillary leak/pulmonary edema). This *in vivo* assay quantifies neutrophil recruitment on the surface of alveolar macrophages, an established metric for the MIF-induced activation of the pro-inflammatory CD74 receptor.⁵⁸ The assay is indirect, using chemokines released by activated macrophages to stimulate CXCR2 receptors on neutrophils (that

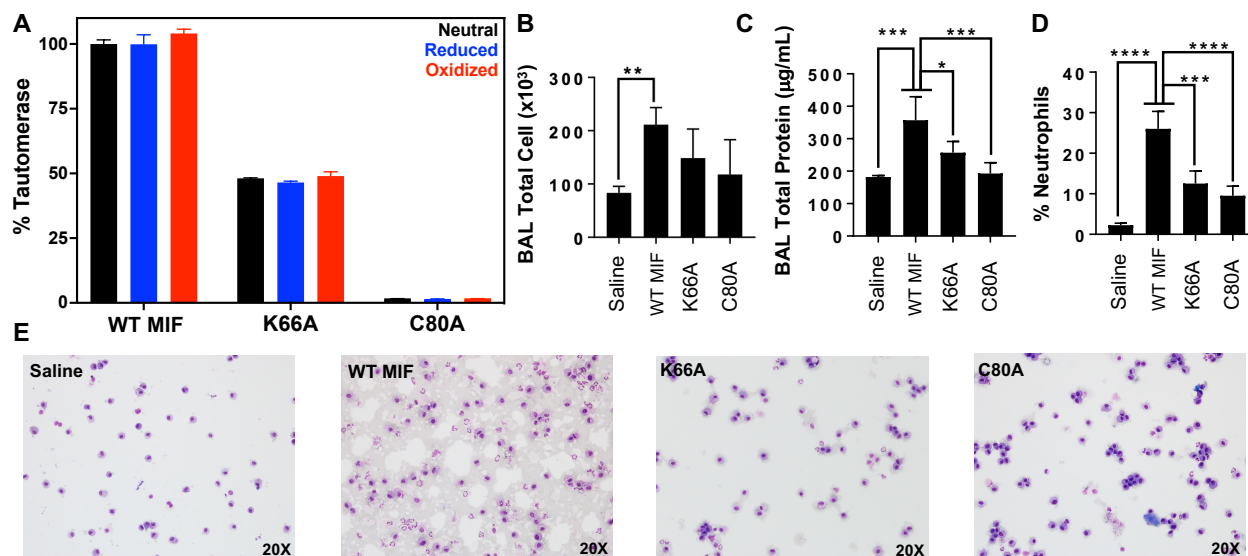


Figure 8. Mutation of the redox-sensitive K66 and C80 residues alters MIF enzymatic and pro-inflammatory function. (A) HPP keto/enol tautomerase activity of WT MIF, K66A, and C80A under redox-neutral (black), reduced (blue), and oxidized (red) conditions. Bars represent the mean of triplicate data sets for each condition $\pm$ SEM. Data are normalized such that the mean of the neutral WT MIF activity is equal to 100%. Mutations at K66 and C80 significantly decreased total immune cell recruitment (B), pulmonary edema protein levels (C), and CD74-dependent neutrophil recruitment (D) in murine lungs *in vivo*. (E) Representative images of Hema 3 staining in BAL fluid cell pellets show decreased neutrophil recruitment in murine lungs by K66A and C80A variants as compared to WT MIF. Data are expressed as mean $\pm$ SEM (n = 4 in each group, *p<0.05, **p $\leq$ 0.01, ***p $\leq$ 0.001 and ****p $\leq$ 0.0001).

do not directly express CD74) to induce their migration to murine lungs.⁵⁹ We found that WT MIF significantly increased the total BAL fluid cell counts over saline controls, while the K66A and C80A variants showed a reduced number of total BAL fluid immune cells (Figure 8B). The pulmonary edema protein levels and neutrophil influx were significantly increased in the WT MIF group relative to vehicle controls (Figure 8C-E). In contrast, we observed significant decreases in the groups to which the MIF variants were delivered, as compared to WT MIF. Together, our data indicate that the K66A and C80A variants have a functional effect in reducing the inflammatory response of MIF in the murine lung. Although this assay does not directly test MIF-induced CD74 activation under redox conditions, the results suggest that the conformational sensitivity to oxidizing conditions observed in WT MIF and attenuated in the K66A and C80A variants can impact biological function, implying an allosteric role for MIF residues K66 and C80. Here, we

demonstrate that assessing environmental perturbations through a structural and dynamic lens can provide a novel route for achieving functional control beyond the known MIF active sites. Several other redox affected residues, such as those exhibiting μs – ms flexibility only under oxidizing conditions (**Figure 4C**), can be tested for similar functional impact as latent allosteric sites in future studies.

Discussion

We used solution redox potentials to modulate the MIF structure and dynamics in order to reveal conformational changes and redox-sensitive residues that may be latent functional sites within its structure. This work builds upon prior studies implying redox sensitivity of MIF,^{11,27,28,60-62} as well as our recent paper highlighting a series of allosteric residues linking the enzymatic (tautomerase) and CD74 binding sites of MIF.⁴¹ Here, we demonstrate that examination of MIF redox sensitivity is another useful avenue for probing function beyond its established active sites. Since MIF participates in both intracellular and extracellular interactions,⁶³ where the redox environments differ, understanding the implications of its redox dependence may also inform its pro-inflammatory mechanisms and functional promiscuity, which has been widely investigated biochemically, but less so at the structural level. A previous characterization of the association of MIF with p53 tumor suppressor suggested the interaction to be redox-dependent, having shown a significant decrease in coprecipitation in the presence of a reductant.⁶⁴ Further, interaction of MIF with ribosomal protein S-19 (RPS-19) was shown to occur more strongly under reducing conditions.⁶⁵ In addition, MIF is known to be post-translationally modified by glycation in the brain tissue of Alzheimer's patients. Most notably, an altered electrophoretic migratory behavior of glycated MIF was observed and demonstrated *in vitro* by incubating MIF with glucose under oxidizing conditions.⁶⁶

Redox signatures of inflammatory diseases, most of which implicate MIF, can also be markedly different from those of healthy humans. The redox potential of epithelial lining fluid is -110 to -40 mV in the lungs of asthmatics, which is significantly more oxidizing than that of healthy humans (-150 to -200 mV).³³ Further, the synovial fluid of rheumatoid arthritis patients has been shown to be highly oxidizing relative to that of a non-arthritic control group.^{67,68} A significant body of literature suggests that when overexpressed at sites of inflammation,²¹ MIF can endure large swings in environmental redox potential. NMR spin relaxation studies show redox-dependent modulation of local flexibility in MIF, with motion observed on multiple timescales. oxMIF, in particular, exhibits the most significant structural and dynamic changes, with widespread NMR spectral perturbations and CPMG relaxation dispersion that show oxidizing potentials stimulate flexibility in ~34% of the MIF structure, including its redox-active residues. However, the majority of these flexible regions have not been confirmed to harbor any functional impact in prior work.

It remains unclear how this relatively compact (12.5 kDa monomer) and symmetrical homotrimer accommodates a wide range of non-overlapping activities. A 2018 study showed that nanosecond dynamics were important for organization of the MIF-CD74 binding interface,⁶ and studies with the BaxB01 anti-MIF antibody suggested selective binding to the MIF ⁵⁶CALC⁵⁹ motif only after rearrangement of the Cys sulfhydryls induced by oxidizing conditions.²⁸ Oxidizing conditions appear to promote a more flexible solvent channel, which we recently showed to be central to an allosteric mechanism regulating tautomerase and CD74 activation involving motion on multiple timescales.⁴¹ Given the number of MIF functions that have yet to be explored at the molecular level and the fact that ~100 X-ray crystal structures of MIF mutants, substrate-, and inhibitor-bound complexes have negligible variation in static structure but vastly different NMR

spectra, it is plausible, if not likely, that additional regions of functional importance exist in MIF.

Here, we asked if changes in solution redox potential could reveal some of these latent sites by modulating the structural and/or dynamic signatures of MIF. We experimentally altered the redox potential of WT MIF NMR samples, enabling us to identify redox-dependent structural changes in residues that may be important for function. We mutated two such residues, K66 and C80, that showed redox-sensitivity in WT MIF and observed structural effects on MIF redox behavior as well as altered functional responses. Residues that display μs – ms flexibility exclusively under oxidizing conditions, as highlighted in **Figure 4**, would also be interesting targets for future studies of MIF redox sensitivity and function. Having further confirmed the propensity of C80 for redox-driven modification by mass spectrometry, we speculate that MIF could act as a sensor that toggles its conformation depending on its cellular environment to engage with varied binding partners, as was previously suggested in a conformational control scheme for the $^{56}\text{CALC}^{59}$ motif. Though beyond the scope of this work, detailed cellular studies of WT and C80A MIF under various redox conditions may reveal this mechanism in the future. The more extensive but partially overlapping set of residues affected by the redox environment suggests the regulatory network controlling non-overlapping MIF functions may be larger than previously reported, where additional “allosteric nodes” may be revealed only when MIF is exposed to stimuli, such as environmental factors associated with inflammation, serving as a natural means of activating latent residues to expand MIF functions under altered cellular conditions. Speculating further, targeting small molecules to redox sensitive residues may prevent MIF from engaging in certain downstream cascades. Future studies of protein-protein interactions with supposed binding partners of MIF, such as thioredoxin^{69,70} or ribosomal protein S19,⁶⁵ as well as with small molecules, can evaluate this mechanism. Another interesting avenue warranting further

exploration is the redox-dependent interaction of MIF with its known inhibitors or with novel compounds, as it presents an opportunity to preferentially target oxMIF, a form of the protein that may appear predominately within the inflammatory state.

Materials and Methods

Protein expression, purification, and redox sample preparation

Wild-type (WT) and/or human MIF variants cloned into a pET11b vector were grown in lysogeny broth (LB) for biochemical studies and in isotopically enriched M9 minimal medium supplemented with CaCl_2 , MgSO_4 , and MEM vitamins for NMR, after adapting BL21(DE3) cells to D_2O . Small cultures of MIF were grown overnight in LB at 37°C and used to inoculate cultures containing 50% D_2O the following morning, which were grown 8 – 10 hours at 37°C and then used to inoculate cultures containing 95% D_2O , which were incubated at 37°C for 12 hours. The cells were collected by centrifugation and resuspended in M9 medium (1 L, 100% D_2O) supplemented with $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotope Laboratories) and $^{13}\text{C}_6\text{H}_{12}\text{O}_6$ as the sole nitrogen and carbon sources, respectively. Cultures were grown to an OD_{600} of 0.8 – 1.0 and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). Following 16-18 hours of post-induction growth at 20°C , the cells were harvested by centrifugation.

The cells were resuspended in ~30 mL of an ice-cold buffer of 20 mM Tris-HCl, 20 mM NaCl, and 1 mM EDTA at pH 7.4 supplemented with protease inhibitors, lysed by ultrasonication, clarified by centrifugation, filtered, and purified with a 120 mL Macro-Prep High Q column (BioRad) pre-equilibrated with the lysis buffer. MIF does not bind the Q resin and is found in the flow-through. MIF was purified to its final form on a HiLoad 26/600 Superdex 200 size exclusion column (GE Healthcare). For NMR samples, MIF was dialyzed into a buffer containing 20 mM sodium phosphate, 1 mM EDTA, and 7.5% D_2O at pH 7.0. Final concentrations of MIF were 0.5

– 1 mM determined using $\epsilon_{280} = 12,950 \text{ M}^{-1} \text{ cm}^{-1}$.⁷¹

Oxidation of MIF was achieved by addition of H_2O_2 to a final concentration of 6 mM in an aliquot of 1 mM MIF. Reduced MIF was prepared with a 6 mM final concentration of DTT. Stocks of the oxidizing and reducing agents were freshly prepared in a degassed buffer of 20 mM sodium phosphate, 1 mM EDTA, and 7.5% D_2O at pH 7.0. Following addition of oxidant or reductant to MIF samples, the pH of all samples was monitored and adjusted to pH 7.0 as needed. NMR tubes were briefly degassed with nitrogen and sealed with Parafilm (Bemis). The samples were equilibrated in the presence of redox reagents for at least six hours. Redox-neutral samples were prepared with equimolar amounts of DTT/ H_2O_2 or in buffer containing no oxidizing or reducing reagents. We observed no appreciable differences between the redox-neutral preparations by NMR, and subsequently used oxidant and reductant-free buffer at pH 7.0 for analysis of redox-neutral MIF.

Circular Dichroism (CD) Spectroscopy

CD spectra and thermal unfolding experiments were recorded on a JASCO J-815 spectropolarimeter equipped with a variable temperature Peltier device. Denaturation curves of 10 μM MIF were recorded at 218 nm in a 2 mm quartz cuvette. The temperature range for each scan was 20 – 100 °C (293 K – 373 K). Thermodynamic parameters were extracted via nonlinear curve fitting of CD data to Equation 1 in GraphPad Prism:

$$\text{Ellipticity } (T) = \frac{[(m_f T + b_f) + (m_u T + b_u)] \exp\left[\left(-\frac{\Delta H_{D,vH}}{R}\right)\left(\frac{1}{T} - \frac{1}{T_m}\right)\right]}{1 + \exp\left[\left(-\frac{\Delta H_{D,vH}}{R}\right)\left(\frac{1}{T} - \frac{1}{T_m}\right)\right]} \quad (1)$$

where m_f , b_f , and m_u , b_u are the slopes and y-intercepts of the folded (low temperature) and unfolded (high temperature) regions of the melting curve, R is the gas constant, T_m and $\Delta H_{D,vH}$ are the unfolding midpoint and van't Hoff enthalpy of denaturation at T_m , respectively. Free energy

analysis was performed as described elsewhere,⁷² using values of ΔC_p , the unfolding heat capacity, estimated from the reports of Privalov and Makhatadze.^{73,74} The differences in apparent unfolding enthalpies, entropies, and free energies at were calculated as:

$$\Delta H_{D,app} = \Delta H_{D,vH} + \Delta C_p(T_{REF} - T_m) \quad (2)$$

$$\Delta S_{D,app} = \frac{\Delta H_{D,vH}}{T_m} + \Delta C_p \ln \left(\frac{T_{REF}}{T_m} \right) \quad (3)$$

$$\Delta G(T) = \Delta H_{D,vH} \left(1 - \frac{T_{REF}}{T_m} \right) + \Delta C_p \left[(T_{REF} - T_m) - T_{REF} \ln \left(\frac{T_{REF}}{T_m} \right) \right] \quad (4)$$

These data are reported per trimer using $\Delta C_p = 4.8$ kcal/molK (114 residues per monomer, 342 residues total) and a reference temperature of 351.7 K, the T_m of redox-neutral MIF (thus, $\Delta \Delta G_{app}$ for redox-neutral MIF = 0). These data are also reported as “apparent” values due to the ~90% reversibility of the MIF unfolding transition.

NMR Spectroscopy

NMR experiments were performed on a Bruker Avance NEO 600 MHz or Bruker Avance III HD 850 MHz spectrometer at 30 °C. NMR data were processed using NMRPipe⁷⁵ and analyzed in Sparky⁴⁴ along with in-house scripts. The pH of NMR samples was monitored after addition of redox reagents to ensure that NMR spectral changes were due to reduction/oxidation. NMR assignments for WT MIF and the K66A and C80A variants were confirmed from standard triple resonance experiments. Combined chemical shift perturbations ($\Delta\delta$) were determined by

$$\Delta\delta = \sqrt{\frac{1}{2}((\delta_{HN}^2) + (\delta_N^2/25))} \quad (5)$$

The HSQC of oxidized MIF was processed with an exponential window function in both the direct and indirect dimensions in NMRPipe. NMR chemical shift perturbations were analyzed as the 10% trimmed mean of all data sets (n=3), where significant perturbations were determined as shifts that

are 1.5 standard deviations above the 10% trimmed mean. Peak intensity and volume for each pair of slow exchanging peaks were calculated in SPARKY, where peak volume was calculated by integrating each peak as a Gaussian. The minor state populations were estimated by taking the volume of the minor peak and dividing by the total volume of the slow exchanging pairs.

NMR spin relaxation experiments were performed using TROSY-based pulse sequences adapted from Palmer and coworkers.⁷⁶ Relaxation data were acquired with the ^1H and ^{15}N carriers set to the water resonance and 120 ppm, respectively. Longitudinal relaxation rates (R_1) were measured with T_1 delays of 0, 20, 60, 100, 200, 600, 800, and 1200 ms. Transverse relaxation rates (R_2) were collected with 1.0 ms spacing between 180° CPMG pulses at total relaxation delays of 0, 1, 2, 4, 8, and 10, and 12 ms. The recycle delay in these experiments was 2.5 s. Longitudinal and transverse relaxation rates were extracted by non-linear least squares fitting of the peak heights (major peaks in cases of slow exchange) to a single exponential decay using in-house software. Uncertainties in these rates were determined from replicate spectra. The heteronuclear cross-relaxation rate (^1H - ^{15}N NOE) was obtained by interleaving pulse sequences with and without proton saturation and calculated from the ratio of peak heights from these experiments. Carr-Purcell-Meiboom-Gill (CPMG) NMR experiments were performed at 600 MHz and 30°C with a constant relaxation period of 40 ms, a 2.0 s recycle delay, and ν_{CPMG} points of 0, 25, 50, 75, 100, 150, 250, 500, 750, 800, 900, and 1000 ms. Relaxation dispersion profiles were generated by plotting R_2 vs. $1/\tau_{\text{cp}}$ and exchange parameters were obtained from fits of these data carried out with in-house scripts. Uncertainties were obtained from replicate spectra.

NMR spin-relaxation rates were fit to one of five semi-empirical forms of the spectral density function using model-free analysis.^{77,78} Fitting of motional parameters was performed in RELAX.^{79,80} The criteria for inclusion of residues in the diffusion tensor estimate relied on the

method of Tjandra and coworkers.⁶⁹ N-H bond lengths were assumed to be 1.02 Å and the ¹⁵N chemical shift anisotropy was set to -160 ppm. During the model selection process, the diffusion tensor parameters were optimized simultaneously, and model selection was repeated until the optimized tensor parameters and order parameter (S^2) did not differ from those of the previous iteration.

Mass Spectrometry

Purified samples of MIF equilibrated under oxidizing and redox-neutral conditions were incubated with a final concentration of 15 mM iodoacetamide (IAA, room temperature, 1 hour in the dark) to alkylate any reduced cysteine residues. Subsequently, cysteines that were in an oxidized state were reduced with a final concentration of 10 mM DTT (room temperature, 30 minutes). Prior to alkylation with *N*-ethylmaleimide (NEM), the sample solution was adjusted to pH 6.5 with 1% formic acid to minimize over-labeling of lysine and N-termini.⁸¹ These samples were then incubated with a final concentration of 15 mM NEM (room temperature, 1 hour in the dark). The reaction was quenched by the addition of DTT to a final concentration of 10 mM. Purified MIF samples equilibrated under reducing conditions were treated with only IAA or only NEM at 15 mM (room temperature, 1 hour in the dark) and then quenched with 10 mM DTT. Labeled MIF samples were dried using vacuum centrifugation, resuspended in 200 mM EPPS, pH 8.5, and digested overnight at 37°C with Trypsin/Lys-C protease mixture at a 50:1 protein to protease ratio. Digested samples were again dried and resuspended in a 5/1 (v/v) solution of acetonitrile and formic acid prepared in ultrapure water. The samples were subjected to C18 solid-phase extraction with columns prepared in-house. The final samples were dried and reconstituted in a solution of 5/2 (v/v) of acetonitrile and formic acid in ultrapure water to a final concentration of 12.5 ng/μL for liquid chromatography-mass spectrometry (LC-MS) analysis.

Mass spectrometric data was collected on an Orbitrap Fusion Eclipse (ThermoFisher Scientific, San Jose, CA) coupled to a FAIMSPro (ThermoFisher Scientific, San Jose, CA) and a Proxeon Easy-nLC 1200 (ThermoFisher Scientific, San Jose, CA). Peptides were separated on a 100 μ m inner diameter microcapillary column packed with $\sim$ 35 cm of Reprosil-Pur 120 C18 resin (3 μ m, AQ, ESI Source Solutions, Woburn, MA). For each analysis, 12.5 ng of MIF sample was loaded onto the column and separated using a 30 min linear gradient from 100% A (5% ACN, 0.125% formic acid) to 50% B (59% ACN, 0.125% formic acid). DDA analyses began with an MS1 spectrum (Orbitrap resolution 120,000) followed by MS2 scans of the 6 most abundant precursor ions. Precursors were isolated in the quadrupole with a 1.2 m/z wide window and fragmented using higher-energy collision dissociation (HCD) set at 35%. Fragments were detected in the ion trap using the rapid scan rate and a maximum injection time of 50 ms. MS1 and MS2 scans were collected using alternating FAIMS compensation voltages of 40 and -60 V and a dispersion voltage of -5000 V. PRM analyses used an inclusion list of 23 targets generated from Skyline software that included precursor mass-to-charge ratio (m/z) and precursor charge state for alkylated and unmodified MIF peptides. All targeting was unscheduled and occurred throughout the duration of the experiment. The scan sequence began with an MS1 spectrum (Orbitrap resolution 120,000) followed by targeted MS2 scans of peaks corresponding to precursors in the target list. Settings for MS2 scans were identical to DDA experiments with the exception of the maximum injection time, which was set to 25 ms. Alternating FAIMS compensation voltages of -40 and -60 V and a dispersion voltage of -5000 V were also used in the PRM experiments.

Oxidation of methionine residues located at the 2, 49, and 101 positions was detected by mass spectrometry. The details of these identifications can be found in **Table 1**. Corresponding MS2 spectra for each peptide identification are shown in **Appendix Figure 1**.

All raw files were imported into the Skyline software package (Version 20.2.0.343)⁸² without modification. Peak areas corresponding to peptide 78-LLCGLLAER-86 with +57 Da (IAA) or +125 Da (NEM) modification at the C80 position were determined using the built-in peak-boundary adjustment tool. Relative amounts of C80 oxidation were determined against the intensity of all peaks in the chromatogram for each condition. Five replicates from two sample preparations were analyzed for experimental variance.

Enzymatic Assay

4-hydroxyphenyl pyruvate (4-HPP) was prepared in 0.5 M ammonium acetate, pH 6.2, and equilibrated overnight at room temperature to generate the predominant keto isomer. A reaction mixture containing final concentrations of 0.425M boric acid (pH 6.2), 100 nM MIF, and 1 mM HPP (saturating) was measured at 306 nm in a 1.5mL quartz cuvette using a Cary 60 UV-Vis spectrophotometer, where an increase in absorbance is due to the formation of the enol-borate complex between HPP and boric acid as MIF catalyses the tautomerization of HPP to the enol form. Absorbance was measured every 10 seconds for 5 minutes, and each reaction was conducted in triplicate per MIF sample. Reduced and oxidized MIF samples were prepared with a final concentration of 1 μ M DTT or H₂O₂, respectively, and equilibrated for at least 6 hours before being added to the reaction mixture.

In Vivo Neutrophil Recruitment Assay

9-10 week old male mice (C57BL/6J) were administered a one-time intratracheal instillation of 100 μ l of normal saline alone (vehicle) or normal saline solution containing 1 μ g of WT MIF, K66A MIF, or C80A MIF. These mice were sacrificed after 6 h of vehicle only or vehicle with experimental agent administration. Bronchoalveolar lavage (BAL) was performed by

cannulating the trachea with a blunt 22-gauge needle and lavaging both lungs with 800 µl of sterile PBS solution. Bronchoalveolar lavage fluid (BALF) was harvested, and total cell counts in BALF were determined using the TC20 automated cell counter (Bio-Rad Laboratories, Inc., Hercules, CA). The differential cell counts were performed on cells cytocentrifuged onto glass slides (Fisher Scientific) stained with the Hema 3 Staining System (Fisher Healthcare, Middletown, VA) and cell differential was tabulated using light microscopy. Total protein concentration in the BAL fluid was measured using the Pierce™ BCA assay kit (Thermo Scientific, Rockford, IL) per the manufacturers microplate protocol and referenced to BSA standards.

All animal studies were approved by the Institutional Animal Care and Use Committee of Cooper University Healthcare, Camden, NJ. The wt mice of genetic background strain (C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed at the pathogen-free animal facility at Cooper University Healthcare. All experiments were done in 9-10 week old male mice. Age, strain and sex differences are known to occur in response to LPS-induced injury.⁸³⁻⁸⁶ We used similar aged adult mice (9-10 weeks old), of the same background strain (C57Bl6/J), and the same sex (male) in order to minimize variability in the biological responses to MIF and mutants that might occur due to differences in the age, strain, and sex of mice.

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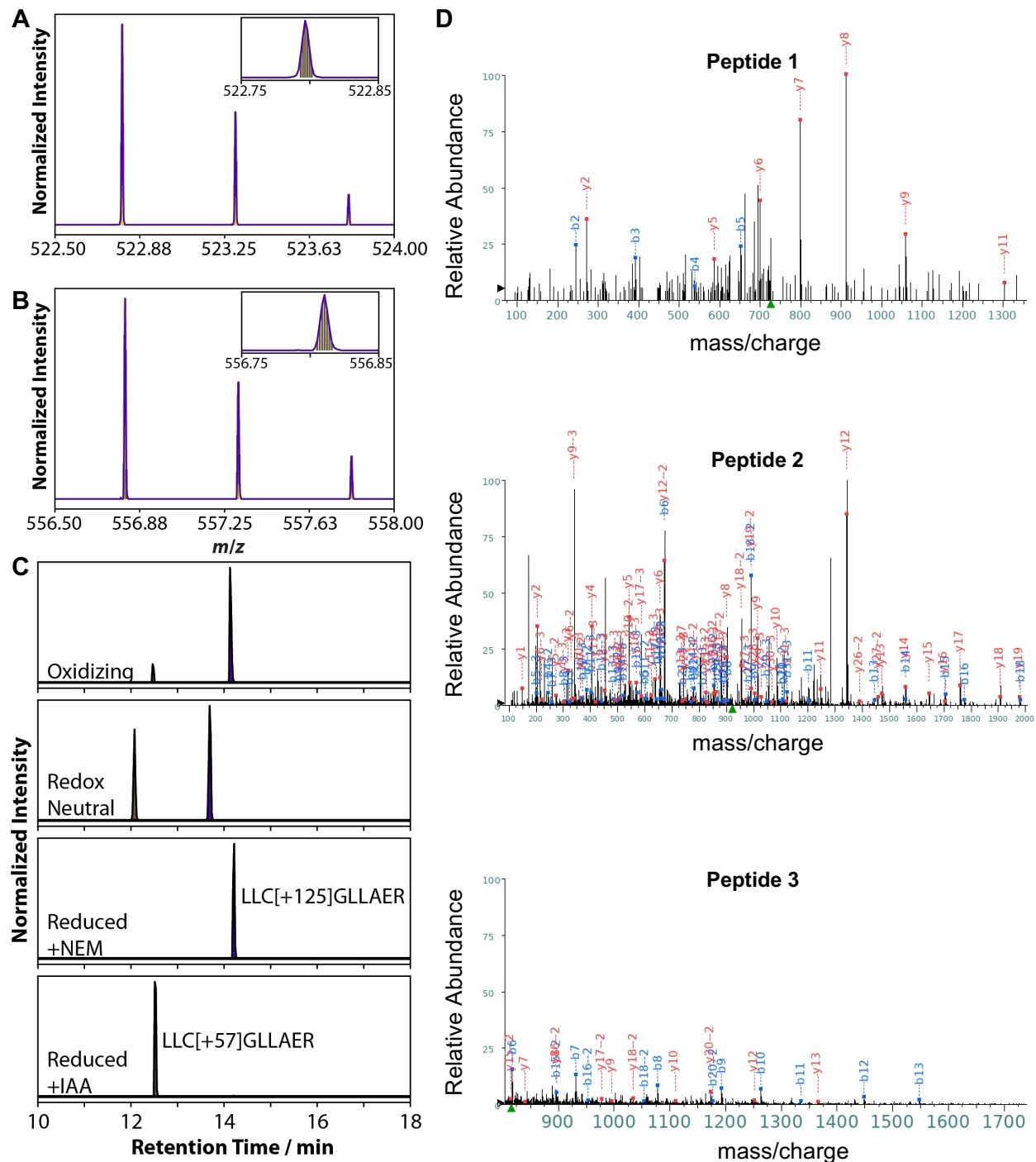
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Appendix



CHAPTER 5

Redox-dependent Plasticity of oxMIF Facilitates its Interaction with CD74 and Therapeutic Antibodies

This chapter is adapted from a published manuscript in Redox Biology (2024) titled:

Redox-dependent Plasticity of oxMIF Facilitates its Interaction with CD74 and Therapeutic Antibodies

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Author Contributions: **E.S.** expressed and purified protein, prepared oxidized protein samples, and conducted and analyzed NMR spectroscopy experiments. **S.S.** prepared oxidized protein samples, and conducted and analyzed ELISA and tautomerase data. **M.F.**, **I.M.**, **J.M.**, and **G.R.** purified recombinant protein and cultured cells for cell-based assays. **A.S.** developed experimental methodology. **M.T.** and **G.P.L.** conceptualized and supervised the project. The manuscript was written through contributions from all authors.

Abstract

MIF is a ubiquitous protein involved in proinflammatory processes, which undergoes an oxidation-driven conformational change to oxidized (ox)MIF. We demonstrate that hypochlorous acid, produced by neutrophil-released myeloperoxidase (MPO) under inflammatory conditions, effectively oxidizes MIF into the oxMIF isoform, which is specifically recognized by the anti-oxMIF therapeutic antibody, ON104. NMR investigation of MIF oxidized by the MPO system revealed increased flexibility throughout the MIF structure, including at several catalytic and allosteric sites. Mass spectrometry of MPO-oxMIF revealed methionines as the primary site of oxidation, whereas Pro1 and Tyr98/99 remained almost unmodified. ELISA, SPR and cell-based assays demonstrated that structural changes caused by MPO-driven oxidation promoted binding of oxMIF to its receptor, CD74, which does not occur with redox-neutral MIF. These data reveal the environment and modifications that facilitate interactions between MIF and its pro-inflammatory receptor, and a route for therapeutic intervention targeting the oxMIF isoform.

Introduction

Macrophage migration inhibitory factor (MIF) was the first discovered lymphokine¹⁻³ and has since been described as a proinflammatory cytokine with glucocorticoid overriding activity that is ubiquitously present in healthy and diseased states.⁴⁻⁶ MIF is a highly conserved protein in mammals, plants, protozoans, nematodes, and invertebrates,⁷ and together with its functional and structural paralog D-dopachrome tautomerase (D-DT, also called MIF-2), constitutes the MIF superfamily.⁷⁻¹⁰ The first crystal structure of MIF revealed a unique fold, not found in any other cytokine, where three 12.5 kDa subunits form a homotrimer arranged around a solvent-accessible, positively charged channel, suggesting MIF can interact with negatively charged moieties.¹¹ The promiscuous cellular interactome of MIF has prompted questions about the molecular factors that

facilitate its behaviour. Since MIF is overexpressed at sites of inflammation and injury, oxidation has been explored as a means to toggle the MIF structure for various interactions. Recent work showed the MIF structure to be increasingly flexible in the presence of oxidants.¹²

Monoclonal antibodies that recognize linear epitopes of MIF located in its solvent channel are not able to access this region under redox-neutral conditions, but were previously shown to selectively interact with MIF species oxidized by sulfhydryl-specific reagents *in vitro*.^{13,14} Chemical oxidation of MIF *in vivo* is likely, given the rise in local concentration of reactive oxygen, nitrogen, sulfur, and chlorine species at sites of inflammation.¹⁵⁻¹⁸ The therapeutic value of such "oxMIF"-specific antibodies, which now include second-generation human IgGs such as ON104, a proprietary human anti-oxMIF antibody, has been demonstrated in rodent models of arthritis, nephritis, colitis, sepsis, and contact hypersensitivity - all conditions where oxMIF levels were found to be elevated.^{14,19,20} We therefore conclude that a common determinant of ON104-detectable oxMIF species is the exposure of the linear ON104 epitope within the MIF solvent channel^{19,20} due to structural rearrangements upon its oxidative modification.

Computational predictions and *in vitro* and *in vivo* experiments suggest that CD74 is a receptor for MIF.²¹⁻²³ CD74 is a type II transmembrane receptor,²⁴⁻²⁶ which acts as a MHC class II chaperone and also participates in non-MHC II protein trafficking.²⁴⁻²⁸ Depending on the isoform, human CD74 contains a 29-46 amino acid cytoplasmic N-terminus, a 26 amino acid transmembrane region, and a 160 amino acid extracellular domain, which is N- and O-glycosylated.^{25,26} CD74 is expressed on the cell surface of antigen-presenting cells, such as B-cells or macrophages^{29,30} and under inflammatory conditions, endothelial and epithelial cells were also shown to express CD74.^{25,27} Historically, detecting the MIF-CD74 interaction has been difficult, perhaps due to the fact that only 2-5% of total CD74 is expressed on the cell surface, regardless of

MHC II expression,^{31,32} and also that CD74 is internalized from the cell surface of B-cells within 10 minutes.³³ Binding of MIF to the CD74 extracellular domain is considered an activation stimulus for intramembrane cleavage and release of the cytosolic intracellular domain of CD74.³⁴ Thus, questions about the molecular interaction between MIF and CD74 remain, including the environmental conditions under which a complex forms.

In the present study, we further examine the oxidation of MIF and its consequences for ON104 and CD74 binding. A prominent feature of the acute and chronic inflammatory response is neutrophil infiltration,³⁵ activation, and release of various mediators including proinflammatory cytokines, proteases and the heme-containing enzyme myeloperoxidase (MPO). Through the formation of reactive oxidating/chlorinating agents, MPO is one of the key neutrophil-derived mediators contributing to organ inflammation and fibrosis in many immune-mediated diseases. In the presence of hydrogen peroxide and chloride, bromide, thiocyanate, tyrosine, or nitrite, MPO catalyses the formation of hypochlorous, hypobromous, and hypothiocyanous acids, tyrosyl radical, and reactive nitrogen intermediates capable of oxidizing a variety of proteins and fatty acids.³⁶ Such oxidative modifications may alter protein activity and, as a consequence, affect cellular functions. Given its abundance in physiological fluids, chloride is believed to be the relevant halide and therefore a preferred substrate for MPO. We demonstrate that activated human neutrophils secrete MIF after stimulation *in vitro*, with both MIF and oxMIF found in the supernatants. Since activated neutrophils also secrete MPO, which catalyzes the production of hypochlorous acid (HOCl) from hydrogen peroxide (H₂O₂) and chlorine,^{15,37-40} we set out to investigate if HOCl is a crucial oxidant of MIF, producing oxMIF at sites of inflammation.⁴¹ We therefore utilized a previously described MPO system (MPO/H₂O₂/Cl⁻) to test the biochemical and structural implications of MIF exposure to MPO-derived HOCl.^{41,42} With a combined approach of

ELISA, surface plasmon resonance (SPR), mass spectrometry (MS), and nuclear magnetic resonance (NMR), we quantified the affinity of our ON104 antibody toward human recombinant MIF oxidized *in vitro*, identified chemical modifications on the MIF trimer that enable its recognition by ON104, and resolved effects of MPO-derived HOCl oxidation on the MIF structure. In addition to our efforts to characterize oxMIF and the mechanism of its transition into a conformation recognized by ON104, we investigated whether the reported interaction between MIF and CD74^{21,22,31,43,44} can be extended to oxMIF with SPR and cell-based assays.

Results

Several distinct MIF protein samples were used in this work to interrogate structural and functional implications across redox states. “MIF” denotes the redox-neutral protein that was not treated with any chemical reagents. “H₂O₂-MIF” denotes a MIF sample containing 5x molar excess of H₂O₂. “NaOCl-oxMIF” denotes a MIF sample treated with 5x molar excess of sodium hypochlorite. “MPO-oxMIF” refers to the species of interest to this work, a MIF sample treated with the MPO/H₂O₂/Cl⁻ reagents (“MPO system”). In the present study, H₂O₂-MIF represented a control for oxidation resulting from the MPO system, where MIF was incubated with H₂O₂ for the same time as the oxidation with the MPO system (20 min), and not for 6+ hours, which was the incubation period previously described to be sufficient for MIF oxidation by H₂O₂.¹²

Human neutrophils generate oxMIF upon in vitro stimulation

To interrogate the role of MPO in the conversion of MIF into oxMIF, freshly isolated human neutrophils were either stimulated with a mixture of 1 ng/mL phorbol myristate acetate (PMA) and 0.5 µg/mL ionomycin, or left unstimulated, and after 18 hours post stimulation, their supernatants were analyzed for MIF and oxMIF by ELISA. Although PMA stimulus decreases

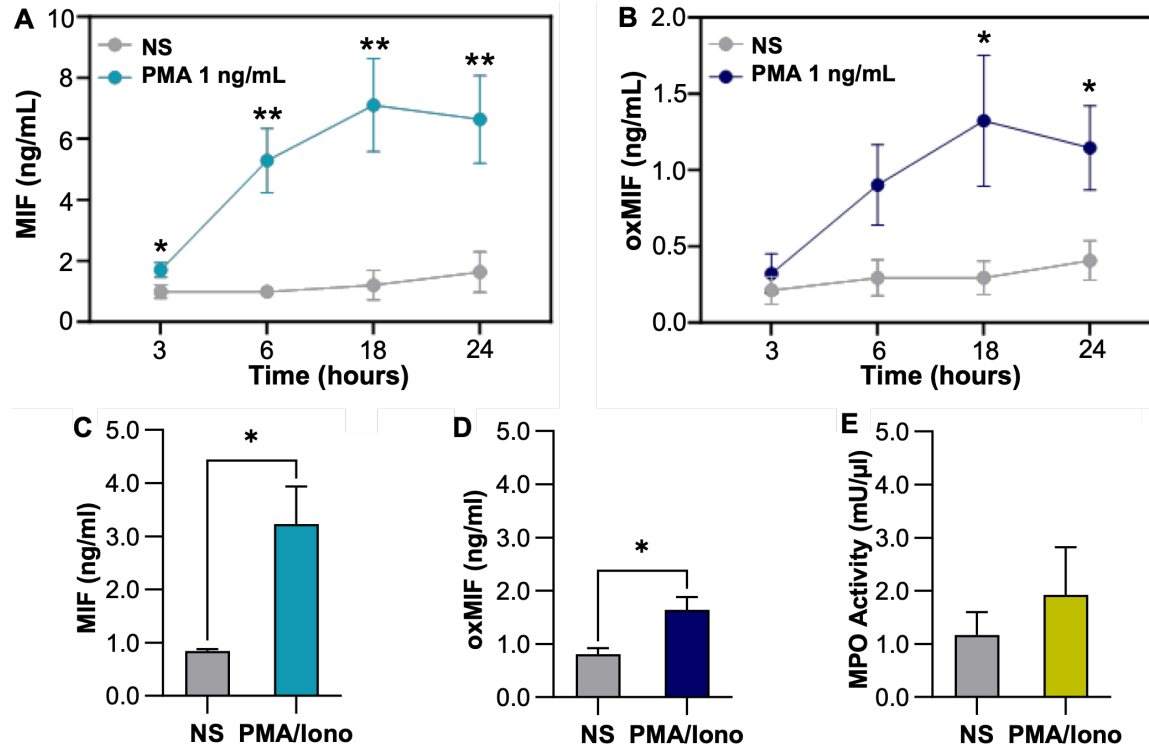


Figure 1. MIF and oxMIF are produced in isolated human neutrophils. (A, B) Time-dependent expression of MIF and generation of native oxMIF in unstimulated (NS; gray) and stimulated neutrophils (PMA; blue) was measured by anti-MIF (A) and anti-oxMIF (B) ELISA 3-, 6-, 18-, and 24-hours post-stimulation. Biological samples $n = 11$ healthy donors, technical replicates $n = 1$, and $*p < 0.05$; $**p < 0.01$. (C, D) Production of MIF and oxMIF by isolated human neutrophils from 11 healthy donors 18 hours post-stimulation with PMA/ionomycin and compared to unstimulated neutrophils. Data are expressed as mean $\pm$ SEM. Biological samples $n = 11$, technical replicates $n = 1$, and $*p < 0.05$. (E) Neutrophils from 7 healthy donors, used in (A-D), were also analyzed for MPO activity based on colorimetric absorbance at 412 nm (Ellman's assay). Unstimulated neutrophils and those stimulated with PMA/ionomycin are compared. Biological samples $n = 7$, technical replicates $n = 1$ per biological sample. Data are reported as mean $\pm$ SD.

neutrophil life span,⁴⁵ our chosen concentration of PMA has been shown to be the minimal required stimulus for initiation of intracellular cascade leading to netosis.⁴⁶ We found the viability of all stimulated neutrophils (i.e. 1 ng/mL of PMA, 1 ng/mL PMA with 0.5 μ g/mL ionomycine, or 1 μ g/mL of LPS) to be at least 70% at 6 hours post-stimulation based on viability checks and comparisons to unstimulated neutrophils.

Time-dependent expression of MIF and generation of native oxMIF in neutrophils stimulated with 1 ng/mL of PMA was monitored in cell culture supernatants collected 3, 6, 18, and

24-hours post-stimulation, and compared to the supernatants collected from unstimulated neutrophils in parallel cell cultures (**Figure 1A**). MIF levels in stimulated neutrophils significantly increased after 3 hours post-stimulation and the highest concentration of MIF was detected at 18 hours post-stimulation. The trend in MIF concentration was reflected with a concurrent elevation of oxMIF concentration (**Figure 1B**), peaking at 18 hours post stimulation. No such trends were observed for MIF and oxMIF levels in unstimulated neutrophils. Based on these findings, we presented the expression of MIF and generation of oxMIF in stimulated neutrophils at the most representative time point, 18 hours post-stimulation with 1 ng/mL of PMA (**Figure 1C-D**).

Endogenous MIF was found in supernatants of unstimulated neutrophils at a basal level of 0.85 ± 0.03 ng/mL, which overlaps with the lowest level of detection. Levels of MIF significantly increased upon stimulation, reaching 3.2 ± 0.7 ng/mL (**Figure 1C**). Importantly, PMA/ionomycin mixture triggered oxMIF generation of 1.6 ± 0.2 ng/mL, while unstimulated neutrophils generated much lower amounts of oxMIF (0.8 ± 0.1 ng/mL, **Figure 1D**). In the same supernatants, MPO activity was checked by a colorimetric assay, which showed slightly, yet non-significantly increased enzymatic activity upon stimulation (**Figure 1E**), suggesting that increased levels of MPO-derived HOCl is responsible for the elevated levels of oxMIF upon *in vitro* stimulation. The principle of the MPO activity assay, known as “Ellman’s assay,” is based on formation of taurine-chloramine and its consequent reaction with Ellman’s reagent, DTNB.^{47,48} Along with HOCl, large quantities of the sulfur-containing β -amino acid taurine, the most abundant intracellular amino acid that is not incorporated into human proteins, are released from stimulated neutrophils.⁴⁹ The formation of taurine-chloramine produces an observable color that is quenched by its subsequent reaction with DTNB and monitored as a loss of absorbance measured at 412 nm, reciprocal to MPO activity. In our samples, the generally low levels of MPO activity could be explained by the

presence of 5% FBS in the supernatants, which is known to suppress MPO function. Serum albumins contained in the FBS have been shown to be powerful scavengers of multiple RNS and ROS species, such as HOCl.⁵⁰ In their presence, the detected MPO activity in the supernatants of stimulated neutrophils could not increase significantly.

ON104 specifically interacts with oxMIF, upon MIF oxidation with hypochlorous acid

MIF and various oxMIF species were tested for ON104 interaction by ELISA and SPR (**Figure 2**). The ELISA clearly showed that ON104 bound to MPO-oxMIF and NaOCl-oxMIF, but not H₂O₂-MIF or untreated MIF, which served as a negative control (**Figure 2A**). MIF oxidants were added at 5x molar excess of the MIF trimer concentration, a ratio selected based on ELISAs with NaOCl-oxMIF and MPO-oxMIF that showed no substantial increase in binding beyond this concentration (**Figure 2D-E**). Further, dynamic light scattering (DLS) studies of oxMIF revealed significant aggregation at concentrations exceeding 5x molar excess of oxidant (**Figure 2F-G**). The H₂O₂ concentration used for oxidation was identical to that used for MPO-driven oxidation, revealing that modifications facilitating ON104 binding to oxMIF originated from hypochlorous acid introduced as a reagent (NaOCl) or produced by MPO. Though structural and dynamic changes were previously reported for H₂O₂-treated MIF, at least 6 hours of incubation were needed to observe such biophysical changes by NMR.¹² The experimental conditions tested here show that the oxidation of MIF by HOCl is a much faster process, as 20 min of incubation were sufficient for MPO and NaOCl to promote the oxMIF conformation required for ON104 binding. The EC₅₀ values obtained for MPO-oxMIF and NaOCl-oxMIF binding to ON104 were highly comparable, at 8.1 ± 0.8 nM and 8.8 ± 1.0 nM, respectively (**Figure 2A**). Nonspecific interactions of ON104 were absent from controls consisting solely of active MPO (i.e., MPO in the presence of H₂O₂ and Cl⁻) or buffer containing the corresponding concentration of NaOCl and H₂O₂. An additional

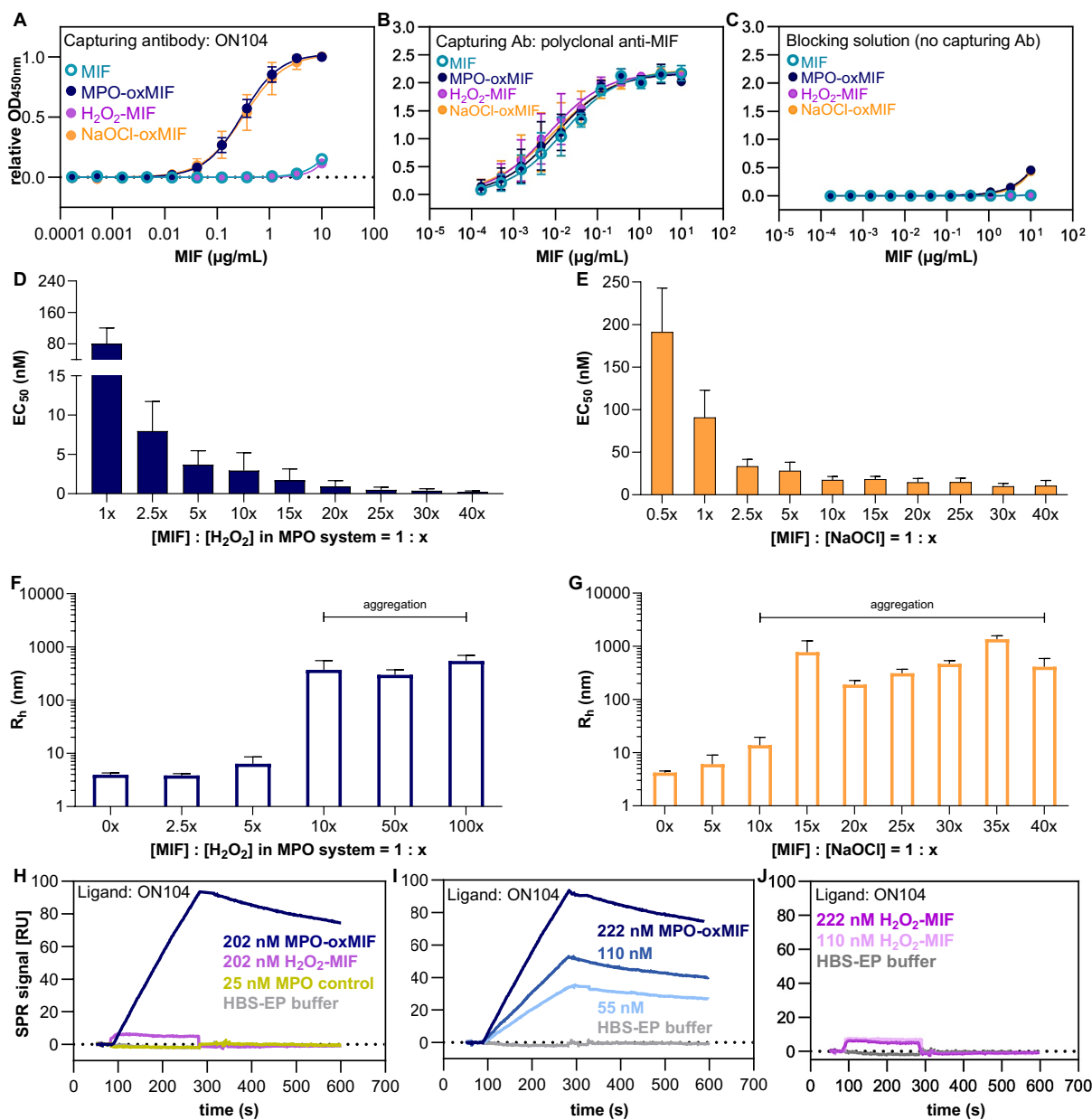


Figure 2. Hypochlorous acid-modified oxMIF specifically interacts with the antibody ON104. (A) MIF, MPO-oxMIF, H₂O₂-MIF, and NaOCl-oxMIF were tested for ON104 binding by ELISA, which was only observed for MPO-oxMIF (EC₅₀ of 8.1 ± 0.8 nM) and NaOCl-oxMIF (EC₅₀ of 8.8 ± 1.0 nM). Data are normalized by highest MPO-oxMIF binding signal and reported as mean ± SEM of n = 3 independent experiments performed in technical duplicates. (B, C) Control ELISAs to confirm the specificity of ON104 toward MIF oxidized by HOCl, using a polyclonal MIF-antibody (B) and blocking solution substituted for an antibody (C). Polyclonal anti-MIF antibody showed robust binding to all MIF-containing samples with EC₅₀ values of 0.18 ± 0.05 nM (redox-neutral MIF), 0.15 ± 0.04 nM (MPO-oxMIF), 0.15 ± 0.04 nM (H₂O₂-MIF), and 0.19 ± 0.06 nM (NaOCl-oxMIF). No binding was observed for blocking solution-only ELISA. Data are shown as mean ± SEM of n = 3 independent experiments performed in technical duplicates. (D, E) Dose-dependent oxidation effects of HOCl on the MIF trimer. EC₅₀ values obtained from ELISA, where ON104 was used as a capturing antibody, and its binding of MIF oxidized with (D) HOCl produced by MPO with 1x-40x molar excess of H₂O₂, and (E) NaOCl-oxMIF, generated by treatment with 0.5x-40x

NaOCl reagent. EC_{50} values are reported as mean $\pm$ SEM measured as technical duplicates in $n = 3$ independent biological experiments. **(F, G)** DLS measurement of MIF and oxMIF samples to assess aggregation of oxMIF via hydrodynamic radius (R_h (nm)). MPO-oxMIF generated with 2.5x-100x molar excess of H_2O_2 is shown in **(F)**, NaOCl-oxMIF generated with 5x-40x NaOCl reagent is shown in **(G)**, and MIF is represented in the 0x data points (no oxidant) in both plots. For each condition tested, biological samples $n = 3$, technical replicates $n \geq 2$. R_h values are reported as mean $\pm$ SEM. **(H-J)** SPR experiments confirm ON104 binding to MPO-oxMIF, with ON104 used as ligand. **(H)** Pilot SPR experiments showing MPO-oxMIF binding at 202 nM to immobilized ON104. The absence of binding is apparent for H_2O_2 -MIF at the same concentration, MPO alone, and buffer controls. **(I)** A K_D of 26.6 ± 1.3 nM from a 1:1 Langmuir model was determined from concentration-dependent analyte binding of MPO-oxMIF to immobilized ON104. K_D reported as mean $\pm$ SD from 3 independent experiments. **(J)** Qualitative SPR experiment was performed at 222 and 110 nM H_2O_2 -MIF to confirm specificity of ON104 toward MPO-oxMIF.

ELISA was performed using a polyclonal anti-MIF antibody, which as expected, bound to all redox-neutral and oxidized MIF samples with low nanomolar EC_{50} values (**Figure 2B**). These data show the specificity of ON104 for HOCl-modified MIF and suggest important therapeutic applications for antibodies like ON104 that can selectively neutralize oxMIF generated by inflammatory conditions. As we have previously observed that MIF can adopt an oxMIF conformation via hydrophobic/electrostatic interactions with ELISA plate surfaces,¹⁴ we performed a second control ELISA, where antibody was omitted and substituted for blocking solution. No promiscuous interactions were observed (**Figure 2C**).

MPO-oxMIF was further interrogated by SPR experiments, with ON104 as a ligand, and MPO-oxMIF, along with controls, as an analyte (**Figure 2H-J**), to confirm the specific interaction of ON104 with MPO-oxMIF as observed from ELISA (**Figure 2H**). The specificity was confirmed by the absence of signals from H_2O_2 -MIF and the MPO and buffer control samples (**Figure 2H, J**). Next, the K_D of ON104 with MPO-oxMIF was determined by applying a 1:1 Langmuir model to the sensorgrams of three independent experiments with different analyte concentrations (**Figure 2I**). The kinetic analysis determined a K_D value of 26.6 ± 1.3 nM, which is in excellent agreement with the EC_{50} from ELISA (**Figure 2A**). Given that the conversion rate of MIF into oxMIF upon treatment with HOCl has not yet been quantified, we estimate the K_D value of this interaction to be even lower.

Met2 and Met101 are the major sites of oxidation in MPO treated oxMIF

MIF, MPO-oxMIF, and H₂O₂-MIF were analyzed by mass spectrometry for intact masses (**Figure 3**). A small portion of N-terminal Met-containing MIF was detected in all samples, consistent with prior observations from MS measurements. In addition to the signal at 12345 Da corresponding to the theoretical mass of the MIF monomer, three additional signals with a mass increment of +16 Da each were observed in the MPO-oxMIF sample, indicating the addition of one, two, or three oxygen atoms of 15.99 Da to the MIF monomeric subunit (**Figure 3**).

To further elucidate the sites of oxidation and other possible modifications, MPO-oxMIF (**Figures 4-5 and Table 1**) and H₂O₂-MIF (**Figure 5A and Table 2**) were digested for peptide mapping, and compared to untreated MIF (**Figure 5A and Table 3**). Complete coverage of the

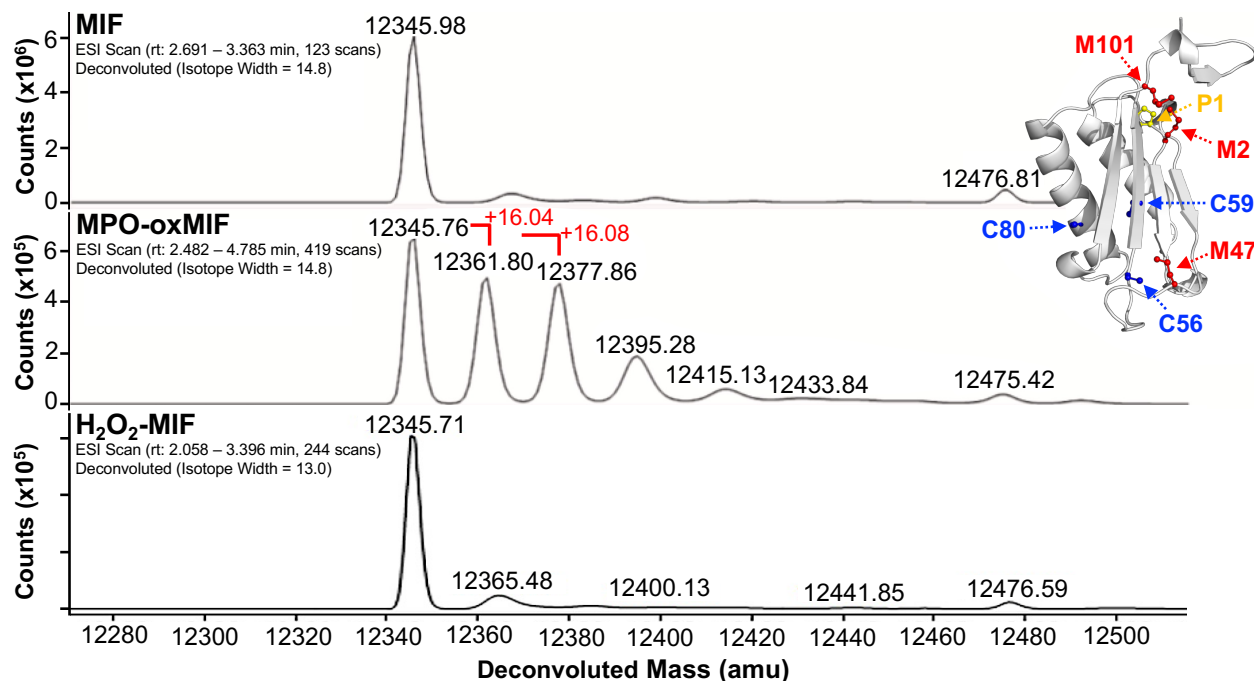


Figure 3. Intact mass analysis of MIF, MPO-oxMIF, and H₂O₂-MIF. Representative spectra are shown for untreated MIF (top), MPO-MIF (middle), and H₂O₂-MIF (bottom). A peak at 12345.98 Da corresponds to the theoretical mass of a MIF monomeric subunit, lacking the N-terminal methionine. Treatment of MIF with the MPO-system produced additional peaks with mass increments of +16 Da, due to the addition of one, two or three oxygen atoms. Biological samples n = 4, technical replicates n = 1 per biological sample (MIF); Biological samples n = 5, technical replicates n = 1 per biological sample (MPO-oxMIF); Biological samples n = 3, technical replicates n = 1 per biological sample (H₂O₂-MIF). Cys and Met residues, and the catalytic Pro1, are highlighted on the MIF monomer structure.

protein sequence, including all Cys-containing peptides, was obtained in all samples. Consistent with prior reports,¹² oxidation was more prevalent at the labile methionine residues. Aside from the cleaved initiating Met1, MIF contains three additional Met residues; Met2, Met47, and Met101. Indeed, the most pronounced peptide-level effect of MPO-driven oxidation of MIF was methionine

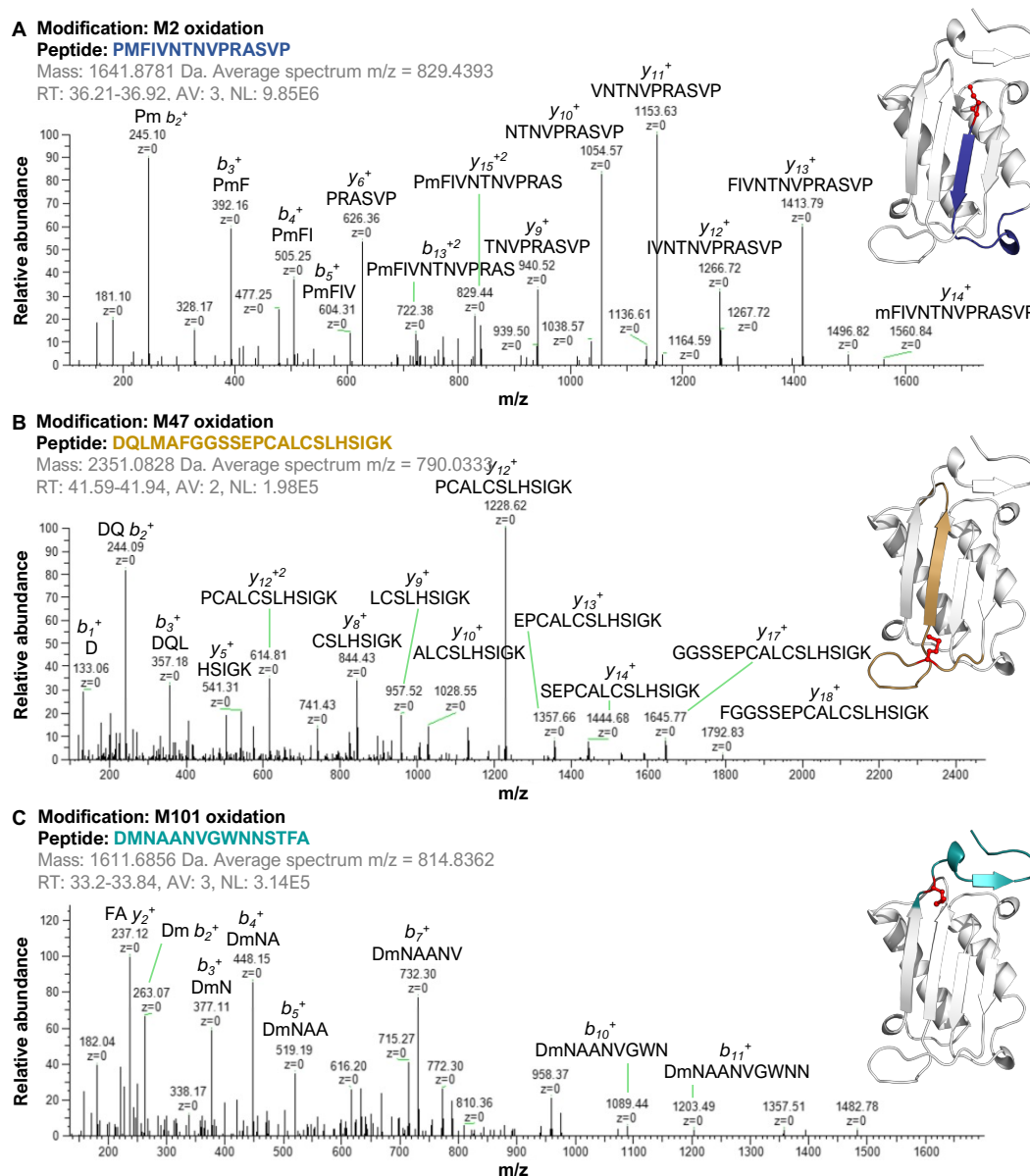


Figure 4. Peptide mass analysis of methionine oxidation in MPO-oxMIF. Peptide mapping MS/MS of MPO-oxMIF, where average spectrum m/z values correspond to peptides with oxidized (A) Met2, (B) Met47, and (C) Met101. Biological samples $n = 5$, technical replicates $n = 1$. One representative spectrum is shown. The peptide mapped in each spectrum is highlighted on the MIF monomer, with the methionine side chain of interest shown in red.

oxidation, where the thioether sulfur atoms can be oxidized once to methionine sulfoxide (mass shift +15.99 Da) or twice to methionine sulfone (+31.98 Da). Interestingly, analyses of Met-containing peptides revealed significant oxidative modifications to Met101 ($66.1 \pm 2.4\%$ sulfoxide and $0.4 \pm 0.3\%$ sulfone) and Met2 ($68.2 \pm 10.6\%$ sulfoxide and $0.2 \pm 0.1\%$ sulfone), and modifications to a lesser extent on Met47 ($10.2 \pm 3.0\%$ sulfoxide and $0.3 \pm 0.5\%$ sulfone) (**Figure 4** and **Table 1**). The same analysis was performed on H₂O₂-MIF, where only a negligible amount of Met oxidation was observed (**Table 2**). Surprisingly, Cys80, previously reported as a redox

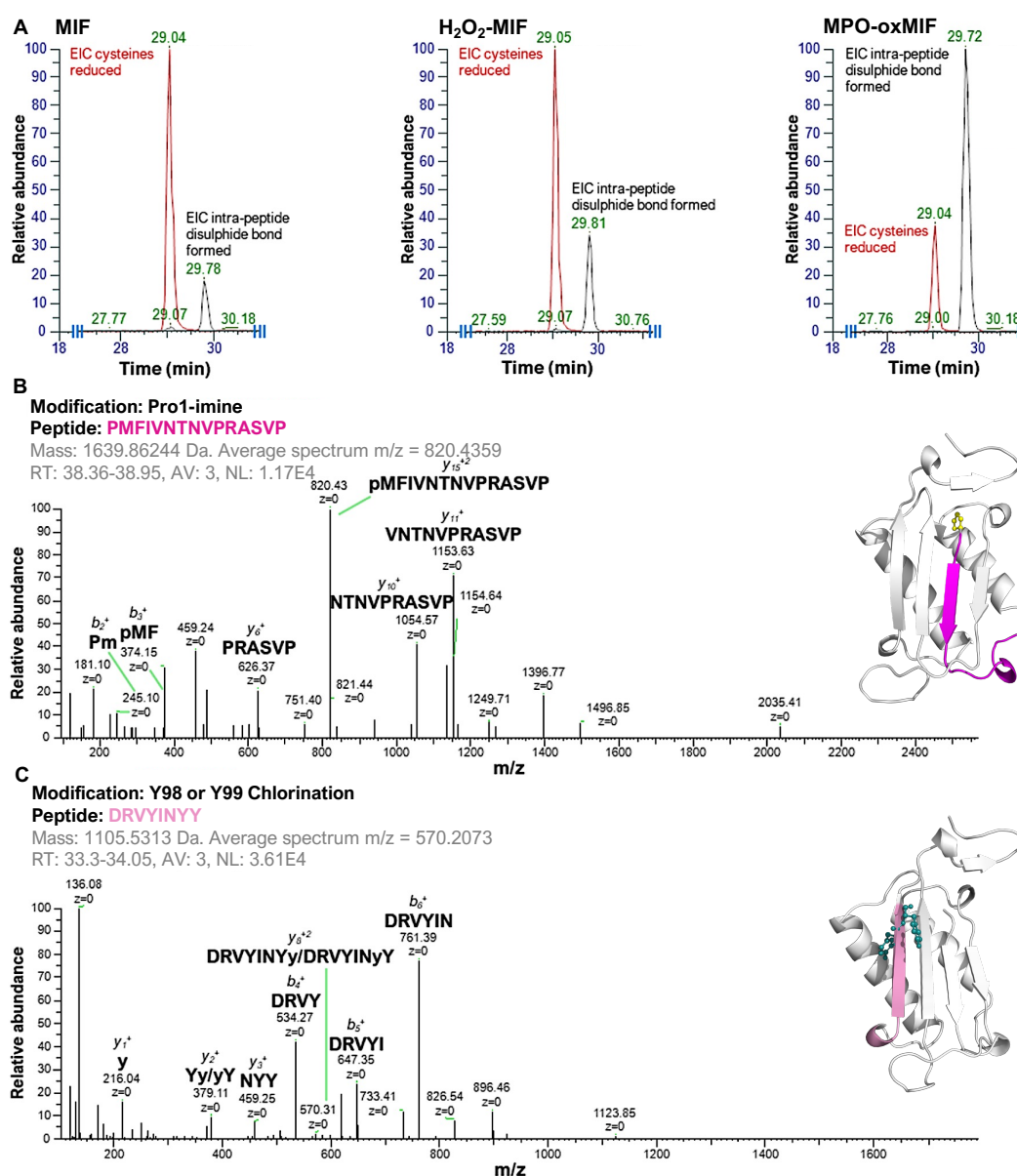


Figure 5. Peptide mass analysis of cysteine, proline, and tyrosine modifications under various redox conditions. (A) Extracted ion chromatograms (EIC) of MIF (left), H₂O₂-MIF (middle) and MPO-oxMIF (right), showing the relative abundance of reduced cysteines and cysteines forming an intra-peptide disulfide bond. For MIF: biological samples n = 3, technical replicates n = 1 per biological sample; H₂O₂-MIF: biological samples n = 4, technical replicates n = 1 per biological sample; MPO-oxMIF: biological samples n = 5, technical replicates n = 1 per biological sample; 1 representative spectrum per condition is shown. (B-C) Peptide mapping MS of MPO-oxMIF, where average spectrum m/z values correspond to the individual peptides where Pro1 was modified to proline-imine (B), and Tyr98 or Tyr99 were chlorinated as chlorotyrosine (C). Biological samples n = 5, technical replicates = 1 per biological sample; one representative spectrum per modification is shown.

switch¹³ and shown to be modified in MIF samples treated with H₂O₂ under longer incubation periods,¹² was found to be unmodified and reduced for all samples. The other Cys residues were partially modified, as Cys56 and Cys59 were found to partially form an intra-peptide disulfide bridge, however the relative abundance of Cys56-Cys59 disulfide bond was higher in the MPO-oxMIF sample (Figure 5A).

Table 1. Peptide mapping MS analysis of modifications in MPO-oxMIF. Modifications identified in MPO-oxMIF included oxidation, chlorination, and proline-imine formation. The average abundance of these modifications is compared to those identified in MIF (Table 3) to highlight changes (Δ modification) under different redox conditions. Modifications of Cys residues are shown in Figure 5A. Biological samples n = 5, technical replicates = 1 per biological sample. Sample 3 is an average of 3 biological samples.

MPO-oxMIF MS/MS Analysis							
Chlorination							
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Sample 3	Average $\pm$ SD	Δ modification vs. MIF
92-99	DRVYINYY	Y98/Y99	3.2%	6.7%	3.9%	4.6 $\pm$ 1.9%	+ 4.6%
Oxidation							
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Sample 3	Average $\pm$ SD	Δ modification vs. MIF
1-15	PMFIVNTNVPRASVP	M2 (sulfoxide)	60.9%	80.3%	63.2%	68.2 $\pm$ 10.6%	+ 56.4%
		M2 (sulfone)	-	0.3%	0.3%	0.2 $\pm$ 0.1%	+ 0.2%
44-66	DQLMAFGGSSEPCALCSLHSIGK	M47 (sulfoxide)	9.8%	13.4%	7.4%	10.2 $\pm$ 3.0%	+ 7.6%
		M47 (sulfone)	-	0.2%	0.9%	0.3 $\pm$ 0.5%	- 0.8%
100-114	DMNAANVGWNNSTFA	M101 (sulfoxide)	68.0%	66.9%	63.3%	66.1 $\pm$ 2.4%	+ 62.6%
		M101 (sulfone)	0.5%	-	0.7%	0.4 $\pm$ 0.3%	- 1.5%
Proline-Imine Formation							
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Sample 3	Average $\pm$ SD	Δ modification vs. MIF
1-15	PMFIVNTNVPRASVP	P1	0.04%	-	0.04%	0.03 $\pm$ 0.02%	-

Table 2. Peptide mapping MS analysis of modifications in H₂O₂-MIF. Oxidation was the only modification identified in H₂O₂-MIF. The average abundance of these modifications is compared to those identified in MIF (**Table 3**) to highlight changes (Δ modification) under different redox conditions. Modification of Cys residues are shown in **Figure 5A**. Biological samples n = 4, technical replicates = 1 per biological sample. Sample 2 is an average of 3 biological samples.

H ₂ O ₂ -MIF MS/MS Analysis						
Chlorination						
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Average ± SD	Δ modification vs. MIF
92-99	DRVYINYY	Y98/Y99	-	-	-	-
Oxidation						
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Average ± SD	Δ modification vs. MIF
1-15	PMFIVNTNVPRASVP	M2 (sulfoxide)	5.6%	13.9%	9.7 ± 5.8%	- 2.1%
		M2 (sulfone)	-	-	-	-
44-66	DQLMAFGGSSEPCALCSLHSIGK	M47 (sulfoxide)	1.1%	3.2%	2.1 ± 1.5%	- 0.5%
		M47 (sulfone)	-	0.8%	0.4 ± 0.6%	- 0.7%
100-114	DMNAANVGWNNSTFA	M101 (sulfoxide)	4.7%	4.7%	4.7%	+ 1.2%
		M101 (sulfone)	0.1%	2.1%	1.1 ± 1.4%	- 0.8%
Proline-Imine Formation						
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Average ± SD	Δ modification vs. MIF
1-15	PMFIVNTNVPRASVP	P1	-	-	-	-

Table 3. Peptide mapping MS analysis of modifications in MIF. Modifications identified in untreated MIF included oxidation and chlorination. The average abundance of these modifications is compared to MPO-oxMIF and H₂O₂-MIF in **Tables 1-2**. Modification of Cys residues is shown in **Figure 5A**. Biological samples n = 3, technical replicates n = 1 per biological sample.

MIF MS/MS Analysis						
Chlorination						
<i>Peptide</i>	<i>Sequence</i>	<i>Modified residues</i>	<i>Sample 1</i>	<i>Sample 2</i>	<i>Sample 3</i>	<i>Average $\pm$ SD</i>
92-99	DRVYINYY	Y98/Y99	-	-	-	-
Oxidation						
<i>Peptide</i>	<i>Sequence</i>	<i>Modified residues</i>	<i>Sample 1</i>	<i>Sample 2</i>	<i>Sample 3</i>	<i>Average $\pm$ SD</i>
1-15	PMFIVNTNVPRASVP	M2 (sulfoxide)	13.7%	10.8%	10.8%	11.8 $\pm$ 1.7%
		M2 (sulfone)	-	-	-	-
44-66	DQLMAFGGSSEPCALCSLHSIGK	M47 (sulfoxide)	3.4%	2.4%	2.1%	2.6 $\pm$ 0.7%
		M47 (sulfone)	1.3%	1.1%	1.0%	1.1 $\pm$ 0.1%
100-114	DMNAANVGWNNSTFA	M101 (sulfoxide)	5.1%	2.7%	2.7%	3.5 $\pm$ 1.4%
		M101 (sulfone)	1.9%	1.8%	2.2%	1.9 $\pm$ 0.2%

Proline-Imine Formation						
Peptide	Sequence	Modified residues	Sample 1	Sample 2	Sample 3	Average $\pm$ SD
1-15	PMFIVNTNVPRASVP	P1	0.04%	0.02%	0.03%	0.03 $\pm$ 0.01%

Table 4. MS/MS analysis of MPO-oxMIF generated with a broad concentration range of MPO-derived HOCl. For this purpose, 1x, 2.5x, 5x, 10x, 25x molar excess of H₂O₂ was included into the MPO system (relative to MIF trimer). Sensitivity of MIF to rising concentrations of MPO-generated HOCl is revealed by rising levels of oxidation and chlorination detected. Formation of Pro1 proline- imine was only observed at 25x oxidant concentration (*this percentage may be overestimated due to the low signal of the peptide). Data reported as mean $\pm$ SD of n = 3 independent experiments.

MPO-oxMIF MS/MS Analysis with Increasing Concentrations of MPO-generated HOCl							
Chlorination							
Peptide	Sequence	Modified residues	Samples oxidized with x molar excess of MPO-generated HOCl				
			1x	2.5x	5x	10x	25x
92-99	DRVYINYY	Y98/Y99	2.60 ± 2.51%	4.12 ± 2.99%	4.82 ± 1.66%	5.99 ± 2.04%	7.38 ± 1.37%
Oxidation							
Peptide	Sequence	Modified residues	Samples oxidized with x molar excess of MPO-generated HOCl				
			1x	2.5x	5x	10x	25x
1-15	PMFIVNTNVPRASVP	M2 (sulfoxide)	38.70 ± 7.79%	62.61 ± 20.85%	81.51 ± 1.13%	91.26 ± 3.00%	94.62 ± 3.31%
		M2 (sulfone)	0.13 ± 0.06%	0.19 ± 0.05%	0.21 ± 0.07%	0.96 ± 0.59%	4.73 ± 2.49%
44-66	DQLMAFGGSSEPCALCSLHSIGK	M47 (sulfoxide)	15.01 ± 2.50%	14.53 ± 6.63%	17.73 ± 4.25%	28.17 ± 10.01%	9.37 ± 16.24%
		M47 (sulfone)	0.59 ± 0.40%	0.71 ± 0.57%	0.58 ± 0.42%	0.80 ± 0.01%	6.18 ± 10.71%
100-114	DMNAANVGWNNSTFA	M101 (sulfoxide)	34.69 ± 4.12%	53.01 ± 7.91%	73.84 ± 6.02%	88.76 ± 9.58%	85.50 ± 12.43%
		M101 (sulfone)	0.18 ± 0.16%	0.31 ± 0.27%	0.60 ± 0.50%	3.23 ± 2.77%	13.81 ± 11.87%
Proline-Imine Formation							
Peptide	Sequence	Modified residues	Samples oxidized with x molar excess of MPO-generated HOCl				
			1x	2.5x	5x	10x	25x
1-15	PMFIVNTNVPRASVP	P1	0.07 ± 0.10%	0.04 ± 0.04%	0.41 ± 0.47%	0.71 ± 0.66%	8.10 ± 14.03%

As a next step, we sought to detect the formation of a proline-imine (mass reduction of - 2.017 Da) at Pro1, which has been previously shown for MPO-oxMIF.⁴¹ To our surprise, after treatment of MIF with the MPO system, hardly any Pro1 was converted into proline-imine, and other proline residues remained unmodified (**Figure 5B** and **Table 1**). We also searched for chlorination events (+33.96 Da), which were not observed on any histidines, but 4.6 ± 1.9 % of Tyr98 or Tyr99 (both contained within the same peptide) formed chlorotyrosine (**Figure 5C** and **Table 1**). No chlorination on any residue, and hardly any Pro1 proline-imine formation was

observed for H₂O₂-MIF or MIF (**Tables 2-3**).

We also assessed the dose-dependence of HOCl generated by MPO system containing 1x, 2.5x, 5x, 10x, and 25x molar excess of H₂O₂ (versus the MIF trimer). Oxidation levels of Met2, Met47, and Met101, as well as Tyr98/Tyr99 chlorination levels, rose with increasing amounts of MPO-generated HOCl (**Table 4**). In all samples, Cys80 was found to be reduced, while the Cys56 and Cys59 peptide again displayed partial oxidation, increasing in trend with the increasing concentrations of HOCl. Only 25x molar excess of H₂O₂ contained in the MPO system produced appreciable Pro1-imine ($\sim 8.10 \pm 14.03$ %), though the low signal of this peptide and the high oxidation rate of Met2 may overestimate its prevalence. Despite these findings, we highlighted results with 5x molar excess of H₂O₂ within the MPO system for consistency with ELISAs and intact mass analysis, as well as the fact that proteolysis of MIF in peptide level MS likely precludes aggregation issues at high oxidant concentrations that cloud the interpretation of the prior biochemistry. Collectively, the peptide mapping of MIF, H₂O₂-MIF, and MPO-oxMIF corroborated the results of intact mass analysis, where the observed mass additions in MPO-oxMIF reflected methionine oxidation detected on the peptide level. All other modifications were relatively minor and will be the subject of future investigations.

NMR analysis of MPO-oxMIF reveals its highest conformational flexibility in the areas of purported CD74 interaction

Solution NMR was then employed to characterize structural and/or dynamic changes to MIF upon oxidation by MPO-derived HOCl (**Figure 6**). The ¹H¹⁵N HSQC spectral overlay of MIF and MPO-oxMIF revealed only minor chemical shift perturbations, indicating that the average global structure of MPO-oxMIF is very similar to its untreated counterpart on the timescale of the experiment (**Figure 6A-C**). Notably, MPO-oxMIF exhibited significant decreases in resonance

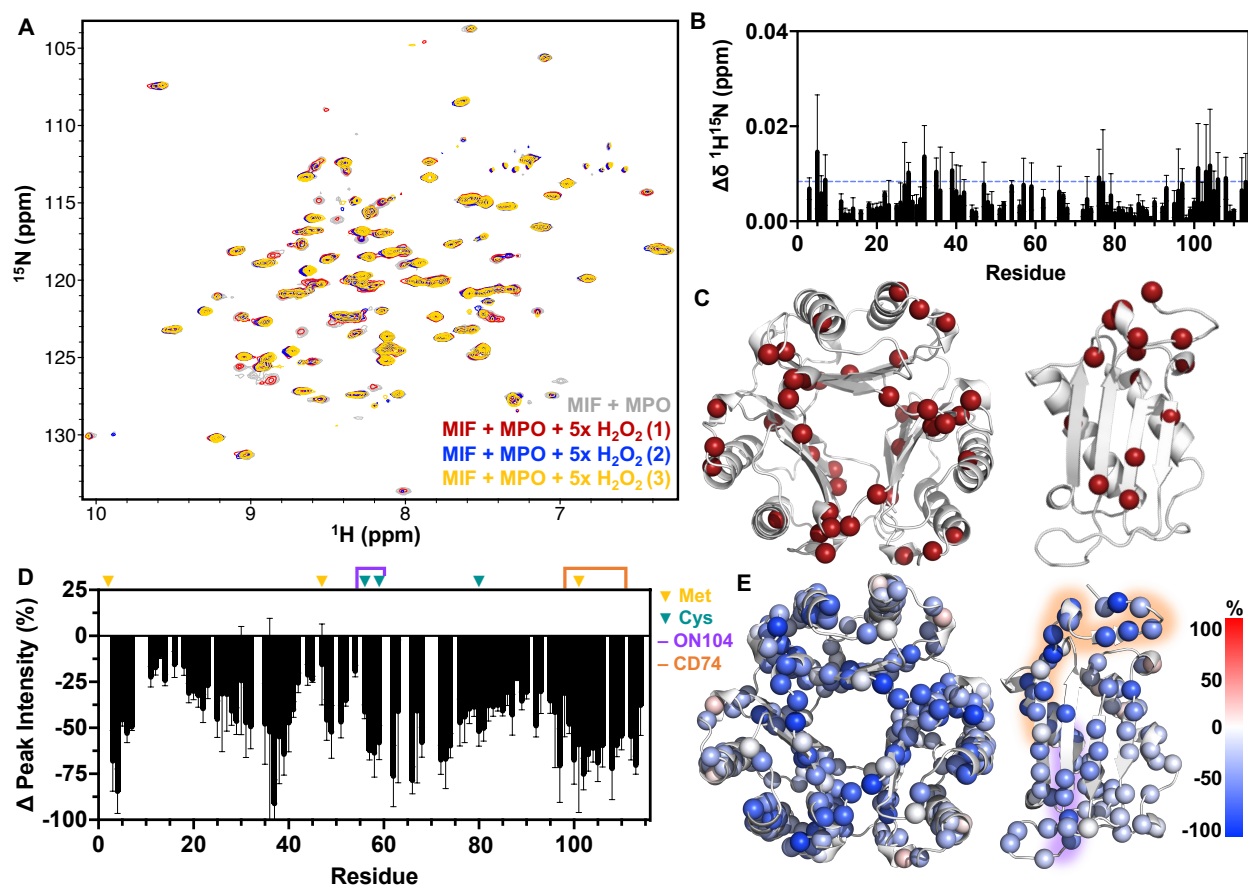


Figure 6. Structural and dynamic effects of MPO-generated HOCl on MIF by NMR. (A) ^1H - ^{15}N HSQC spectral overlay of MIF (grey) with $n=3$ biological samples of MPO-oxMIF (red, blue, and gold). (B) ^1H - ^{15}N combined chemical shift perturbations ($\Delta\delta$) averaged ($\pm$ SD) over $n=3$ biological replicates for MPO-oxMIF, calculated relative to MIF. The blue dashed line denotes 1.5σ above the 10% trimmed mean of all data sets. (C) Significant ^1H - ^{15}N chemical shift perturbations ($+1.5\sigma$) are mapped onto the MIF structure in red spheres. (D) Changes in NMR ^1H - ^{15}N HSQC resonance intensity (Δ peak intensity) for MPO-oxMIF, calculated relative to untreated MIF. Met and Cys residues are denoted by yellow and teal arrows, respectively. The ON104 binding epitope and purported CD74 binding region are highlighted by purple and orange brackets, respectively. Error bars were determined from $n=3$ biological samples, $n=1$ technical replicates per sample. (E) The per-residue Δ peak intensity is mapped onto the MIF structure, with blue and red spheres denoting decreases and increases in resonance intensity, respectively. The color gradient represents the magnitude of Δ peak intensity. The ON104 binding epitope and predicted CD74 binding region are highlighted in purple and orange, respectively.

intensity due to dynamically-driven line broadening, clearly indicating that the MIF structure becomes significantly more flexible when oxidized by the MPO system (Figure 6D). When changes in NMR resonance intensity (Δ peak intensity) for MPO-oxMIF are mapped onto the MIF structure, the residues with the largest enhancement of flexibility (i.e., lowest Δ peak intensities) surround the opening of the central solvent channel (Figure 6E). Therefore, the conformational

plasticity in this region may allow the antibody to gain access to the solvent channel and the central β -sheet, which contains the ON104 epitope.¹⁹ Further, this region is also predicted to encompass the CD74 interaction site, suggesting that oxidation of MIF by HOCl may alter binding to its receptor *via* increased flexibility. Oxidation of MIF could therefore toggle its molecular interactions as a mechanism to modulate function under specific redox conditions.

oxMIF selectively binds to the extracellular domain of CD74

We performed SPR experiments with MPO-oxMIF, H₂O₂-MIF, and MIF as the analytes and the recombinant extracellular domain (ECD) of human CD74 (aa 73-232), attached to the sensor chip as the ligand (**Figure 7**). We have shown that under conditions described here, H₂O₂-MIF behaves the same as MIF, thus H₂O₂-MIF was used for comparison to MPO-oxMIF. MPO in the absence of MIF was also used as a control and a minor background signal was observed when tested for CD74 ECD binding (**Figure 7D**). Surprisingly, MPO-oxMIF, but not H₂O₂-MIF and MIF, bound to CD74 ECD (**Figure 7A, C**). Though the concentration of MPO used to oxidize MIF was decreased by half (25 nM) from prior SPR studies to limit the minor background signal, MPO-oxMIF still bound to CD74 ECD with the same affinity as oxMIF generated with 50 nM MPO (**Figure 7E**). MPO-oxMIF analyte was applied to the CD74 ECD at concentrations ranging from 25 – 200 nM and sensorgrams were analyzed using a 1:1 Langmuir model (**Figure 7B**). The obtained K_D of MPO-oxMIF and CD74 ECD was 11.8 ± 3.7 nM, in good agreement with the previously published K_D for MIF and CD74 ECD, also determined by SPR.³¹

MPO-oxMIF interacts with CD74 in cell-based assays

We initiated *in vitro* studies of the oxMIF-CD74 interaction using HT29 colon carcinoma cells as a model for epithelial cells, which are known to upregulate CD74 under inflammatory

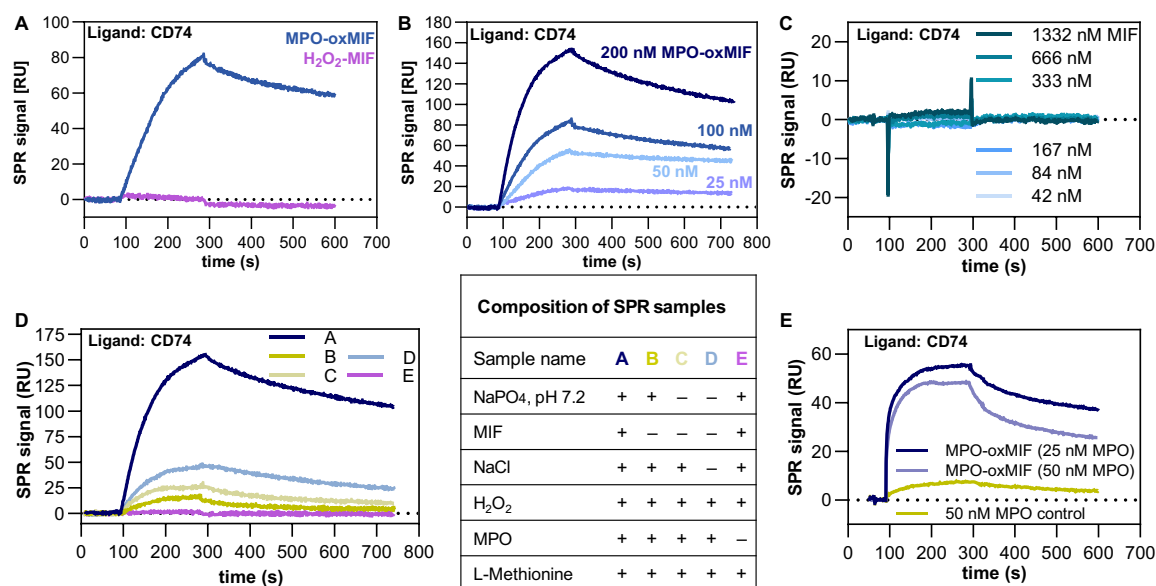


Figure 7. Redox-dependent binding of MIF to the CD74 receptor via SPR. (A) Pilot SPR experiment with 100 nM H₂O₂-MIF and 100 nM MPO-oxMIF showed that analyte MPO-oxMIF, and not H₂O₂-MIF, bound to immobilized CD74 ECD. (B) A K_D of 11.8 ± 3.7 nM from a 1:1 Langmuir model was extracted from concentration-dependent binding of MPO-oxMIF to CD74 ECD. K_D reported as mean $\pm$ SD from $n = 4$ independent experiments. (C) Redox-neutral MIF does not interact with CD74. SPR experiment where untreated MIF was used as an analyte, serially diluted from 1332 nM to 42 nM, and CD74 ECD was used as a ligand, clearly showed no binding in $n = 6$ independent experiments. (D) Qualitative SPR experiment to confirm CD74 interaction with MPO-oxMIF. CD74 ECD was used as a ligand and control samples, where MIF and different components of MPO system were used as analytes. A low background signal for MPO alone was observed (yellow, curve B). The highest background signal assigned to MPO alone was observed when phosphate buffer, MIF and NaCl were excluded from the sample (light blue, curve D). MPO-oxMIF (dark blue, curve A) strongly bound to CD74 ECD, while MIF did not (magenta, curve E), even in the presence of H₂O₂ (but in the absence of MPO). (E) Qualitative SPR experiment at concentrations of 25 nM (dark blue trace) or 50 nM MPO (light blue) confirm sufficient MPO was used to oxidize MIF and enable its binding to CD74 ECD. Sample containing 50 nM MPO in the absence of MIF was used as a control and produced a negligible background.

conditions (Figure 8). Upon incubation with IFN- γ , HT29 cells demonstrated an unambiguous induction of CD74 expression on the cell surface, as shown by FACS (Figure 8A). In the absence of IFN- γ , these cells do not express CD74. We also analyzed the expression of CD44, since CD44 was reported to be an integral member of CD74/MIF receptor complex.⁵¹ In contrast, expression of CD44 on the cell surface of HT29 was very high and did not change upon incubation with IFN- γ (Figure 8B). In the next FACS experiment, HT29 cells $\pm$ IFN- γ were incubated with H₂O₂-MIF and MPO-oxMIF (Figure 8C). After multiple washing steps, MPO-oxMIF was detected on the

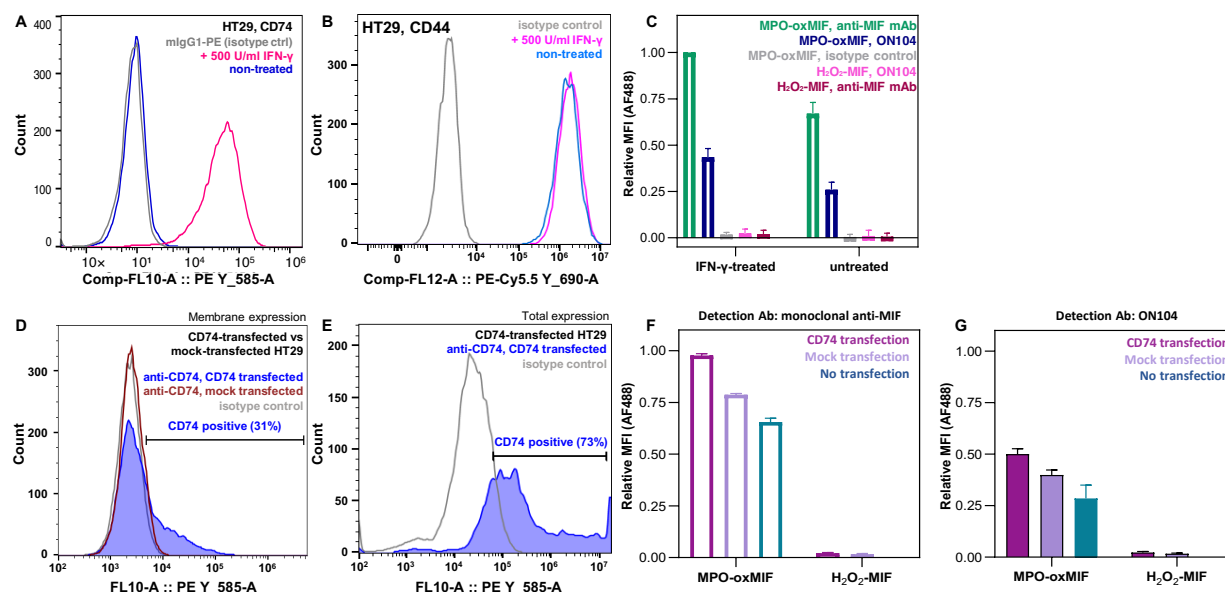


Figure 8. Redox-dependent binding of MIF to HT29 cell-surface expressed CD74. (A) FACS analysis of IFN- γ -treated HT29 cells confirmed the presence of CD74 by anti-CD74-PE antibody (pink). In the absence of IFN- γ , cells did not express CD74 (blue). (B) IFN- γ treatment of HT29 cells reveals that CD44 expression is independent of stimulation, as signals obtained by staining with anti-CD44-PE-Cy5 overlapped for treated (pink) and non-treated cells (blue). One representative FACS histogram of $n = 3$ independent experiments is shown for (A-B). (C) FACS analysis of IFN- γ -treated HT29 cells incubated with MPO-oxMIF or H₂O₂-MIF. Exogenous MPO-oxMIF was detected on the cell surface. MPO-oxMIF was also detected on the surface of non-treated cells. Antibodies used to detect each species are detailed in Materials and Methods. Data are expressed as relative geometric mean fluorescence intensity (MFI) after normalizing to the MFI values of MPO-oxMIF binding to IFN- γ -treated HT29 detected with anti-MIF mAb and upon the subtraction of the MFI of secondary antibody alone (mean of $n = 3$ independent experiments $\pm$ SEM). (D) Membrane exposure of CD74 in CD74-transfected (blue) versus mock-transfected (red) HT29 cells. Cells were stained with anti-CD74-PE monoclonal antibody, clone LN2, or with isotype control mouse IgG1,k-PE (grey), followed by staining with fixable viability dye eFluor780. (E) Total (membrane plus intracellular) expression of CD74 in CD74-transfected HT29 cells. After the FACS acquisition of membrane CD74-stained HT29, cells were fixed, permeabilized and stained with anti-CD74-PE (blue-filled histogram overlay) or isotype control mouse IgG1,k-PE (grey line histogram overlay) to assess intracellular expression of CD74. One representative histogram from $n = 3$ independent biological experiments is shown for (D-E). (F, G) FACS analysis shows increased binding of exogenous MPO-oxMIF to CD74-transfected HT29 cells (maroon) compared to mock-transfected (purple) and non-transfected HT29 cells (teal). H₂O₂-MIF does not bind to HT29 cells. Binding was detected with monoclonal anti-MIF antibody (G), or with ON104 (F). Data expressed as relative MFI values after normalizing to the MFI values of MPO-oxMIF binding to CD74-transfected HT-29 detected with anti-MIF mAb and upon the subtraction of the MFI of secondary antibody alone (mean of $n = 3$ independent experiments $\pm$ SEM).

cell surface of both IFN- γ -treated and non-treated cells *via* ON104 and monoclonal anti-MIF antibody, respectively. Detection with monoclonal anti-MIF antibody also probes binding of H₂O₂-MIF, as this antibody recognizes both MIF and oxMIF.

As we cannot exclude that treatment with IFN- γ upregulates other cell surface receptors beyond CD74, we next introduced CD74 into the untreated HT29 cells by transient transfection with plasmid encoding the full-length human CD74. While non-transfected and mock-transfected HT29 cells did not significantly present CD74 on the cell surface, HT29 cells transfected with CD74 demonstrated modest cell surface exposure of CD74 (around 31% of CD74 positive cells from a population of total viable cells) 48 hrs post-transfection (**Figure 8D**). Intracellularly, CD74 showed a strong expression in non-transfected HT29 cells, and transfecting with CD74 cDNA further increased CD74 levels (**Figure 8E**). We next assessed the binding of MPO-oxMIF and H₂O₂-MIF to the cell surface of CD74-transfected *versus* mock-transfected and non-transfected HT29 cells, where the binding was detected with monoclonal anti-MIF antibody, a total MIF binder, as mentioned above (**Figure 8F**), or with ON104 (**Figure 8G**). As evident from the **Figure 4F-G**, MPO-oxMIF, but not H₂O₂-MIF, bound to HT29 cells. Binding of MPO-oxMIF was stronger for CD74-transfected HT29 cells when compared to mock-transfected or to non-transfected cells. The 21-26% increase in MPO-oxMIF binding to CD74-transfected cells mirrors the cell surface exposure of CD74 in those cells (around 31% CD74-positive cells versus 9.8% in mock-transfected cells, **Figure 8D**). In agreement with its function in assisting assembly and subcellular trafficking of the MHCII complex,²⁵ only a modest percentage of CD74 was exposed on the cell membrane of CD74-transfected HT29 (see **Figure 8D**). This prompted us to redesign the cDNA of CD74 by removing most of its intracellular region to enable the enhanced cell surface presentation. We next forced the expression of CD74 in the cell lines-HCT116 and ExpiCHO-S, both of which provided much higher transfection efficiency than HT29. Removal of the CD74 intracellular domain resulted in remarkably high cell surface presentation of CD74 in both cell lines (**Figure 9A-B**). Consistent with high membrane expression of CD74 in transfected cells, we

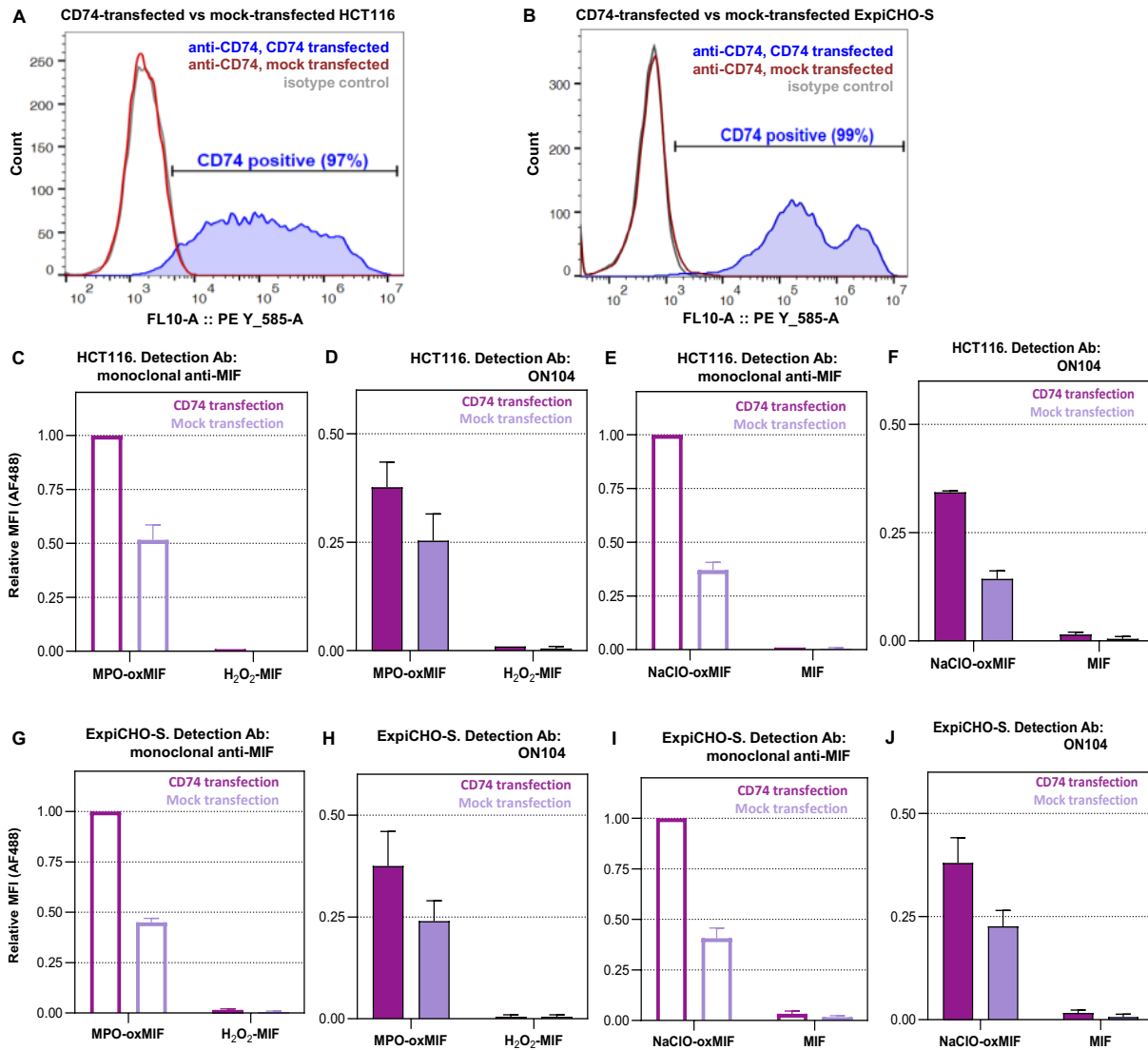


Figure 9. Additional assays to study the oxMIF-CD74 interaction on the surface of HCT116 and ExpiCHO-S cells. (A, B) Membrane exposure of CD74 in CD74-transfected (blue) versus mock-transfected (red) HCT116 cells (A) and ExpiCHO-S cells (B). Cells were stained with anti-CD74-PE monoclonal antibody, clone LN2, or with isotype control mouse IgG1,k-PE (gray), followed by staining with fixable viability dye eFluor780. One representative FACS histogram from n=3 independent experiments is shown for each cell line. (C-J) FACS analysis shows increased binding of exogenous MPO-oxMIF (C, D, G, H) and NaClO-oxMIF (E, F, I, J) to CD74-transfected (maroon) compared to mock-transfected (purple) HCT116 (C-F), and ExpiCHO-S (G-J) cells. In contrast, H₂O₂-MIF and MIF do not bind to the cells (C-J). Data are expressed as relative MFI values after normalizing to the MFI values of MPO-oxMIF and NaClO-oxMIF binding to CD74-transfected HCT116 and ExpiCHO-S cells detected with anti-MIF mAb and upon the subtraction of the MFI of secondary antibody alone (mean of n = 3 independent experiments ± SEM).

observed a substantial increase in oxMIF binding to CD74-transfected HCT116 and ExpiCHO-S cells compared to mock-transfected cells (Figure 9C-J). Remarkably, both MPO-oxMIF and

NaOCl-oxMIF, evaluated head-to-head, demonstrated increased binding to CD74-transfected *versus* mock-transfected HCT116 and ExpiCHO-S cells, while MIF showed no binding (**Figure 9C-J**). Together with the increased binding of MPO-oxMIF to IFN- γ -stimulated HT29 cells (**Figure 8C**), enhanced binding of oxMIF to two additional CD74-transfected cell lines versus mock transfections points to CD74 as the oxMIF receptor in three distinct cases (HT29, HCT116 and ExpiCHO-S cells).

We also explored the expected interaction between endogenous oxMIF and CD74 in human neutrophils isolated from healthy donors (**Figure 10**). To trigger the generation of oxMIF, neutrophils were first stimulated with LPS *in vitro*, and the presence of oxMIF on the cell surface was detected with the dye-labeled ON104 (ON104-AF488) (**Figure 10A**). After 18 hours of incubation with LPS, a significant increase of oxMIF on the cell surface of neutrophils was observed (**Figure 10B**).

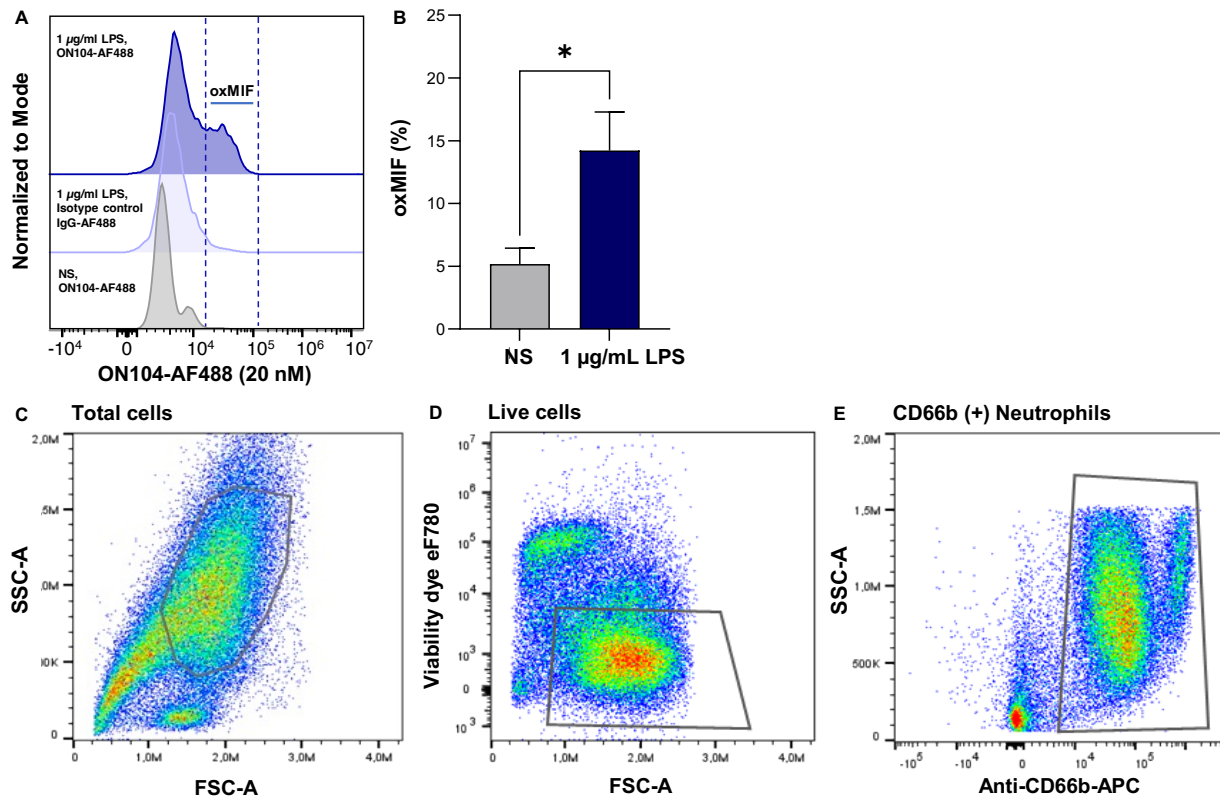


Figure 10. Endogenous oxMIF binding to CD74 on human neutrophils. (A) Representative FACS histograms showing oxMIF presence on the cell surface of unstimulated (NS; gray) versus LPS-activated (blue) neutrophils from a healthy donor. oxMIF levels were detected and quantified with ON104-AF488 and plotted in (B). Data are expressed as a mean $\pm$ SEM of $n = 5$ individual donors and $*p < 0.05$. (C-E) Representative FACS gating strategy used to analyze oxMIF and CD74 expression on stimulated human neutrophils. After excluding doublets in FCS-A versus FSC-H plots, cell debris in SSC-A versus FSC-A plot (C), and gating on viable (eFluor780 negative) cells (D), the human neutrophil population was identified as CD66b(+) (E). One representative plot is shown of $n = 3$ individual donors performed with $n \geq 2$ technical replicates.

Discussion

HOCl-driven oxidative modifications promote the oxMIF conformation

Antibody binding studies demonstrate that oxidation of MIF by HOCl promotes a conformation that is selectively bound by ON104, which was not observed for H₂O₂-MIF or redox-neutral MIF. The specificity of ON104 for HOCl-oxidized MIF correlates well with its impressive preclinical efficacy.¹⁹ Interestingly, the reduction potential of H₂O₂ is higher than that of HOCl (1.32 V vs 1.28 V, respectively), making it a stronger oxidant.¹⁵ However, HOCl acts much more rapidly, oxidizing methionine one million-fold faster than H₂O₂.⁵² Due to this extreme difference, cells appear to utilize HOCl as a defence mechanism requiring fast and non-selective protein oxidation, and H₂O₂, when a slow, but highly selective messenger molecule is needed for redox-dependent signalling.^{15,39,53,54} The production of HOCl is a key innate immune defense against invading pathogens⁵⁵; however, its excessive production during chronic inflammation is strongly linked to disease progression in conditions such as rheumatoid arthritis⁵⁶ or atherosclerosis.⁵⁷ Since MIF is a key mediator of acute systemic inflammation and autoimmune disorders, it will be found in the same environment as that of HOCl, thus its oxidation via HOCl suggests a critical mechanism to alter its biological and pathological activities.

Exposure of proteins to HOCl can result in a broad spectrum of possible modifications at the peptide level, such as the oxidation of Cys, Met, Trp, or Phe residues, chlorination of Tyr and His residues, and formation of proline-imine.^{15,16,18,41} We investigated the prevalence of such

modifications in MPO-oxMIF by intact mass and peptide mapping MS. Our prime interest was the detection of oxidized cysteines, specifically Cys56, Cys59, and Cys80. It has been shown previously that sulfhydryl-specific reagents react with Cys80 and trigger a conformational change within the MIF trimer, making it recognizable to anti-oxMIF antibodies.¹³ Oxidoreductase motif-related Cys56 and Cys59, which are within the ON104 epitope E₅₄PCALC₅₉, were found as free thiols, whereas Cys80 was suggested to act as a redox molecular switch, enabling transition of MIF into oxMIF.¹³ Cys80 was also shown to be a critical allosteric node that attenuated MIF redox sensitivity when mutated.¹²

In the present study, prior to digestion with AspN/LysC protease, MPO-oxMIF was treated with an AccuMAP kit (Promega) under nonreducing conditions to preclude artificial nonenzymatic posttranslational modifications (deamidation, disulfide bond scrambling, and oxidation) during sample preparation. In MPO-oxMIF, Cys80 was surprisingly unmodified, while Cys56 and Cys59 were found as partially disulfide bonded. Nevertheless, nitrite (NO₂⁻) has been identified as an additional substrate for MPO under physiological conditions, leading to the generation of nitrogen dioxide radical (NO₂•).⁵⁸ While NO₂• is known to be highly unstable,^{59,60} it is exceptionally potent in catalyzing protein nitration/nitrosylation, resulting in nitrotyrosine formation and the S-nitros(yl)ation of cysteines. Recent findings have demonstrated that MIF can undergo S-nitros(yl)ation, with Cys80 being identified as the sole target.⁶¹ However, we note that our analysis here focused solely on the HOCl pathway and further investigations into S-nitros(yl)ation will be considered in future studies. Given that the MIF crystal structure shows bond distances between the sulfur atoms of Cys56 and Cys59 of ~10 Å (measured in PyMoL), a substantial conformational change is expected to take place upon oxidation, allowing the formation of a disulfide bond. The localization of Cys56 at the beginning of an unstructured loop seems to facilitate this

reorganization. Collectively, our findings suggest that the redox state of MIF cysteines represents a reagent-specific signature of oxMIF. Though reports of the reactivity of HOCl toward methionine side chains vary,^{16,62} the more labile nature of methionine, relative to cysteine, informs our data and previous kinetic studies with this oxidant.

In contrast to cysteines, all three methionines within the MIF monomer (Met2, Met47, and Met101), were found to be oxidized to methionine sulfoxide in various amounts, with negligible methionine sulfone observed. The Met residues are located at the interface of two adjacent MIF subunits and are generally solvent-exposed. The presence of Phe3, Phe49, and Tyr99 adjacent to Met2, Met47, and Met101, respectively, could also indicate that these pairs form S-aromatic interactions, which are known to diminish the propensity of sulfur atoms to oxidize when the distance between the sulfur and the center of an aromatic ring is within 7 Å.⁶³ Measurement of these distances in PDB: 1MIF with PyMoL revealed that such interaction is indeed possible for the Met101 – Tyr99 pair. Met47 and Phe49 appear too far in this crystal structure (~8 Å), but the possibility of S-aromatic contact cannot be completely excluded, as they are both located within a flexible loop. The same applies to Cys80 – Trp108, where Trp108 is positioned within the loop of the adjacent subunit. Cys56 – Phe49 and Cys59 – Phe3 (Phe3 from the adjacent subunit) also represent candidate pairs for S-aromatic interaction. The involvement of MIF methionine and cysteine residues in S-aromatic interactions could explain why we observed substantial, but not complete, oxidation of methionines, and a very limited oxidation of cysteines. However, this point is merely speculative since the orientations of amino acid side chains in a crystal structure do not fully reflect solution dynamics.

The hydrophilicity of an oxidized methionine side chain is higher than that of a reduced methionine, and oxidized methionines have long been known to introduce substantial changes to

protein tertiary and quaternary structures, protein stability, hydrogen bonding, and lead to extreme events such as unfolding or aggregation.⁶³⁻⁶⁶ Therefore, the oxidized methionines in MPO-oxMIF contribute to the conformational changes needed to expose the E₅₄PCALC₅₉ epitope in the solvent channel to allow binding of ON104. A mutation-induced loosening of the MIF trimer, resulting in a more flexible structure with lower thermodynamic stability, has been observed by NMR and circular dichroism, respectively.²³ This likely explains the difficulty in crystallographically capturing such a lowly-populated MIF conformer, or for that matter, any MIF-protein complex.

We also draw an analogy to so-called guardian methionines described for *E. coli*-originating glutamine synthetase and other systems, such as recombinant human stem cell factor, interferon alpha-2b, tissue plasminogen activator, keratinocyte growth factor, alpha-2-macroglobulin, and calmodulin.⁶⁷ In these examples, solvent-exposed methionines are strategically positioned around the entry to the enzyme active sites, where they serve as a front-line protection against events such as ROS-mediated protein oxidation. As they readily oxidize, the guardian methionines absorb the otherwise inevitable oxidation of the crucial residues in or around the active site, thereby preventing oxidation-dependent deactivation and/or destruction. We can envision such a mechanism being applicable to MIF, where under inflammatory conditions MIF undergoes its transition to oxMIF. Peptide mapping of MPO-oxMIF shows Met2, Met47, and Met101 are oxidized, while Pro1 and Tyr99, which are directly or allosterically involved in tautomerase enzymatic activity and/or receptor binding, remained almost completely unmodified^{9,12,21-23,41,44} (**Figures 4-5** and **Tables 1, 4**). NMR studies of MPO-oxMIF also show substantial flexibility in regions surrounding Pro1-Met2 (N-terminus), Tyr98/99-Met101 (allosteric solvent channel), and the C-terminus (**Figure 7D-E**). Contrary to our findings, others have reported a substantial modification of Pro1 when MIF is treated with 5x molar excess of

HOCl, either added as household bleach or generated by the MPO system.⁴¹ Furthermore, an almost complete loss of tautomerase activity in MIF samples exposed to 5x or higher molar excess of HOCl was observed. We encountered only mild (~10%) attenuation of tautomerase activity for MIF oxidized with 5x molar excess of NaOCl and no effect with H₂O₂ alone (**Figure 11**). The very low amount of Pro1-imine detected in our MPO-oxMIF samples is consistent with its essentially intact tautomerase function.

The oxidative modifications to the MIF structure described here are driven by HOCl and contribute to the conformational plasticity of oxMIF that facilitates its selective binding to ON104, and further, to CD74 as discussed below. However, in a cellular environment, many other post-translational modifications are possible and have been characterized for MIF (Schindler et al., 2018), such as Pro1 carbamylation,^{41,68} Cys59 cysteinylolation,⁶⁹ Cys80 S-nitrosylation,^{61,70} Ser90 phosphorylation,^{69,71} S-glycosylation at Ser111 and/or Thr112,⁶⁹ and glycation.⁷² These modifications hold biological significance for MIF, with many resulting in altered function,⁷³ and

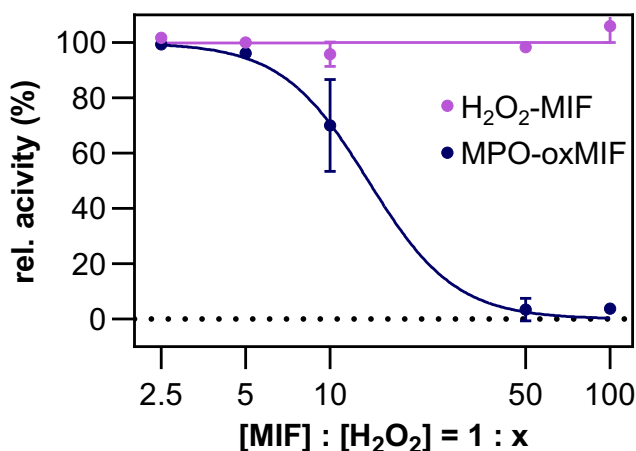


Figure 11. MIF tautomerase assay after treatment with H₂O₂ alone or with the MPO system. The ability of MIF to convert the orange-colored L-dopachrome methylester into a transparent solution served as a readout for its tautomerase activity. An approximate 10% drop in the enzymatic activity was observed when MIF was treated with 25 nM MPO and 5x molar excess of H₂O₂ (blue) and continuously decreased as rising concentrations of the MPO system components were added. Tautomerase activity remained intact when MIF was treated only with H₂O₂ (purple). Biological samples n = 3, technical replicates n ≥ 1. Relative enzyme activity reported as mean ± SEM

may be additional avenues for modulating MIF via environmental factors. In addition to the modifications characterized in this study, those that have been shown to occur in the extracellular space (i.e. carbamylation and glycation), where interactions with CD74 take place and oxMIF is targeted, could be factors that alter antibody and receptor binding.

MPO-oxMIF interacts with CD74 ECD

Many reports detail MIF interacting with its cognate receptor.^{21-23,31,43,44,74-76} Our SPR experiments investigating whether MPO-driven oxidation of MIF interferes with CD74 binding showed, surprisingly, that recombinant CD74 ECD (aa 73-232) did not interact with H₂O₂-MIF and MIF, but bound MPO-oxMIF with a K_D of 11.8 ± 3.7 nM. This value agrees with a K_D of 9 nM for MIF and CD74 ECD determined in a similar SPR experiment.³¹ An even higher affinity (K_D of 2 nM) was reported when MIF was used as a ligand and CD74 ECD as an analyte. We have demonstrated that H₂O₂-MIF used in this study behaves as a redox-neutral MIF and did not observe any interaction between CD74 ECD and MIF/H₂O₂-MIF analyte, nor for any experiment using MPO-oxMIF as a ligand and CD74 ECD as an analyte.

We considered several explanations as to why our SPR data differ from those reported in prior work. It should be noted that redox-sensitive proteins can be complex and such systems reconstituted *in vitro* are prone to interference. Such a caveat also applies to cell-based assays, which may be especially complex in co-culture settings, where secreted reactive oxygen or nitrogen species, or differing enzyme activities, can contribute to the unique oxidative environment. Recombinant human MIF used in previously published SPR experiments was purified under denaturing conditions with 60% acetonitrile/water.⁷⁷ Although MIF was then refolded, it is worth noting that effects of 40% (w/v) acetonitrile on protein secondary structure may include aggregation, conformational changes, and an increase in helical content.⁷⁸ However,

circular dichroism studies of refolded recombinant human MIF revealed an α -helical content of only 15%,⁷⁷ a lower-than-expected percentage that might be a consequence of elution conditions during sample preparation. Indeed, the predicted α -helical content of MIF (UniProt P14171) calculated by JPred4⁷⁹ is 26%, and that extracted from the PDB:1MIF crystal structure is 36%. Thus, the MIF protein preparation itself may have contained some population of structurally altered MIF resulting from purification or refolding procedures.

Earlier preparations of refolded MIF were inspected by laser desorption MS.⁷⁷ As this technique would not resolve mass differences of 16 Da, it is thus not possible to unambiguously conclude if the refolded protein was oxidized or not. Further, we have observed that adsorption of MIF on a solid surface, such as an ELISA plate, results in conformational change(s) resembling oxMIF, as surface-attached MIF can be detected by ON104 antibody. This may explain strong binding between surface-adsorbed MIF and CD74 analyte.³¹ Further, the K_D measured for MPO-oxMIF binding to CD74 ECD matches that published for MIF, leading us to believe that the latter interaction originates from a structurally altered MIF, representing a minor population of the total MIF sample. We therefore expect this K_D value to be even lower, as 1) more than one CD74 molecule may be interacting with one MIF trimer,⁴⁴ and 2) not all MIF is necessarily converted into oxMIF upon MPO-treatment.

In our cell-based assays, we showed the binding of exogenous MPO-oxMIF to CD74-expressing IFN- γ -treated HT29 cells. Moreover, the binding of MPO-oxMIF to HT29 cells which were transfected with CD74 was higher than to mock-transfected and non-transfected cells. In agreement with the main role of CD74 as a chaperone of MHC class II D α and D β chains, only a small proportion of CD74 can be found on the cell surface independent of the MHC class II complex. Others have attempted to establish human CD74-overexpressing melanoma cells by

transfecting them with CD74-expressing plasmids.⁸⁰ Although those transfections showed overexpression of CD74 mRNA and protein, they did not show upregulation of cell surface CD74 levels, and the only way of delivering CD74 to the cell surface was stimulation with IFN- γ . We were able to detect an increase in cell surface CD74 by transfecting HT29 cells with CD74-encoding plasmid (**Figure 8D**), although consistent with prior work,⁸⁰ the cell surface expression of CD74 was still very modest, possibly due to the limited transfectability of HT29. We therefore engineered the cDNA of CD74 by removing most of its intracellular region and expressed it in the two additional cell lines that allow high efficiency transfections: HCT116 and ExpiCHO-S. In a good agreement with prior work,⁸¹ removal of the CD74 intracellular domain resulted in a very high cell surface presentation of CD74 in transfected HCT116 and ExpiCHO-S cells (Fig 9A, B). This enhanced cell membrane expression of CD74 translated into a notable increase in oxMIF binding to CD74-transfected HCT116 and ExpiCHO-S compared to mock transfected cells (**Figure 9C-J**).

Furthermore, we also detected oxMIF on the cell surface of activated neutrophils. Hence, our data indicate that in activated neutrophils, IFN- γ -treated HT29 cells, and CD74-transfected HT29, HCT116 and ExpiCHO-S cells CD74 acts as a receptor for oxMIF. However, binding of MPO-oxMIF to untreated, mock-transfected, or non-transfected cells that do not express CD74 was also observed and suggests that receptors other than CD74 might also be involved in the interaction. One plausible candidate could be CD44, a known signalling component of the CD74-MIF receptor complex.⁵¹ CD44 was strongly expressed on the surface of HT29 cells independently of IFN- γ treatment (**Figure 8B**), and it is also reported to be expressed by neutrophils and many other cell lines and primary cells.⁸² Further efforts are warranted to validate the role of CD44 as a possible oxMIF (co)-receptor.

In summary, our data demonstrate that MPO-oxMIF, but not redox-neutral (untreated) MIF and H₂O₂-MIF, interacts with anti-oxMIF antibody ON104 and CD74 ECD. We show that oxidation of methionines impacts the conformation of the MIF trimer in subtle ways, as each of these residues is positioned at the interface of the MIF subunits. When oxidized, heightened flexibility in these regions may loosen the compact MIF structure, enabling access of oxMIF-specific antibodies and interaction between the crucial residues of oxMIF and extracellular portion of CD74. These studies further support the assumption that pathological activities of MIF are mediated *via* oxMIF in a structure-dependent manner. This is plausible as MIF, which is ubiquitously present even in healthy individuals unlike other inflammatory cytokines, could be detrimental to human health if its reported inflammation- or cancer-promoting properties were not subject to further regulation. Microenvironments with high MPO activity and HOCl concentrations that could favor the generation of oxMIF are an integral part of inflammatory processes. Despite the fact that many cytokines possess small and unremarkable structures, their functional diversity is widely noted. Such observations implicate structural plasticity as a critical determinant for the capacity of cytokines to bind with different partners and thus exert unique biological effects.

Materials and Methods

Expression and purification of recombinant human MIF

Expression and purification procedures tightly followed those described by Rossmueller and Mirkina.⁸³

Human neutrophil isolation and cell culture

Human neutrophils were isolated from anticoagulated whole blood obtained from eleven (11) healthy volunteers (local RedCross Vienna, Austria) using an immunomagnetic-based

approach. The isolation procedure was performed using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL Technologies, Canada) according to manufacturer's protocol. Purified neutrophils were washed in Hank's Balanced Salt Solution (HBSS without Ca^{2+} and Mg^{2+} , Gibco, US), counted and plated in a U-shaped 96-well plate at a density of 0.25×10^6 cells per well in RPMI medium 1640 Glutamax™ containing 5% FBS (Gibco, US) and 1% PS (Gibco, US). Cells were then incubated for 18 hours with a mixture of phorbol myristate acetate (PMA) and ionomycin at final concentrations of 1 ng/mL and 0.5 $\mu\text{g/mL}$, respectively (both Sigma Aldrich, catalog # P8139 and I0634), or with lipopolysaccharide (LPS) at a final concentration of 1 $\mu\text{g/mL}$ (Sigma Aldrich, catalog # L5293, serotype O111:B4). After stimulation, the cell culture medium was collected and stored at -80°C for subsequent oxMIF and total MIF ELISA analysis, and for MPO activity analysis.

ELISA for detection of MIF and oxMIF in supernatants of stimulated neutrophils

A monoclonal anti-oxMIF antibody with an increased affinity for oxMIF (property of OncoOne R&D GmbH, deriving from BaxA10) was used to determine both total MIF and oxMIF levels in supernatants. To detect total MIF, it was converted to oxMIF by ProClin300 (Sigma-Aldrich, catalog # 48912-U), a known MIF oxidant.¹⁴ The minimum amount of ProClin300 required to convert MIF into its “oxidized-like” conformation was determined by ELISA with MIF samples treated with 0.5%, 1%, and 2% ProClin300. Binding of a BaxA10-derived monoclonal anti-oxMIF antibody did not improve with ProClin300 concentrations greater than 0.5% (**Appendix Figure 1**). Intact mass analysis further confirmed a +115.1 Da addition of the ProClin300 methylisothiazoline moiety to $43.3 \pm 4.6\%$ of MIF subunits (**Appendix Figure 1**), which appears sufficient for its transition to an oxidized-like conformation.

For oxMIF ELISAs, an in-house anti-oxMIF capture antibody was diluted to a concentration of 2 µg/mL in 1x DPBS and added to each well of a 96-well MaxiSorp plate (Thermo Scientific, catalog # 442404) for 1 hour at room temperature (RT) with constant shaking. Wells were then blocked with 250 µl/well of 2% (w/v) fish gelatine (FG) in 1x TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween20 (v/v), pH 7.5; 2% FG/TBST) at 4 °C, overnight. On the next day, the supernatants were diluted 1:2 in 2% FG/TBST for oxMIF detection, and in 2% FG/TBST containing 0.5% ProClin300 for total MIF detection. A standard sample consisting of 30 ng/mL ProClin300-treated recombinant human MIF was also used. After washing steps, 100 µl of the supernatants and standard were serially diluted in 2% FG/TBST, with or without 0.5% ProClin300, and applied to 96 well-plate and incubated for 1.5 hours at RT with continuous shaking. After second round of washing, 100 µl/well of polyclonal rabbit anti-MIF antibody (property of OncoOne R&D GmbH) was diluted to 1 µg/mL in 2% FG/TBST and added to the plate and incubated for 1 hour at RT with continuous shaking. After a third round of washing, 100 µl/well of goat anti-rabbit IgG-horseradish peroxidase (HRP, Thermo Scientific, catalog # A16110) diluted 1:10.000 in 1x TBST was added and incubated for 1 hour at RT with continuous shaking. The solution was discarded, and plates were washed and revealed by 100 µl/well of 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Scientific, catalog # 34029). The chromogenic reaction was stopped by the addition of 50 µl/well of 30% (v/v) sulfuric acid (H₂SO₄). Absorbance was measured at 450 and 650 nm using a Tecan Infinite M200 PRO microplate reader. OxMIF levels were calculated by a linear regression curve fit in GraphPad Prism 9.0, where the choice of fitting model (linear vs nonlinear regression) was performed by Akaike's interpolation criterion (AICc). The OD values recorded for supernatants treated with ProClin300 or untreated were then interpolated and the concentrations of total MIF/oxMIF levels were extracted. Results are

presented as mean (ng/mL) $\pm$ SEM. Comparison between two groups was determined using the two-tailed paired and non-parametric Wilcoxon test. Results were considered significant at a p value lower than 0.05. For time-dependent expression of MIF in stimulated vs. unstimulated neutrophils, a Shapiro-Wilk normality test, two-way repeated measures ANOVA, followed by a Fisher's LSD test, were performed to identify significant differences between the groups. The number of subjects is provided in the figure legends.

MPO activity analysis

Supernatants collected from isolated human neutrophils stimulated with a PMA/ionomycin mixture (1 ng/mL and 0.5 μ g/mL, respectively) were assessed for MPO activity using a colorimetric myeloperoxidase activity assay (Abcam, catalog # ab105136) according to the manufacturer's protocol. MPO activity in the test samples was calculated as follows: MPO activity = $B \cdot \Delta T \cdot V \cdot \text{sample dilution factor}$, where B is the nanomolar concentration of TNB calculated from the $\Delta A_{412\text{nm}}$, T is the time of the first incubation (in min), and V is the pre-adjusted sample volume (in ml) added into the reaction well. $\Delta A_{412\text{nm}}$ is calculated as a difference between the absorbance of a background reference and the absorbance of a sample. Data are expressed as mU/ μ mol where one unit (U) is the amount of MPO that hydrolyzes the substrate and generates taurine chloramine to consume 1.0 μ mol of TNB per minute at 25 °C. The data derives from seven healthy human donors (n=7).

Oxidation of recombinant human MIF in vitro

The protocol for *in vitro* oxidation of recombinant human MIF followed a prior report.⁴¹ In brief, MIF was oxidized with recombinant human myeloperoxidase (MPO, Planta Vienna), hypochlorous acid (NaOCl, 10% aqueous solution, technical grade, PanReac AppliChem), and

hydrogen peroxide (H_2O_2 , 30% aqueous solution, PerhydrolTM for analysis EMSURE ISO, Supelco, Merck). Enzymatic oxidation of MIF was performed in 50 mM phosphate buffer at pH 7.2, supplemented with 140 mM NaCl. H_2O_2 was added at 5x molar excess in respect to the concentration of MIF trimer. Unless stated otherwise, MPO was used at 1/20 of the MIF trimer concentration, which resulted in 50 nM MPO. Chemical oxidation was performed in 1x DPBS, where MIF was incubated with either 5x molar excess of H_2O_2 or NaOCl in respect to the concentration of MIF trimer. All reactions were performed for 20 min at RT with 1400 rpm of shaking. L-Methionine was then added to the mixtures at a final concentration of 1 mM to scavenge residual hypochlorous acid. Oxidized samples were freshly prepared for each experiment.

ELISA for testing specificity of ON104 anti-oxMIF antibody toward MPO- and NaOCl-oxidized MIF

96 well MaxiSorp plates were coated with 100 μl /well of ON104 and rabbit polyclonal anti-MIF antibody (both property of OncoOne R&D GmbH) at 2 $\mu\text{g}/\text{mL}$ in 1x DPBS and the plates were gently shaken for 1 hour at RT. Antibodies were discarded and plates were blocked with 250 μl /well of 2% FG/TBST and gently shaken overnight at 4 °C. The blocking solution was discarded the next day, the plates were washed 4 times with 1x TBST, and MIF samples (non-treated MIF and MIF oxidized as described above) were added to the plates. Serial 1:3 dilutions of samples were done in 2% FG/TBST, with a starting concentration of 10 $\mu\text{g}/\text{mL}$, and 100 μl /well of sample was incubated with coated antibodies for 1.5 hours at RT with gentle shaking. Along with MIF samples, MPO, H_2O_2 and NaOCl in reaction mixtures where MIF was substituted with equal volume of 1x DPBS were used as controls. The samples were then discarded, the plates were washed 4 times with 1x TBST, and 4 $\mu\text{g}/\text{mL}$ of biotinylated polyclonal rabbit anti-MIF antibody (property of OncoOne R&D GmbH) in 2% FG/TBST was applied at 100 μl /well and incubated

for 1 hour at RT with gentle shaking. The antibody was then discarded, the plates were washed 4 times with 1x TBST, and 100 µl/well of Pierce™ Streptavidin Poly-HRP (Thermo Scientific, catalog # 21140) was applied at 1:10.000 dilution in 1x TBST and incubated for 1 hour at RT with gentle shaking. The solution was discarded, the plates were washed 4 times with 1x TBST, and 100 µl/well of 1-Step™ Ultra TMB-ELISA Substrate Solution (Thermo Scientific, catalog # 34029) was applied. The reaction was stopped after ~ 2 min by addition of 30% (v/v) H₂SO₄ at 50 µl/well. Absorbance was measured at 450 nm and 650 nm on a Tecan plate reader and for the final representation of the OD values, a difference between OD_{450nm} and OD_{650nm} was calculated. Biotinylation of polyclonal rabbit anti-MIF antibody was performed in advance, using the EZ-Link Sulfo NHS-LC-LC-Biotin kit (Thermo Scientific, catalog # A35358), according to the manufacturer's protocol.

Surface plasmon resonance (SPR)

SPR was conducted at NBS-C BioScience & Consulting GmbH, in Vienna, Austria. To determine the specificity of ON104 anti-oxMIF antibody toward MPO-oxMIF and to examine the interaction between CD74 ECD and H₂O₂-MIF/MPO-oxMIF, ON104 and CD74 were immobilized on Biacore CM5 sensor chips (Cytiva) ID 010623/Lot # 10331615 and ID 101022/Lot # 10308836, respectively. The extracellular domain of CHO-derived human CD74 protein, covering residues Gln73-Met232, with an N-terminal HA (YPYDVPDYA) tag (R&D Systems, catalog # 3590-CD-050, Lot # THY1122031) was used. Coupling was performed with the Biacore amine coupling kit (Cytiva, catalog # BR100050), which included NHS and EDC cross-linkers, according to the manufacturer's protocol. In the ON104 – oxMIF binding experiment, coupling of ON104 resulted in a density of 3947 response units (RU) on the flow cell FC3, and in the CD74 ECD – oxMIF binding experiment, coupling of CD74 ECD resulted in a

density of 4259 RU on the FC3. FC1 was used only to react with HBS-EP running buffer (catalog # BR100188). Reaction mixtures containing redox-neutral MIF (untreated MIF in 1xPBS), MIF treated with H_2O_2 in the absence of MPO (H_2O_2 -MIF), MPO-oxMIF, or MPO alone were used as analytes. MPO-oxMIF was prepared as described above, with the final MPO concentration at 25 nM. Each analyte (100 μl) was applied to the ligand-coated chip at a flow rate of 30 $\mu\text{l}/\text{min}$ for 3 min, using a BiacoreTM 3000 Instrument (Cytiva). The dissociation time was 300 s and was followed by a 3 min regeneration with 100 μl of 10 mM HCl. Equimolar concentrations of H_2O_2 -MIF and MPO-oxMIF (202 nM) as analytes were used for a qualitative pilot experiment to assess ON104 binding. Equimolar concentrations of H_2O_2 -MIF and MPO-oxMIF (100 nM) as analytes were used for a qualitative pilot experiment to assess CD74 ECD binding. Further, a concentration series was prepared for kinetic binding analysis of MIF/ H_2O_2 -MIF/MPO-oxMIF to ON104 and CD74, respectively. Analyte concentrations of 222, 110, 55 nM MPO-oxMIF and 222, 110 nM H_2O_2 -MIF were flown over covalently coupled ON104. The CD74 ECD-coupled chip was incubated with 200, 100, 50, and 25 nM MPO-oxMIF, 1332, 666, 333, 167, 84 and 42 nM redox-neutral MIF, and various combinations of the MPO system components as additional controls. Double referencing of the obtained sensorgrams included subtraction of the background signal originating from the amine activated surface and running buffer. To obtain binding parameters for the interactions, background-corrected sensorgrams were analyzed by local simultaneous association/disassociation fitting, using the 1:1 Langmuir model with mass transport limitation corrections in BiaEvaluation 4.1 and Biacore T200 software (Cytiva). The obtained values are represented as a mean $\pm$ SD of the calculated equilibrium constant (K_D), in nM units.

Peptide mapping mass spectrometry

MPO-oxMIF, H₂O₂-MIF and MIF were subjected to peptide mapping MS. 1 mg/mL of MPO-oxMIF was oxidized with the MPO system containing 50 nM MPO, 140 mM NaCl and 5x molar excess of H₂O₂, as described above. 1 mg/mL of H₂O₂-MIF was prepared in the same mixture as MPO-oxMIF, with the exception that MPO was substituted with an equal volume of 1x DPBS. MIF in 1x DPBS was diluted to 1 mg/mL in the same buffer. All samples were incubated at room temperature for 20 min with shaking at 1400 rpm. Subsequently, 1 mM methionine (as a final concentration) was added to the MPO-oxMIF and H₂O₂-MIF samples. Since detection of disulfide bonds was also within the scope of this analysis, no reduction or alkylation of the samples were performed.

Samples were denatured with a buffer containing guanidine hydrochloride under nonreducing conditions. The procedure is described in the technical manual of ACCUMap Kit (Promega, catalog # VA1050) under “Sample Preparation with guanidine HCl Under Nonreducing Conditions, point 4.A.”, with the main difference that the N-Ethylmaleimide (NEM) alkylation was not performed, and that the acetone precipitation was introduced. In brief, the samples were denatured with AccuMAP™ Denaturing Solution (containing GuHCl) and diluted in water (1:5 v/v) prior to protein precipitation with acetone (4:1 v/v). Predigest of the samples was performed with an AccuMAP™ Low pH Resistant rLys-C for 1 hr at 37 °C in the AccuMAP™ Low pH Reaction Buffer, in a sample:enzyme ratio of 1:10. The final digest was then performed with another addition of rLys-C (sample:enzyme ratio of 1:9) and AspN (Promega, catalog # V162A) in a sample:enzyme ratio of 1:60. Samples were digested for 3 hours at 37°C.

Approximately 0.5 µg of treated sample was then injected onto an LC-MS nanoEase C18 column (nanoEase M/Z HSS T3 Column, 100 Å, 1.8 µm, 300 µm X 150 mm, Waters) using 0.1% formic acid as the aqueous solvent. Within the first 50 min, a gradient from 1% - 40% B (B: 80%

acetonitrile, 0.1% formic acid) was applied and was then followed by a 10 min gradient from 40% - 95% B at a flow rate of 6 μ l/min. The latter gradient facilitated the elution of large peptides. Detection was performed with an Orbitap MS (Exploris 480, Thermo) equipped with the standard H-ESI source in positive ion, DDA mode (switching to MS/MS mode for eluting peaks). MS-scans were recorded in the range of 350 - 1200 Da, and the 20 highest peaks were selected for fragmentation. Instrument calibration was performed using Pierce FlexMix Calibration Solution (Thermo Scientific, cat. nr. A39239). Results were analyzed using PEAKS (Bioinformatics Solutions Inc.). Manual analysis was performed with Freestyle (Thermo Scientific). Quantitation and percent oxidation were calculated with Skyline,⁸⁴ where EIC areas of identified peptide on the MS1 level were compared (more than one charge state summed up, 4 isotopic peaks).

Intact mass analysis

MPO-oxMIF, H₂O₂-MIF, and MIF were analyzed for their intact mass. Preparation of the samples is described under peptide mapping. 2 - 10 μ g of respective protein solution was directly injected into a LC-ESI-MS system (LC: Agilent 1290 Infinity II UPLC), respectively. A gradient from 15% to 80% acetonitrile in 0.1 % formic acid at a flow rate of 400 μ L/min was applied over 15 min time frame, using a Waters BioResolve column, 2.1 x 5 mm. Detection was performed with a LC-TOF instrument (Agilent Technologies 6230 B) equipped with the Jetstream ESI source in positive ion, MS mode (range: 100 - 3200 Da). Instrument calibration was performed using ESI calibration mixture (Agilent Technologies, catalog # G1969-85000). Data was processed using MassHunter BioConfirm B.08.00 (Agilent) and the spectrum was deconvoluted by MaxEnt (MaxEnt Solutions, Ltd).

Nuclear magnetic resonance (NMR) spectroscopy

^{15}N -labeled samples of MIF for NMR studies were expressed in M9 minimal media containing MEM vitamins, MgSO_4 , and CaCl_2 , and supplemented with $^{15}\text{NH}_4\text{Cl}$ (Cambridge Isotopes Laboratories) and $^{12}\text{C}_6\text{H}_{12}\text{O}_6$ as the sole nitrogen and carbon sources, respectively. BL-21 (DE3) cells were grown to an OD_{600} of 0.8 – 0.9, induced with 1 mM IPTG, and incubated for 16-18 hours at 20°C. Cells were then harvested by centrifugation and purified as described previously.¹² Briefly, cells were resuspended in 20 mM Tris-HCl, 20 mM NaCl, and 1 mM EDTA at pH 7.4, lysed by ultrasonication, and clarified by centrifugation. The supernatant was purified with a 120 ml Macro-Prep High Q column (BioRad) pre-equilibrated with the lysis buffer, where MIF was eluted in the flow-through. MIF was further purified on a HiLoad 26/600 Superdex 200 size exclusion column (GE Healthcare) with lysis buffer. MIF samples were then dialyzed into a buffer containing 40 mM sodium phosphate, 50 mM sodium chloride, and 7.5% D_2O at pH 7.0. Samples of HOCl-treated MIF *via* the MPO system were prepared by combining 65.7 μM MIF with 3.28 μM MPO and 328.5 μM H_2O_2 . For comparison, untreated MIF was prepared as described above without the addition of H_2O_2 . The pH of NMR samples was monitored after the addition of MPO and/or H_2O_2 to ensure that the samples remained at pH 7.0. NMR experiments were performed on a Bruker Avance NEO 600 MHz spectrometer at 30°C. ^1H - ^{15}N HSQC were collected with the ^1H and ^{15}N carriers set to the water resonance and 120 ppm, respectively. NMR data were processed using NMRPipe⁸⁵ and analyzed in NMRFAM-SPARKY.⁸⁶

FACS analysis of oxMIF, CD74 and CD44 expression

To induce the expression of endogenous CD74 on the cell surface, HT29 human colon carcinoma cells (RRID:CVCL_0320) were treated with IFN- γ (500 U/ml, PeproTech, catalog # 300-02) for 24 hours, or left untreated in tissue culture flasks. The next day, cells were dislodged from flasks by trypsinization, counted, and seeded at 2×10^5 cells/well, in 50 μl of growth

medium/well (McCoy's 5a Medium Modified +10% FBS) in a U-bottom 96-well plate. MPO-oxMIF was generated as described above, and H₂O₂-MIF (in the absence of MPO) was used as a negative control. A total of 50 µl of MPO-oxMIF (or negative control) was added to 50 µl of cell suspension in a 96-well plate. After incubating at 37°C for 30 min, cells were pelleted by centrifugation, washed with FACS staining buffer (SB, composed of PBS + 5% FBS), and resuspended in 100 µl of ON104, rabbit monoclonal anti-MIF antibody (property of OncoOne R&D GmbH) and isotype control (all antibodies at 70 nM). After incubation for 40 min on ice, cells were pelleted by centrifugation and, after washing with FACS SB, incubated with goat anti-human IgG-AF488 (H+L specific, Thermo Fisher Scientific) for detection of ON104 and isotype control, and goat anti-rabbit IgG-AF488 (H+L specific, Thermo Fisher Scientific) for detection of rabbit monoclonal anti-MIF antibody. In separate dedicated wells, cells were stained with 3 µl of the anti-human CD74-PE (clone LN2, BioLegend), anti-human CD44-PE-Cy5 (clone IM7, BioLegend), or their isotype controls (PE-labeled mouse IgG1,k, or PE-Cy5-labeled rat IgG2b, k, respectively), for 40 min on ice. After washing, cells were stained with eFluor780 viability dye (eBioscience), and washed and resuspended in FACS SB.

Alternative to the induction of endogenous CD74 on the cell surface by stimulation with IFN- γ , HT29 cells were transiently transfected with pCMV6-AC plasmid DNA encoding the full-length human CD74 transcript variant 2, accession number NP_004346.1 (Origene, catalog # SC319602), or with mock plasmid CAG/HaloTag®-HiBiT/BlastR (Promega, catalog # CS1956B17) encoding the intracellular HiBiT-tagged HaloTag® protein by using Lipofectamine 3000 reagent (Thermo Fisher Scientific, catalog # 11668027) according to manufacturer's protocol. Briefly, cells were seeded into 75 cm² tissue culture flasks at 3.5x10⁶ cells per flask in 15 ml of growth medium (without antibiotics) and allowed to adhere overnight. On the following day, in each flask cells

were transfected with 25 µg of plasmid DNA, 50 µl of P3000 reagent, and 68 µl of Lipofectamine 3000, using OPTI-MEM Reduced Serum Medium (Thermo Fisher Scientific, catalog # 31985062) for diluting Lipofectamine 3000 and DNA plus P3000 reagent before complexing. 48 hours post-transfection, cells were dislodged by trypsinization, counted, and binding of MPO-oxMIF and H₂O₂-MIF to CD74-transfected *versus* mock- and non-transfected cells was studied as described above. In separate dedicated wells, cells were assessed for the expression of CD74. Cell surface staining with anti-human CD74-PE (clone LN2, BioLegend) and isotype control (mouse IgG1,k-PE) followed by the staining with eFluor780 viability dye was performed as described above. Next, to assess the total (cell surface + intracellular) expression of CD74, after the cell surface expression of CD74 was measured, the left cells were fixed with IC fixation buffer (eBioscience) for 15 min, washed with PBS, permeabilized by incubation for 10 min with ice-cold methanol, and stained with anti-human CD74-PE, or isotype control.

LPS-activated neutrophils isolated from 3-5 healthy donors were washed in SB, centrifuged at 300× g for 10 min, and stained with viability dye (1:1000; FVD-eFluor780, eBioscience) for 15 min at RT. Cells were washed and incubated for 40 min at 4 °C with a fixed concentration of ON104-AF488 (20 nM) or its isotype IgG-AF488 (20 nM). After a washing step, cells were incubated with a mix of conjugated antibodies anti-human CD66b-APC (clone 6/40c, BioLegend) at 1:100, and anti-human CD74-PE (clone LN2, BioLegend) at 1:50 in SB, for 40 min at 4 °C. Cells were then fixed with 100 µl of IC fixation buffer (eBioscience) for 10 min, washed and resuspended in SB. Forward Scatter Height (FSC-H) *vs.* Area (FSC-A) as well as Side Scatter Area (SSC-A) *vs.* FSC-A plots allowed the discrimination of singlet events and total cells respectively. Cells were analyzed for FVD- eFluor780 signal and high events were excluded, as they correspond to dead or dying cells. Neutrophil (CD66b⁺) subset was identified and oxMIF and

CD74 expression was assessed. Statistical analysis was performed using paired and non-parametric Wilcoxon t-test.

Data acquisition was performed on CytoFLEX S flow cytometer (Beckman Coulter, US) and data were analyzed with FlowJo software (BD). Frequencies and geometric mean fluorescence intensity values (geoMFI) for AF488 and PE channels were transferred into GraphPad Prism software.

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Appendix

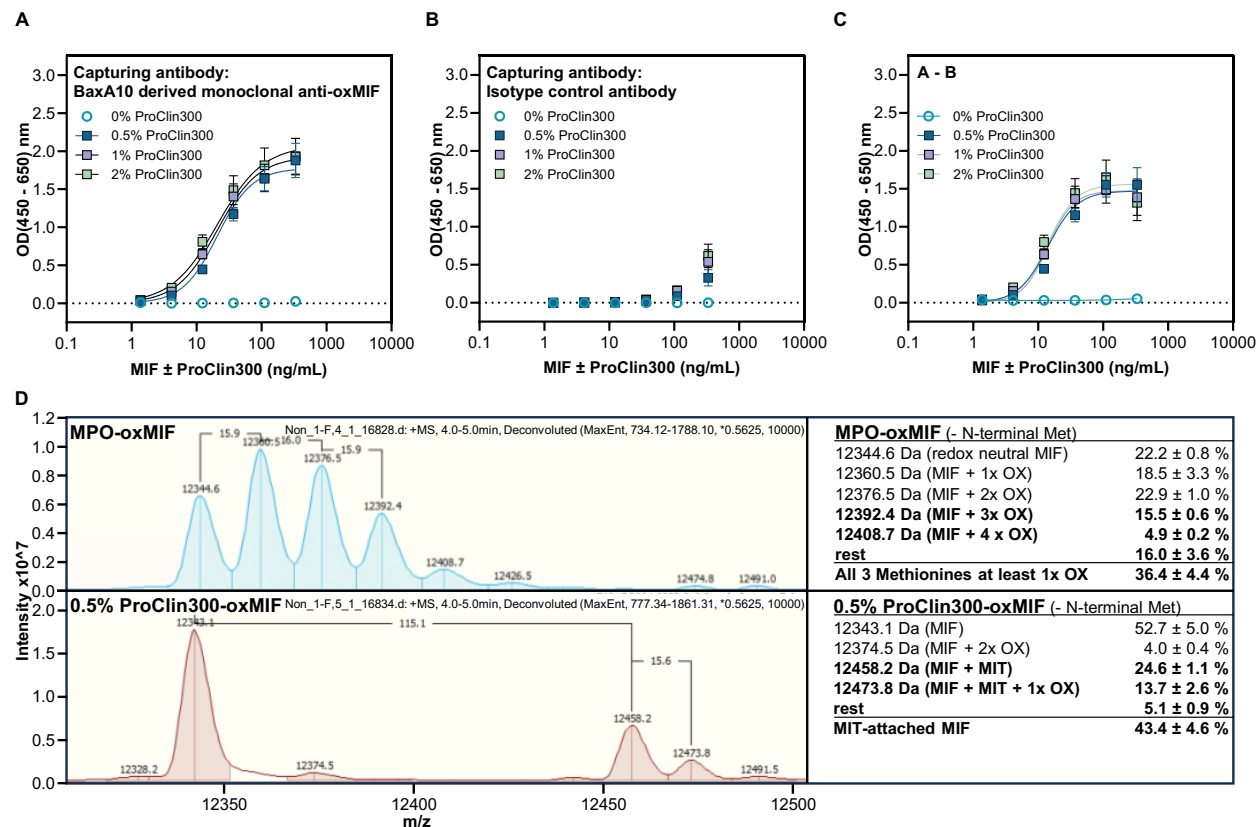


Figure 1. Validation of 0.5% Proclin300-oxMIF as a standard for oxMIF ELISA. (A) The effect of 0.5%, 1% and 2% Proclin300 on MIF was tested by ELISA, where our anti-oxMIF antibody was used to capture. No difference in binding was observed with greater than 0.5% Proclin300 applied to MIF, suggesting that the conversion to oxidized-like MIF was complete. (B) No binding between Proclin300-MIF and an isotype control capturing antibody was observed. At higher concentrations of Proclin300 in the treated MIF samples, a contribution to the background signal was observed, however. Signals from (B) were then subtracted from (A). After background correction (C), no differences to (A) were observed. In all cases, no binding was observed for MIF. For each condition tested: biological samples $n = 3$, technical replicates $n \geq 2$. (D) Intact mass MS analysis of MPO-oxMIF and 0.5% Proclin300-MIF. The MPO-oxMIF sample was prepared with 5x H_2O_2 within the MPO system. Total oxidation of MPO-oxMIF was defined as an addition of at least 3 oxygen atoms (+ 3x 15.99 Da) per MIF subunit, where 1 subunit contains 3 methionines. The MS signal intensities of 3x (or more) oxidized MPO-oxMIF sum to $36.4 \pm 4.4\%$. In the Proclin300-MIF sample, the attachment of Proclin300 component methylisothiazoline (MIT, MW = 115.1 Da), was observed for $43.4 \pm 4.6\%$ of MS signal intensities. The extracted percentages for both samples suggest that oxidation or modification of one-third of MIF subunits is sufficient for its transition to oxidized (or oxidized-like) conformation and that the levels of oxidation between the two samples were comparable. MPO-oxMIF: biological samples $n = 5$, technical replicates $n = 1$; Proclin300-oxMIF: biological samples $n = 3$, technical replicates $n = 1$ per biological sample. Relative amount reported as mean $\pm$ SD.

CHAPTER SIX

Discussion

Studies of the Allosteric Regulation of MIF

Macrophage Migration Inhibitory Factor (MIF) is a ubiquitous and multifunctional human protein with pro-inflammatory and enzymatic activities.¹ Given its compact structure, the mechanistic principles that facilitate its non-overlapping functions remain elusive. Further, MIF is implicated in a broad range of diseases marked by chronic inflammation, including arthritis,² sepsis,^{3,4} cancers,⁵⁻¹⁰ and neurodegeneration,¹¹⁻¹⁵ positioning MIF as a compelling target for the development of therapeutics that selectively target its disease state structures. However, the role that MIF plays in these diseases, and the impacts of disease pathophysiology to the structure and function of MIF, are not well understood, necessitating the elucidation and characterization of the structure-function axis of MIF under healthy and disease-linked conditions to inform drug discovery efforts.

Allostery, a regulatory process that functionally couples distant sites within a protein,¹⁶⁻¹⁸ has emerged as a key determinant of the underlying mechanisms of MIF biochemistry. This thesis has undertaken foundational studies of the allosteric regulation of MIF, including identification of allosteric residues and networks that control its enzymatic and signaling functions,^{19,20} and the impacts of pro-inflammatory stimuli on the biophysical properties and biomolecular interactions of MIF.^{21,22} The sensitivity of solution NMR spectroscopy is leveraged to visualize MIF structure and dynamics with atomic resolution that are critical for the propagation of long-range allosteric communication, including lowly-populated conformations and multi-timescale motions that are not captured in static crystal structures and had not yet been explored in the MIF field.²³⁻²⁶ The biophysical insights provided by NMR studies of MIF in this dissertation, coupled with additional synergistic techniques such as MD simulations, SPR, X-ray crystallography, and mass spectrometry, and in conjunction with enzymatic and pro-inflammatory assays that demonstrate

the functional effects of allostery, have contributed important findings to the understanding of the allosteric regulation of MIF function by elucidating novel allosteric sites to target for translational outcomes.

Key Findings and Future Directions

Chapters Two and **Three** provide foundational studies of MIF allostery through the elucidation and characterization of allosteric pathways within the MIF structure and how modulation of these pathways impacts function. **Chapter Two** identified an allosteric pathway that controls MIF tautomerase function, comprised of the catalytic Pro1, Met2, His62, and Tyr99.¹⁹ When the allosteric pathway residues His62 and Tyr99 were mutated to alanine, phenylalanine, and glycine, the enzymatic activity of MIF was altered, as expected for allosteric sites. Interestingly, a correlation was observed between the size of the central solvent channel and altered tautomerase activity. When mutated to phenylalanine, where the solvent channel opening is decreased relative to WT MIF, an increase in tautomerase activity is observed. Conversely, when the solvent channel opening is increased due to alanine and glycine residues, tautomerase activity decreases. MD simulations of solvent channel water molecules explored this phenomenon, and found that the Phe variants have a dense and highly ordered hydrogen bond network proximal to residue 99 when compared to WT, while Ala and Gly mutants exhibited little-to-no water-mediated hydrogen bonds proximal to residue 99. These data suggested that water molecules are important for bridging His62 and Tyr99, and disruption of the hydrogen bond networks results in a decoupling of communication between them, leading to decreased enzymatic activity. It also established an important functional role for the central solvent channel of MIF. NMR studies showed that mutation of His62 and Tyr99 caused structural changes to the other allosteric pathway residues, providing atomic evidence of the allosteric coupling. Further, mutation of residues at

Pro1 and Met2 caused structural perturbations at His62 and Try99, demonstrating the inherent reciprocity of allosteric signaling. The NMR results also demonstrated an inverse correlation between protein dynamics and function, where mutation of His62 and Tyr99 modulated the dynamics of MIF, via line broadening, in a way that is inversely proportional to catalytic activity. Intriguingly, Tyr99 was previously implicated in an allosteric pathway that control CD74 activation. Thus, this work identifies Tyr99 as an important allosteric node that links its tautomerase and pro-inflammatory function, thus establishing a regulatory network that mediates functional control.

Chapter Three delves further into the role of Tyr99 as a critical allosteric mediator in MIF.²⁰ Tyr99 was mutated to a cysteine (Y99C), where the sulfhydryl group is the same molecular distance from the active site as C γ of Tyr99, but eliminates the π - π stacking of Tyr99 and His62. Further, it was hypothesized that the cysteines would form inter-monomer disulfides within the central solvent channel and provide insight into Tyr99 inter-subunit communication. To sterically encourage disulfide formation, engineered heterotrimeric variants containing WT and Y99C monomeric subunits (Y99C/WT mixed trimers) were studied alongside Y99C homotrimers. X-ray and NMR analyses did not suggest the formation of disulfides; however, distinct differences were observed structurally, dynamically, and functionally between the homo- and hetero-trimeric assemblies. The Y99C variant resulted in structural perturbations at the allosteric pathway residues described in Chapter Two and at the C-terminus, where CD74 receptor activation has been mapped. Y99C/WT mixed trimers also showed perturbations in these regions, though to a lesser extent than the respective homotrimers. Given the fewer structural perturbations observed and the presence of WT monomers within the heterotrimers, it was postulated that Y99C/WT mixed trimers would inhibit tautomerase activity to a lesser extent than Y99C homotrimer. However, the

Y99C homotrimer only reduced tautomerase activity by 55%, while the Y99C/WT mixed trimers exhibited a 77% reduction in catalytic activity. To rationalize these results, NMR spin relaxation studies reveal that the Y99C/WT mixed trimers exhibit increased flexibility at critical regions of the protein relative to WT and the Y99C homotrimer. Dynamics and subunit symmetry may therefore be important factors in Tyr99-mediated allosteric communication.

Building on these foundational studies of MIF allostery, future work will expand the molecular picture of the allosteric regulatory network that has emerged as a result of this work. In addition to CD74 activation and tautomerase activity, MIF is also believed to be an oxidoreductase and a nuclease.^{27,28} Mapping the allosteric pathways that regulate these enzymatic functions, and characterizing if and how they intersect with the pathways already described, will provide a comprehensive understanding of the allosteric regulation of MIF function and clarify how the MIF structure toggles to accommodate its functions within a compact molecular space. These studies should also be expanded to the binding interactions of MIF with its expansive interactome.²⁹⁻³⁵ An important direction of this work on the allosteric regulation of MIF is to also characterize the spatial, temporal, and environmental conditions under which these allosteric pathways are activated or deactivated. Therapeutics can then be intuitively designed to target the allosteric network to yield tunable control.

Chapters Four and Five investigate the impacts of pro-inflammatory stimuli on the biophysical properties and functions of MIF given its expression at sites of inflammation, which are known to be oxidizing cellular environments due to increased levels of reactive oxygen species. In **Chapter Four**, the properties of hydrogen peroxide-treated MIF are explored relative to redox-neutral and reduced MIF.²¹ NMR studies revealed that MIF is structurally perturbed and becomes increasingly dynamic under oxidizing conditions. Mass spectrometry found that Cys80, previously

identified as a redox switch, is selectively modified under oxidizing conditions, where an increase in cystine abundance is observed. This modification may be important for the conversion of MIF to its oxidized conformation. Mutation of redox-sensitive residues that are modified (C80) or highly dynamic (K66) under oxidizing conditions resulted in an attenuation in redox-dependent structural changes. Further, mutation of K66 and C80 reduced tautomerase activity and CD74-activated neutrophil recruitment *in vivo*. Thus, K66 and C80 are identified as latent allosteric sites, revealed in this work under oxidizing conditions, that alter enzymatic function and CD74 activation. Further, they allosterically modulate the conformational landscape of MIF under oxidizing conditions.

Chapter Five extends the studies of biologically-relevant oxidants and their impacts on MIF to hypochlorous acid (HOCl).²² HOCl, generated by neutrophil-released myeloperoxidase, was found to promote the oxMIF conformation and selectively bind to anti-oxMIF antibodies. HOCl appears to more efficiently alter the MIF structure than hydrogen peroxide, suggesting that it is a major player in acute inflammation, whereas hydrogen peroxide-mediated changes may be more relevant under sustained inflammatory conditions. Leveraging the anti-oxMIF antibodies, it was found that human neutrophils generate the distinct oxMIF conformation upon stimulation. NMR studies revealed biophysical signatures of HOCl-modified oxMIF that are distinct from those observed for redox-neutral MIF, including increased flexibility across the MIF structure and, notably, at the opening of the central solvent channel where MIF is proposed to bind the CD74 receptor. Mass spectrometry revealed that the major sites of chemical modification by HOCl oxidation occur at the MIF methionine residues, which line the monomer-monomer interface and may facilitate a ‘loosening’ of the MIF trimer through increased dynamics, as observed by NMR, to allow the anti-oxMIF antibodies to bind its epitope within the central solvent channel. SPR

investigations of the MIF-CD74 receptor interaction reveal that oxMIF, but not redox-neutral MIF, binds to CD74. Collectively, these data demonstrate that pro-inflammatory oxidative conditions modulate the conformational plasticity of MIF and facilitate its interaction with the CD74 receptor, suggesting a redox-driven mechanism to its inflammatory signaling function, which is an important finding for the field of MIF biology.

An important future direction to the study of MIF under pro-inflammatory, oxidizing conditions is to provide a comprehensive molecular picture of the disease-relevant oxMIF conformation. Computational docking is a high-throughput method to preliminarily assess binding of small molecules to proteins. However, this structure-guided approach necessitates a realistic model as input, which is currently lacking for the oxMIF conformation. The added lack of structural perturbations in any crystallographic snapshots of MIF, despite abundant evidence to the contrary in solution, compounds this issue. Structure determination by NMR may be difficult due symmetric and trimeric structure of MIF. However, measurement of residual dipolar couplings (RDCs) by NMR, and comparison to MD-simulated conformational ensembles, may be a viable route to generating a three-dimensional picture of oxMIF. RDCs report on the orientation of internuclear vectors, which can provide highly sensitive information on the tertiary and quaternary structure of a protein.³⁶ RDCs can be back-calculated from structural models and compared to the experimental RDC measurements for structural refinement. Sedinkin *et al.* recently demonstrated the utility of comparing experimental RDCs with those calculated from molecular dynamics (MD)-simulated conformational ensembles to determine which ensemble is the most consistent with the experimental data.³⁷ The conformational ensemble that best captures oxMIF can then be used for docking of small molecules that selectively target this disease-relevant conformation of MIF.

Another possible route for solving the structure of oxMIF is through cryo-EM of oxMIF bound to an anti-oxMIF antibody, and attempts to do so are ongoing.

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