Novel Role for *Macrophage Migration Inhibitory Factor* in the Regulation of Inflammation

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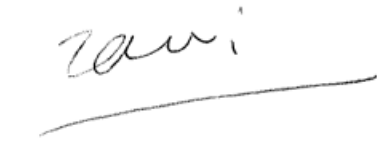
A. Declaration

I hereby declare that the work presented within this thesis is the result of my own investigation except where a reference has been made to published literature or acknowledgement is made for unpublished research. While registered as a research degree student at Monash University, I have not been a registered or enrolled for another award of this university or of any other academic or professional institution.

Shahrzad Zamanitaghizadeh Rabe

5.1.2020



B. List of abbreviations

4-IPP	4-Iodo-6-phenylpyrimidine
AKT	Protein kinase B
ALPS	Autoimmune lymphoproliferative syndromes
ALR	AIM2-like receptor
ALS	Amyotrophic lateral sclerosis
ASC	Apoptosis-associated Speck-like
BMDM	Bone marrow-derived macrophages
CpG-ODNs	cytosine-phosphate-guanosine-oligodeoxynucleotides
D-DT	D-dopachrome tautomerase
DAMP	Danger-associated molecular pattern
DHICA	5,6-dihydroxyindole-2-carboxylic acid
dsRNA	double-stranded RNA
ER	Endoplasmic reticulum
EV	Extracellular vesicle
GM-CSF	Granulocyte macrophage colony-stimulating factor
HMGB-1	High mobility group box protein 1
HSP90	Heat shock protein 90
HTS	High-throughput screening
IFN	Interferon
Ii	Invariant Chain
iBMM	Immortalized bone marrow-derived macrophages
IRF3	Interferon Regulatory Factor 3
ISO-1	(S, R)-3-(4-hydroxyphenyl)-4, 5-dihydro-5-isoxazole acetic acid methyl ester
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases
M-CSF	Macrophage colony-stimulating factor

MDH	Malate dehydrogenase
MIF	Macrophage migration inhibitory factor
MSU	Monosodium urate
NF-κB	Nuclear factor kappa B
NLR	Nod-like receptor
NO	Nitric oxide
oxMIF	Oxidised MIF
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
pDC	Plasmacytoid dendritic cell
PEC	Peritoneal exudate cells
PI3K	Phosphoinositide 3-kinases
PS	Phosphatidylserine
PTM	Post-transnational modification
RIPK1	Receptor-interacting protein kinase 1
RIPK3-MLKL	Mixed lineage kinase domain-like
SLE	Systemic lupus erythematosus
SMAC	Second mitochondria-derived activator of caspases
SOD1	Superoxide dismutase 1
TLR	Toll-like receptor
TNF-α	Tumour necrosis factor-α
TNFR1	Tumour necrosis factor receptor 1
TPOR	Thiol-protein oxidoreductase
UPEC	Uropathogenic Escherichia *coli*
XIAP	X-linked IAP

C. Acknowledgement

First, and most of all, praises and thanks to the God for the strength he gives me each day and without his blessings, this study would not have been possible.

My heartfelt gratitude to my family for their love, prayers, caring, and sacrifices throughout my study. I am very much thankful to my daughter, Mehrnoosh, who experienced all of the ups and downs of my research and for her valuable prayers.

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I would gratefully acknowledge the financial, academic and technical support of Monash University, particularly in the award of two prestigious Postgraduate Research Scholarships to undertake my research.

Last but not least, I am proud of myself for successful management of many challenges I faced during my candidature to complete my study.

D. List of publications during candidature

1. Shahrzad Zamani, Tali Lang, Jacinta P.W. Thomas, Eric F. Morand, James Harris. Migration inhibitory factor (MIF) is released following lytic cell death: Possible role as a DAMP. Poster presented at: 46th ASI conference; November, 2017; Brisbane, Queensland.
2. Shahrzad Zamani, Tali Lang, Jacinta P.W. Thomas, Eric F. Morand, James Harris. Migration inhibitory factor (MIF) is released by dying monocytes like a danger-associated molecular pattern (DAMP). Poster presented at: 8th Annual ASMR Victorian Student Research Symposium; June, 2017; Melbourne, Victoria.
3. Shahrzad Zamani, Eric F Morand and Jacqueline K Flynn. "Assays for Measuring Cell Death". James Harris and Eric F. Morand (eds.), Macrophage Migration Inhibitory Factor, Methods and Protocols, Methods in Molecular Biology, vol. 2080. Springer Nature, 2019, 173-183.
4. Mitophagy and the release of inflammatory cytokines. James Harris, Nadia Deen, Shahrzad Zamani, Md Abul Hasnat. Mitochondrion; Volume 41, July 2018, Pages 2-8.

- A part of this published article is extracted from the results I obtained in my research work:

Necrotic cell death increases the release of Macrophage migration inhibitory factor (MIF) by monocytes/macrophages. Wendy Dankers, Md Abul Hasnat, Vanesa Swann, Arwaf Alharbi, Jacinta P.W. Lee, Megan A. Cristofaro, Michael P. Gantier, Sarah A. Jones, Eric F. Morand, Jacqueline K. Flynn, James Harris. Immunology and cell biology. doi:10.1111/imcb.12376.

E. Table of figures

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Abstract

BACKGROUND: Macrophage migration inhibitory factor (MIF), produced by monocytes/macrophages, has been reported to be involved in inflammatory responses and wound healing. Endogenous and exogenous danger associated molecular pattern (DAMP) molecules possess either beneficial tissue repair function or provoke detrimental uncontrolled inflammation responses. The release of DAMPs following cell lysis or tissue damage is implicated in the pathogenesis of multiple auto-inflammatory diseases. An increasing number of studies link MIF release to conditions in which cell membrane leakage and tissue damage occur. However, the mechanism by which MIF is released has not been fully elaborated.

AIMS: The aim of this study was to seek evidence of MIF as a DAMP molecule. Also, MIF tautomerase activity as a key mechanism involved in this function was explored.

METHODS: In the first set of studies, MIF release following cell death was studied. For this purpose, various forms of cell death including apoptosis, primary necrosis, pyroptosis and necroptosis were induced in THP-1 monocytes. Cell death inhibitors were also employed. Cell death was confirmed using Annexin V-FITC and PI staining and lactate dehydrogenase (LDH) release. MIF release was measured using ELISA. Next, MIF modulatory role in inflammatory responses induced by TLR4 activation was investigated. For this, wild-type (WT) and MIF gene deplete (*Mif*$^{-/-}$) macrophages of different sources and classical dendritic cells were employed. Pro-inflammatory responses in WT and *Mif*$^{-/-}$ cells were induced by TLR4 activation using bacterial lipopolysaccharide (100 ng/ml) for various time points (2, 6 and 24 h). WT cells in separate groups were pre-treated with an inhibitor of MIF tautomerase activity (4-IPP). Produced levels of type I interferon and TNF-α were measured using luciferase reporter bioassay and ELISA, respectively.

RESULTS: Data indicated a significant correlation between MIF release and lytic but not apoptotic forms of cell death in monocytes. In addition, findings demonstrated a variable role for MIF in TLR4 induced type I interferon and TNF-α production in both macrophages and dendritic cells. In addition, a possible role for MIF tautomerase function was suggested.

CONCLUSION: Together, these results for the first time unveil a correlation between MIF release and lytic modes of cell death in monocytes, suggesting passive release following cell lysis. These findings identify lytic cell death as a novel mechanism involved in MIF release. Importantly, MIF can regulate TLR-dependent production of type I interferon differently in macrophages and dendritic cells. These findings shed new light on the novel role of MIF as a potential DAMP molecule.

Keywords: MIF, DAMP, Cell death, Type I interferon, Inflammation, Macrophage, Dendritic cell

Chapter 1. Literature Review

1.1. Macrophage migration inhibitory factor – structure and variants

Macrophage migration inhibitory factor (MIF) is a multi-functional molecule with hormone, cytokine, chaperone and enzyme like activities (1-3). MIF is a small protein (12.5 kDa) composed of 114 amino acids (4, 5). The MIF family comprises the MIF-1 isoform and the recently recognised *D*-dopachrome tautomerase isoform (D-DT, MIF-2) that have similar but not identical structural and functional characteristics (6, 7).

Investigation of three dimensional structure of MIF family proteins suggested conflicting results regarding the presence of single form or a mixture of monomer, dimer and trimer forms *in vivo* (2). MIF monomer subunits associate to form homodimer or homotrimer units via C-terminus which could also dissociate into the monomeric state (8). This dynamic oligomer interconversion may be associated with MIF's distinct functional diversity. Both the trimeric and monomeric units exert chaperone-like function (9, 10). The trimeric MIF form has a solvent-permeable cavity in the centre and the catalytic site of its enzymatic activity located between two monomeric units (8). MIF possesses intrinsic dopachrome/phenylpyruvate tautomerase catalytic activity and also thiol oxidoreductase enzymatic functions.

MIF is expressed by a wide range of tissues and cells of the immune system including dendritic cells, monocytes and macrophages (11, 12). In addition to chaperone-like and intrinsic enzymatic activities, MIF can directly interact with various intracellular and extracellular molecules and receptors (12).

MIF was historically recognized in 1966 as a cytokine that regulates macrophage migration during type IV hypersensitivity responses (13, 14). MIF was later "re-discovered" as it appeared to have the capacity to act as a pituitary hormone and also to counteract the immunosuppressive and anti-inflammatory responses of glucocorticoids (15, 16).

MIF-2 (D-DT) is another family member introduced as a structural homolog (Figure 1) of MIF in 1998 (17). Both similar and different properties between D-DT and MIF-1 have been reported (6, 18-20). Gene encoding MIF-1 and D-DT proteins are located next to each other on chromosome 22. Based on amino acid sequence similarity of about 35%, MIF-1 and D-DT are considered as structural homologous proteins (18). Three-dimensional structure of both proteins is similarly a barrel-shaped homotrimer (21). Several recent studies implied significant multi-functional similarity between MIF-1 and D-DT (22). Both proteins have been reported to be expressed ubiquitously in many cell types and tissues during physiological and inflammatory conditions (23). When they bind to the same cell surface receptor (CD74/CD44) complex, intracellular ERK1/2 MAP kinase is activated that results in pro-inflammatory responses (7, 22). In terms of their tautomerase enzymatic function, MIF-1 exerts stronger activity than MIF-2. During the last years, various inhibitors of tautomerase activity were identified that suppress MIF-1 alone (*S,R*-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester, ISO-1) or both MIF-1 and MIF-2 (4-iodo-6-phenylpyrimidine, 4-IPP) simultaneously (24, 25).

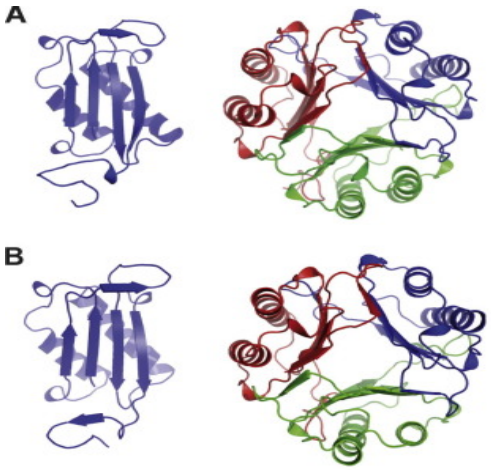


Figure 1. Three-dimensional structure of human MIF proteins.
X-ray crystallography of (A) D-DT and (B) MIF-1 proteins in the forms of monomer (left panel) and homotrimer (right panel). Each monomer consist of two antiparallel α-helix and a β-sheet. Three identical monomers of human MIF isoforms assemble into a homotrimer structure with rotational symmetry. This figure adapted from Merck et al., 2012 (6).

Oxidised MIF (oxMIF) is a recently discovered disease-related isoform of MIF that circulates in the plasma under certain conditions. oxMIF expression by several immune cells that play important roles in acute infections, cancers and chronic inflammatory disorders is reported (26). Recently, targeting oxMIF by employing a neutralising antibody was shown to successfully suppress the tumorigenic effect of MIF and enhance the response of tumor cells to anti-cancer therapies (27). Moreover, the application of monoclonal antibodies against oxMIF lessened the severity of acute and chronic enterocolitis, cooperatively with glucocorticoids, in mouse models (26). These findings indicate that oxMIF can be a valuable biomarker and therapeutic target in different cancers (26-28).

1.2. Mechanisms of MIF action

Unless otherwise specified, here the term MIF is used for the described "original" isomer (MIF-1). MIF can act as cytokine, enzyme, chemokine, hormone and chaperone in autocrine, paracrine and endocrine modes of action (29). MIF biological functions including binding to receptors and interactions with protein partners, both of which could be affected by its intrinsic catalytic activities (12).

1.2.1. Intrinsic enzymatic functions

1.2.1.1. Dopachrome tautomerase activity

X-ray crystal structure analysis (Figure 2) reveals that MIF contains a tautomerase motif on the interface between monomer subunits of trimeric form; the catalytic site located on the pocket encompasses proline (Pro1) at the N-terminal residue (30). The tautomerase pocket is involved in conversion of keto and enol forms of D-dopachrome (d-isomer of 2-carboxy-2,3-dihydroindole-5,6-quinone) into DHICA (5,6-dihydroxyindole-2-carboxylic acid) (30). The relevant physiological MIF substrate is still unclear, since currently known substrates, D-dopachrome and its precursor (D-Tyrosine), are not produced in vertebrates (1).

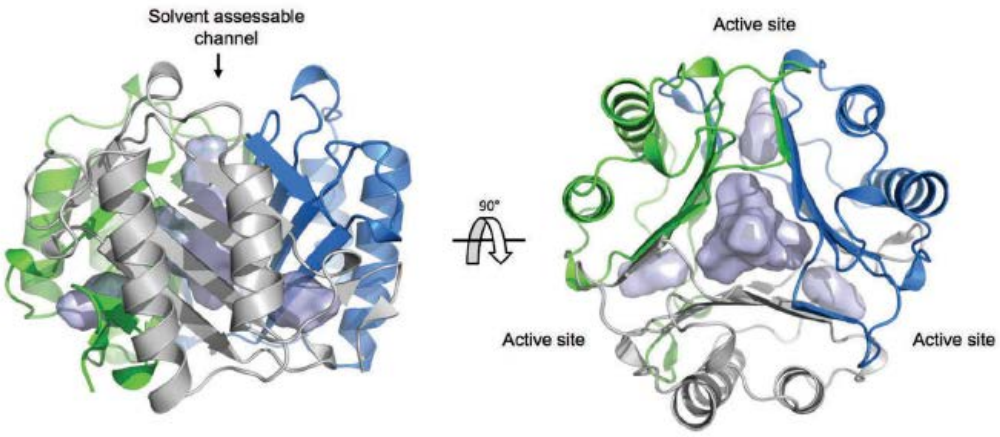


Figure 2. The flexibility of the three-dimensional structure of MIF.
One tautomerase active site located between the interfaces of monomer subunits in each MIF homotrimer molecule that are surrounded by a central hydrophobic channel. This figure adapted from Bloom et al., 2016 (12).

During recent years, several classes of competitive, allosteric, or covalent/suicide inhibitors of MIF tautomerase activity are reported to antagonise its biological functions (31). However, there continues to be controversy about the exact biological importance of the MIF tautomerase active site (12).

1.2.1.2. Thiol-protein oxidoreductase (TPOR) activity

MIF molecules contain one pseudo-(E)LR motif (Asp-44-X-Arg-11) that acts as the active site for TPOR function (12). MIF has cysteine-mediated redox function due to the intrinsic thiol (di-sulfide) protein oxidoreductase (TPOR) property that requires a disulphide motif in the centre of its catalytic site (32). The cysteine 81 residue of MIF acts as "switch cysteine" in the central thiol-protein oxidoreductase motif and enables the formation of an enzymatically active form of MIF (28).

MIF participates in controlling cellular redox homeostasis via its TPOR activity (33). In addition, MIF oxidoreductase (redox) activity promotes myocardial protection by reducing oxidative stress during ischemia-reperfusion injury (34,

35). Moreover, MIF TPOR activity could play a role in the suppression of apoptosis and binding to intracellular partners in macrophage stimulation during septic shock (36-38). MIF-2 (D-DT) lacks the pseudo-(E)LR motif (18).

1.2.2. Molecular chaperone-like role

MIF is a heat-stable molecule and exposes a hydrophobic surface that makes it a member of the "small molecular chaperone" family (39). MIF possess ATP-independent chaperone-like action assisting in proper folding of various macromolecules to reach their final active conformation and enable their normal functions (9, 10, 39). Dissociation of MIF oligomers into the monomer form can efficiently drive its chaperone-like function to bind and refold the thermally denatured molecule of malate dehydrogenase (MDH) and glycogen phosphorylase *b* (Ph*b*) into their native conformations (39).

Some studies suggested chaperone-like activity to play a role in pathological conditions. Amyotrophic lateral sclerosis (ALS) disease is manifested by a mutation in the superoxide dismutase (SOD1) gene and the abnormal accumulation of SOD1 protein. It is shown that MIF can play a protective role in ALS by direct binding with misfolded SOD1 and preventing its aggregation (40-43). Also, MIF binds to insulin in pancreatic beta cells to support the proper folding and hexamer formation that ensure normal insulin function (10).

1.2.3. Hormone- and chemokine-like activities

MIF has been described as a hormone derived from the anterior pituitary that counteracts the immunosuppressive function of glucocorticoids (16, 44, 45). Moreover, MIF was historically introduced as the regulator of random migration and chemotaxis of macrophages (46). These findings imply a role for MIF as a cytokine, chemokine or ligand for specific receptors. This will be described in detail below, but a full review of the many reported pro-inflammatory actions of MIF is beyond the scope of this chapter.

1.3. MIF secretion and release

1.3.1. Unconventional mode of secretion

Intracellular MIF is mainly localised in cytoplasmic small vesicles/pools (47, 48), nucleus (49, 50) and is accompanied by Golgi complex-associated protein p115 (51). MIF is secreted by non-classical pathways without targeting to endoplasmic reticulum (ER), as a result of lacking a signal sequence at the amino (N-terminal) terminus of its polypeptide chain (48). Unconventional secretion modes of inflammatory proteins, particularly danger-associated molecular patterns (DAMPs), by extracellular vesicles can be either active by live cells after stimulation, or passive as a result of necrosis (52).

1.3.2. Constitutive and stimulated secretion

MIF is constitutively produced by many cell types and released in small amounts from pre-existing intracellular pools (47, 53). MIF is variously reported to be released following by stimulation with pathogen-associated molecular pattern (PAMP) molecules (bacterial lipopolysaccharide, LPS), cytokines (TNF-α), and hormones (corticosteroids and adrenocorticotrophic hormone) (3, 47, 51, 54). However, studies in this thesis call into question these findings.

1.3.3. MIF in extracellular vesicles

Extracellular vesicles (EVs) can act as an essential route of intracellular and cell-to-cell communication by conveying biological cargoes and signal transduction under physiological and pathological conditions (55). Characterisation of the proteomic content and functional analysis of EVs can provide valuable clues for identifying novel biomarkers and therapeutic targets in disease (56, 57).

It has been reported that MIF can be secreted by non-classical mode via EVs in some cell types in response to particular signals and thus transfer regulatory signals (47, 58, 59). For example, MIF is co-expressed with well-defined biomarkers in the exosomes secreted from lung cancer cells that could improve

the specificity of lung cancer diagnosis (60). In addition, MIF is secreted in exosomes by adipocytes in response to adipokines that affect insulin resistance (61, 62).

1.4. MIF-1 and D-DT signal through receptor binding and endocytosis

MIF binding to different receptor complexes (Figure 3) has been reported to trigger various cell responses such as recruitment of immune cells, production of inflammatory mediators, cell proliferation and survival (63, 64). The diversity of reported cell surface binding partners of MIF is not typical of cytokines, and which of the reported receptors is most important for MIF function is still unresolved.

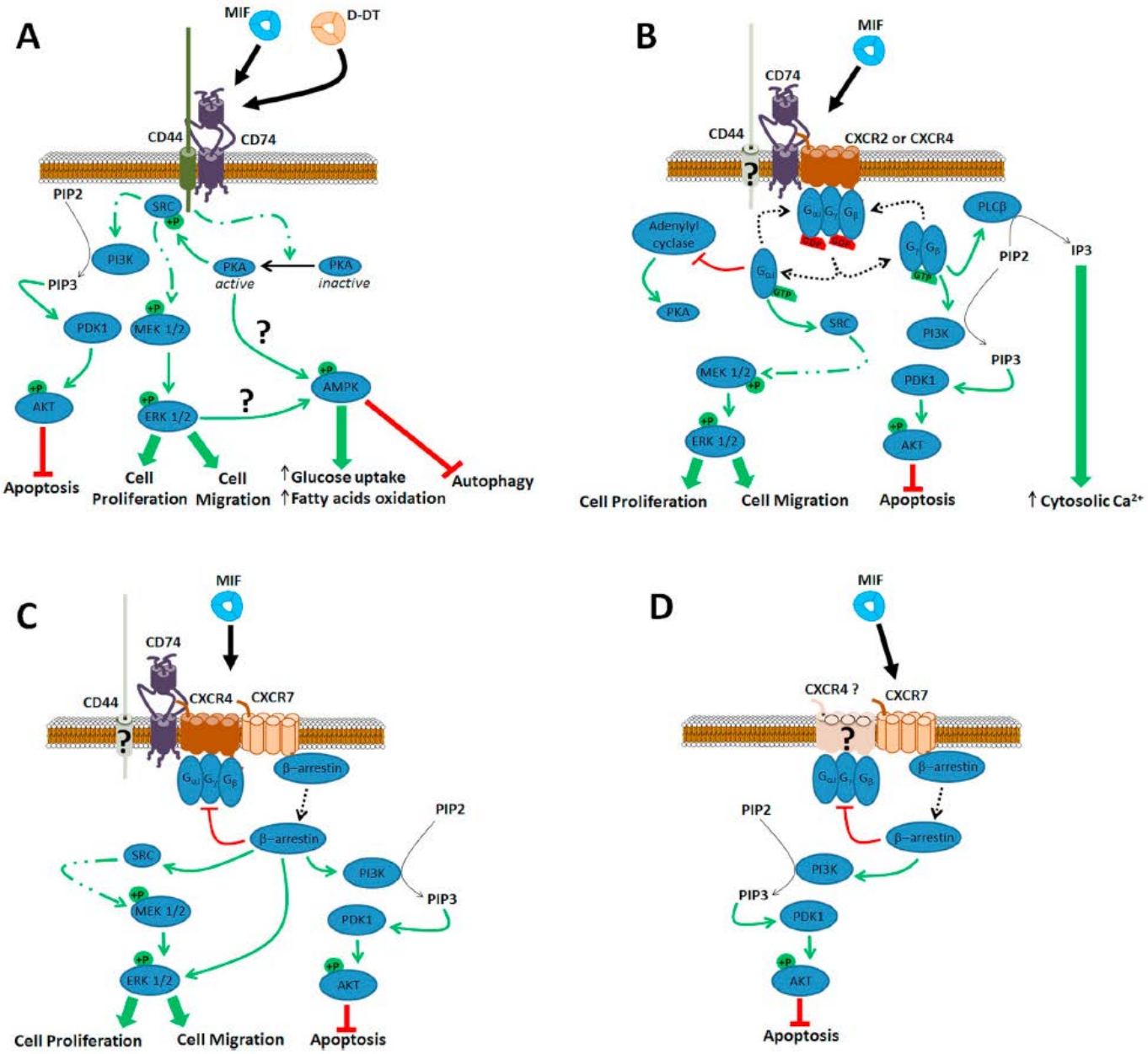


Figure 3. Surface receptor complexes for MIF family cytokines and their triggered signalling pathways.

(A) Interaction of MIF-1 and D-DT (MIF-2) with CD74-CD44 complex result in the phosphorylation of SRC that activates ERK1/2, PI3K-AKT and AMPK signalling cascades regulating cell growth, migration and cell death, glucose uptake and autophagy. This is the only identified pathway for MIF-2 (B) MIF binding to CD44-CXCR2 or CXCR4 complexes resulting in the activation of G-coupled proteins followed by triggering PI3K-AKT, PLC-β and ERK1/2 signalling events. (C) Two potential signal transduction modes following MIF ligation to the receptor complex consisting of CXCR7 involves the activation of PI3K-AKT and ERK1/2 mediate by β-arrestin. (D) Binding of MIF to CXCR4/CXCR7 complex without the involvement of CD74 and CD44 via β-arrestin could only stimulate the PI3K-AKT signals. This figure adapted from Jankauskas et al., 2019 (18). In the graph, green and red lines represent stimulation and inhibition, respectively.

1.4.1. Cognate receptor

CD74 (invariant chain of MHC class II) is referred to the cognate MIF receptor and is reported to be involved in the recruitment and formation of MIF receptor complexes (65). Both MIF-1 and MIF-2 are described to form high-affinity receptor-ligand complexes with CD74 (7). CD74 is involved in some immune-relevant disorders through controlling cell activation, expansion and motility (66, 67).

1.4.2. Non-cognate receptors

The co-receptor molecule CD44 is reported to be a fundamental member of the MIF-CD74 receptor complex (64). Phosphorylation and recruitment of CD44 following by MIF-CD74 binding is described to mediate subsequent activation of several signalling cascades (63, 64). In addition, CXC chemokine receptors (CXCR2, CXCR4, and CXCR7) are associated with MIF receptor complex that promote induction of multiple downstream signalling cascades (64, 68-71). CD74 is identified as an important element of these MIF receptor complexes by immunoprecipitation studies (18).

1.5. MIF biological activities

1.5.1. Diversity of MIF biological functions

MIF can exert a wide range of biological properties (Table 1) according to the producing cell and conditions (72).

Table 1. A summary of various functions and molecular mechanisms of MIF action.

Biological roles	**Mechanisms of action**
Inflammatory cytokine	Induces the expression pro-inflammatory mediators
Chemotactic chemokine	Induces rolling and adhesion of leukocytes to vessel wall Promotes transendothelial migration of leukocytes
Hormone activity	Glucocorticoid antagonist released by the anterior pituitary
Enzymatic activity	Tautomerase activity Thiol-protein oxidoreductase (TPOR) activity
Deregulation of cell cycle	Regulates p53-mediated cell death
Neuronal renewal	Promotes neural stem/progenitor cells proliferation Enhances neural differentiation
Tumorigenic activity	Stimulates proliferation of tumour cells and tumour growth Inhibits apoptosis Suppress anti-tumoral immune responses
Pro-angiogenesis	Induces secretion of pro-angiogenic factors
Cancer metastasis favouring	Reduces E-cadherin expression Increases matrix metalloproteinases expression

Table is adapted from Nobre et al., 2017 (73).

1.5.2. Post-translational modifications affect MIF functions

Post-translational modifications (PTMs) of MIF (Table 2), particularly at proline and cysteine residues of the catalytic site, are suggested to impact MIF functions and may be partly responsible for the diverse functions exhibited by MIF (74).

Table 2. Post-translational modifications in MIF molecule and their biological significance.

Modification type	Chemistry of modification	Biological consequences	Ref
Met-1	Cleavage of N-terminal methionine	Unknown	(15, 75)
Cys-57/60	Conformational change of ß-sheet	Disease-related isoform of MIF in septicemia, psoriasis, asthma, ulcerative colitis, Crohn's disease, Alzheimer's	(76)
Pro-2	Oxidation of proline-imine	Tautomerase function loss Maintained anti-apoptotic activity	(26)
Pro-2	Covalent binding of isothiocyanates epicatechin-quinone, carbamylation	Tautomerase function loss Conformational alteration Inhibition of CD74 binding	(77-80)
Cys-60	Cysteinylation at Cys-60	Regulation of B and T cells responses	(81)
Cys-81	S-Nitrosation	Enhancement of oxidoreductase function Augmenting cardioprotective function	(82, 83)
Ser-112 **Thr-113**	S-Glycosylation	Reduced ERK1/2 and AKT activation	(81)
Ser-91	Phosphorylation	Reduction of cysteinylated MIF function	(81)

Table is adapted from Schindler et al., 2017 (74).

1.6. MIF as a candidate DAMP molecule

1.6.1. DAMP definition – release and function

According to “Danger Theory” model (84), danger-associated molecular pattern (DAMP) molecules can be secreted actively or passively by stressed or necrotic cells. They act as danger signals once they are released following lytic cell death and damage during microbial infections, inflammation and tumour progression (85-87). However, the definition of danger message transporter molecules has been refined since the first description, and the term has caused many controversies (88).

1.6.2. DAMP molecules are released upon necrotic cell death

1.6.2.1. Cell death modes

The ancient dogma about the definition of apoptosis and accidental necrosis has been changed by finding new forms of death. Apoptosis is a programmed form of cell death which aims to maintain homeostasis, remove damaged cells and promote normal tissue development and healing (89, 90). Apoptotic cells are eventually cleared via phagocytosis, termed efferocytosis, without triggering inflammation (91). In comparison, necrotic forms of cell death can be triggered by various external and internal factors and lead to severe tissue damage and cell death (89, 90). Different types of necrotic cell death including necroptosis, pyroptosis, and secondary necrosis end in shared cellular outcomes (92). Table 3 provides a brief description of the various forms of cell death and their association with the release of DAMP molecules (92).

Advanced understanding of relationships between disease development and aberrant cell death pathways may result in strategies that contribute to clinical intervention. Different modes of cell death are linked with tissue injury and dysregulation immune responses in several diseases including cancers, infection, neurodegenerative diseases, and inflammatory and autoimmune disorders (93, 94).

Table 3. Comparison of different cell death forms.

	Apoptosis	**Necroptosis**	**Pyroptosis**	**Necrosis**
Mode of cell death	Programmed	Programmed	Programmed	Accidental
Initiators	TNF-α FasL TRAIL Pathogens Environmental factors	TNF-α FasL TRAIL Microbial infections Ischemic injury	DAMPs Microbial infections	Toxins Infections Inflammation Trauma
Intermediate signalling	Caspase-3, -7, -8-dependent Mitochondrial pathway	Caspase-independent TNFR signalling JNK activation Necrosomes	Caspase 1-dependent Nod-like receptors Pyroptosome Inflammasomes	-
Cellular events	Non-lytic Cell shrinkage DNA fragmentation Apoptotic bodies	Lytic Loss of plasma membrane Swollen organelles	Lytic Pore formation Leak of contents	Lytic Leak of contents
Inflammation	Non-inflammatory	Pro-inflammatory	Pro-inflammatory	Pro-inflammatory
Immunogenicity	-	++	++	+++
DAMPs released	-	Long genomic DNA IL-6	HMGB1 ATP IL-1α/β IL-6 IL-18 TNF-α Chemokines	HMGB1 ATP IL-1α IL-33 mRNA Genomic DNA

Table is adapted from Inoue et al., 2013 (95).

1.6.2.1.1. Apoptosis and primary necrosis

Apoptosis is a programmed cell death that follows a distinct sequences of events including initiation, execution and final stages (96). Apoptosis is tightly regulated through two major (extrinsic and intrinsic) signalling pathways (96). These pathways are tightly regulated through several canonical signals including nuclear factor NF-κB, c-Jun N-terminal kinase (JNK), tumour protein p53, and tumour necrosis factor (TNF) pathways. Also, numerous environmental factors modulate apoptosis-related signalling pathways (91, 97). The final cellular outcome of apoptosis is a set of changes in normal cellular morphological and biochemical features including chromatin condensation, membrane blebbing, and fragmentation of genomic DNA (96, 98). In addition, apoptotic cells/bodies can expose newly expressed or modified molecules such as phosphatidylserine (PS) as "eat me" signals to trigger phagocytosis (99, 100). Cells undergoing apoptosis are eventually cleared via phagocytosis without triggering inflammation (91, 101). Abnormal apoptosis is associated with the pathophysiology of autoimmune diseases (102), microbial infection (103), cancer (104), developmental abnormities (105), and neurodegenerative disorders (106). Understanding the relationships between environmental triggers and compromised signalling is pivotal to the development of therapeutical and preventive modalities (91).

In contrast to apoptosis, necrotic forms of cell death occur as a pathological process following exposure to physical, chemical and physiological damage (107). Morphologically, necrosis can be defined by the swelling of mitochondria, disruption of plasma membrane and explosive release of cellular substances (108). The final outcome is release of danger molecules and elicitation of pro-inflammatory responses (107, 109).

Primary and secondary necrosis (accelerated primary necrosis) have both shared and distinct properties (92, 110). Primary necrosis is induced by toxic substances, depletion of ATP, heat shock response, freeze thaw cycles, toxins, oxidative damage, all of which lead to cell lysis, DAMP release and activation of

inflammatory and immune responses (102, 111, 112). Secondary necrosis is a passive cell swelling that occurs as the consequence of deficient or insufficient removal of apoptotic cells by phagocytes (113, 114).

1.6.2.1.2. Pyroptosis

Pyroptosis is a programmed and caspase-dependent form of necrotic cell death that ends in cellular lysis, and release of pro-inflammatory mediators (115). Inflammasomes are protein complexes involved in pyroptosis induction. They consist of procaspase-1 zymogen and a member of NLR (Nod-Like Receptor) family or AIM2-like receptor (ALR) family, which are linked directly through CARD (Caspase activation and recruitment domain) interaction or indirectly by the adapter ASC (Apoptosis-associated Speck-like) protein (116, 117). The formation of NLRP3 inflammasome complex results in the activation of caspase-1 and ultimately the secretion of mature, bioactive forms of IL-18 and IL-1β, and DAMPs such as IL-1α and HMGB1 (118, 119). Nigericin, a bacterial pore-forming toxin, can induce pyroptosis mediated by NLRP3 inflammasome (120). Pyroptotic cell death is a protective mechanism for macrophages and dendritic cells in response to intracellular microbial pathogens (112, 121). In addition, pyroptosis can be induced by silica, asbestos, monosodium urate (MSU) crystals, and nigericin in macrophages and dendritic cells (122). Moreover, pathogen-associated molecular patterns (PAMPs) can induce pyroptotic death through the stimulation of toll-like receptors (112, 121). Although inflammasome activation during pyroptosis plays a vital role against intracellular pathogens, aberrant or excessive induction may contribute to inflammatory diseases, Alzheimer, diabetes, and cancer (123, 124).

1.6.2.1.3. Necroptosis

Necroptosis is a regulated type of necrotic cell death induced by death receptors, toll-like receptors and interferons (125). Ripoptosome and necrosome complexes

are involved in necroptosis induction via toll-like receptor 3 (TLR3) and the tumour necrosis factor receptor 1 (TNFR1) signal transduction pathway (126). Kinase activity of receptor-interacting protein 1 and 3 (RIP1 and RIP3) is required for necroptosis induced by TNF-α (127). The final stage of necroptosis represents morphological features of both apoptosis and necrosis. The necroptotic form of cell death is associated with DAMP release due to plasma membrane disruption and stimulation of pro-inflammatory responses. Released DAMP molecules act as cytokines and chemotactic factors to contribute to acute and chronic inflammatory reactions (128). Necroptosis can be induced experimentally using artificial second mitochondria-derived activator of caspases (SMAC) mimetics that antagonise X-linked IAP (XIAP) in caspase suppressed cells (129, 130).

Necroptosis is associated with the production of organ-specific DAMPs that could be considered as diagnostic biomarkers (131). In addition, necroptosis could be of clinical importance in the pathogenesis of various inflammatory diseases, cancer and neurodegenerative disorders (131-134).

1.6.2.1.4. Ferroptosis

Ferroptosis is a non-apoptotic regulated mode of cell death and is dependent on the intracellular levels of iron (135-137). Ferroptotic cell death results from lethal accumulation of lipid peroxide arising from reactive oxygen species (ROS) generation (138). Ferroptosis is involved in various physiological and pathological events such as neurodegeneration and elimination of cancer cells (139).

1.6.2.1.5. Autophagic cell death

Autophagy is the main intracellular mechanism responsible for degrading and recycling of unwanted cytoplasmic components and nutrients to provide new essential components or source of energy (140). Autophagy is involved in normal

homeostasis by controlling cellular damage, removal of senescent cells and suppression of tumorigenesis (141). Also, autophagy has been shown to regulate innate and adaptive immune responses against intracellular pathogens (142). Autophagy-mediated cell death is a caspase-independent programmed cell death that occurs during physiologically and pathologically relevant circumstances such as embryogenesis and elimination of tumor cells (143).

1.6.3. Evidences suggesting MIF is released like a DAMP molecule

MIF is found abundantly in the extracellular space during inflammation and tissue injury (144). Some studies linked MIF release to conditions in which cell membrane leakage and tissue damage occur (87, 145-148). A study showed that MIF exacerbates neurologic damage through the induction of cell death in stroke, and MIF deletion reduced neuronal death induced by spinal cord damage in mice (149, 150). Circulating levels of MIF potentially resulting from DAMP-like release are suggested as a valuable prognostic biomarker for neurological injury, ischemia-induced hepatocellular damage, post-cardiac arrest syndrome and renal cortex necrosis (87, 145-148, 151).

In a recent report, remarkable MIF release was reported in human neutrophils following secondary necrosis but not stimulation by microbial agents or inflammatory mediators (152, 153). However, MIF release upon cell death induction in monocytes/macrophages has not been previously shown. This knowledge gap is addressed in my thesis.

1.6.4. Key roles of DAMP molecules

1.6.4.1. DAMP molecules modulate immunity and inflammation

Danger-associated molecular pattern (DAMP) molecules are recognised by multiple intracellular and extracellular pattern recognition receptors (PRRs). The main DAMP-sensing receptors (Table 4) are toll-like receptors (TLRs), RIG-I-

like receptors (RLRs), NOD-like receptors (NLRs), and the receptor for advanced glycation end products (154, 155).

Table 4. Pattern recognition receptors (PRRs) and their relevant DAMP ligands.

PRR family	Major members	DAMP ligands
TLRs	TLR1-9	HMGB1, HSPs, S100 proteins, histones, DNA, RNA, mtDNA, syndecans, biglycan, versican, heparan sulfate, fibrinogen
NLRs	NOD1, NOD2, NLRP family	Uric acid, mROS, histones, biglycan, LMW hyaluronan
RLRs	RIG-I, MDA5, LGP2	RNA
CDSs	AIM2-like receptor	DNA
Scavenger receptors	CD36, CD44, CD68, CD91, CXCL16, RAGE	HMGB1, HSPs, S100 proteins, calreticulin, versican

TLR, toll-like receptor; NLR, NOD-like receptor; RIG-I-like receptors (RLRs); CDS, cytosolic DNA sensor. Table is adapted from Roh et al., 2018(155).

DAMP molecules are of pathological importance for diseases such as cancers, autoimmune disorders, metabolic syndrome, and neurodegenerative diseases (155, 156). Accumulating evidence from clinical studies suggests many DAMP molecules as valuable prognostic/diagnostic biomarkers and potential therapeutic targets in various infectious and inflammatory diseases (155-157).

As summarised in Table 5, DAMPs can exert a vast range of inflammatory functions including conveying the danger message, regulating immune responses, contributing to cellular interactions, and coordination of tissue regeneration processes (86). Although DAMPs contribute to normal host defence and promote repair regeneration, they can serve as danger signals to trigger innate and adaptive immune responses following interaction with their sensing receptors (155, 158). Activation of immune responses leads to the production of cytokines and pro-inflammatory mediators such as interferon type I and TNF-α (159).

Table 5. DAMP molecules involved in sterile inflammation and inflammatory disease.

Sensing receptors	Expression pattern	DAMPs	Pro- inflammatory functions	Inflammatory diseases
TLR2	Ubiquitous DCs Monocytes Macrophages Neutrophils	HMGB1, HSPs, versican, biglycan, decorin, eosinophil- derived neurotoxin, surfactant protein A/D, β- defensin 3, histone	Promotes the production of pro- inflammatory cytokines and chemokines	IRI RA cancer inflammatory diseases
TLR3	Ubiquitous DCs Monocytes Macrophages NK cells	mRNA	Promotes the production of pro- inflammatory cytokines, chemokines and IFN type I	IRI inflammatory diseases
TLR4	Ubiquitous DCs Monocytes Macrophages Neutrophils Endothelial cells	HMGB1, tenascin- C, HSPs, S100s, biglycan, decorin, heparin sulfate, hyaluronic acid, fibrinogen, fibronectin, β- defensin, surfactant protein A/D, lactoferrin, neutrophil elastase, peroxiredoxin, histone, ox- LDL	Promotes the production of pro- inflammatory cytokines, chemokines and IFN type I	IRI RA cancer inflammatory diseases
TLR7	Ubiquitous pDCs Monocytes Macrophages B cells	IgG-ribonucleoprotein complex, microRNAs	Promotes the production of IFNα and other cytokines and chemokines	SLE inflammatory diseases
TLR9	Ubiquitous pDCs Monocytes Macrophages B cells	IgG-chromatin complex, mtDNA, HMGB1	Promotes the production of IFNα and other cytokines and chemokines	SLE inflammatory diseases
NLRP3	DCs neutrophils Monocytes Macrophages	MSU, glucose, cholesterol crystals, ATP, Alu- RNA	Promotes IL-1β and IL-18 secretion initiates pyroptosis	Gout Atherosclerosis
RIG- I	Ubiquitous epithelial cells myeloid cells	Endogenous 5′ppp RNA	Promotes the production of IFN- I and other cytokines and chemokines	SLE cancer
AIM2	Ubiquitous Epithelial cells DCs Monocytes Macrophages B cells NK cells	Cytoplasmic DNA, damaged DNA	Promotes IL-1β and IL-18 secretion Initiates pyroptosis	Cancer chronic kidney disease
RAGE	Ubiquitous	HMGB1, S100s, DNA	Upregulates inflammatory genes Promote migration proliferation apoptosis	Diabetes cancer inflammatory diseases

Alu-RNA, RNA transcribed from Alu elements; DAMP, damage- associated molecular pattern; DCs, dendritic cells; dsRNA, double- stranded RNA; HMGB1, high- mobility group box 1 protein; HSPs, heat shock proteins; IFN-I, type I interferons; MSU, monosodium urate; mtDNA, mitochondrial DNA; NK cells, natural killer cells; NLRs, NOD- like receptors; pDCs, plasmacytoid dendritic cells; RA, rheumatoid arthritis; RAGE, receptor for advanced glycation end products; RIG- I, retinoic acid inducible gene I; RLRs, RIG- I-like receptors; SLE, systemic lupus erythematosus, TLRs, Toll- like receptors. Table is adapted from Gong et al., 2018 (159).

1.6.4.2. DAMP molecules orchestrate the process of tissue repair

Tissue repair refers to a complex molecular and cellular process of regeneration of damaged tissue to restore typical architecture and function (160, 161). Several intracellular and extracellular DAMP molecules such as high-mobility group box (HMGB)-1 and adenosine triphosphate (ATP) are shown to orchestrate the healing process of damaged tissue by controlling the responses of innate and adaptive immunity, and tissue regeneration (162-164). In wound healing, released alarmins and DAMPs can act as chemokines and promote the migration of required cells (mainly phagocytes) to eliminate dead cells and debris (163). Afterwards, several cell types proliferate to replace the normal tissue architecture and extracellular matrix. Finally, pro-angiogenic functions of DAMP molecules can participate in the angiogenesis process to form new blood vessels (165).

1.6.5. Evidences suggesting MIF acts like a DAMP molecule

1.6.5.1. MIF can regulate innate and adaptive immune responses

Once released, MIF can regulate various aspects of immune system. MIF induces the production of pro-inflammatory mediators including TNF-α, IL-1β, IL-6, nitric oxide (NO), prostaglandin E_2 (PGE_2) and H_2O_2 (166), as well as enhancing macrophage activities such as intracellular killing and phagocytosis (167, 168). In addition, MIF triggers autophagy and in turn autophagy can mediate MIF secretion (169, 170). MIF also can regulate tissue homeostasis and wound healing (171, 172).

1.6.5.1.1. Production of type I interferons upon TLR stimulation

Type I interferons (IFN), mainly IFN-α and IFN-β, are part of the first line of host immune defence against microbial invasion (173). They exert biological functions including antiviral action, immunomodulatory and anti-proliferative effects (174). Enhanced activation of type I IFNs is shown in infectious diseases and inflammatory disorders such as AIDS, SLE and tuberculosis (173, 175).

As shown in Table 6, Type I IFNs can be produced by various cell types including dendritic cells, epithelial cells, phagocytes, fibroblasts and synoviocytes (176). The main intracellular pathways involved in their production include RIG-I (retinoic acid-inducible gene I), TRIF (TIR-domain-containing adapter-inducing interferon-β) and IRF7 (Interferon regulatory factor 7) (177).

Table 6. Major pathways of type I interferon production.

Pathways	Main actors	Localisation	Inducers	Cell types	Ref
RIG-I pathway	RIG-I (MDA-5)	Cytoplasmic	Single-strand RNA viruses Double-strand RNA viruses	Conventional DCs Fibroblasts Hepatocytes	(178-183)
TRIF pathway	TLR3-TRIF	Internal vesicles	Unmethylated ds RNA	Macrophages Hepatocytes	(181, 182, 184)
	TLR4-TRIF	Plasma membrane	Viral glycolipids		
IRF7 pathway	TLR9-MyD88-IRF7(IRF5)	Endosome	Unmethylated RNA Injured cells	Plasmacytoid DCs	(185-187)
	TLR7/8-MyD88-IRF7(IRF5)		Unmethylated CpG DNA Chromatin immune complexes		

The table is adapted from https://www.invivogen.com/review-type1-ifn-production.

Toll-like receptor 4 (TLR4) can interact with PAMP and DAMP molecules as binding ligands to trigger immune responses (188, 189). Bacterial lipopolysaccharide (LPS) is a well-defined ligand of TLR4 (189). Following by ligand recognition, TLR4 employs several downstream accessory molecule protein-protein interactions to transfer the stimulatory signal (190, 191). TLR4 signalling is comprised of two well-defined MyD88-dependent and MyD88-independent (TRIF-dependent) pathways (192). The MyD88-dependent pathway is responsible for the expression of several pro-inflammatory cytokines such as

IL-6 and TNF-α (192). The MyD88-independent pathway results in the production of interferon-inducible genes and type I interferon (193).

Toll-like receptor 9 (TLR9) has been shown to be localized in endoplasmic reticulum (ER), endosomes, and lysosomes (194). TLR is expressed in multiple immune cells such as plasmacytoid dendritic cells (pDCs), neutrophils monocytes, $CD4^+$ T cells and B cells as well as several nonimmune cell types (195). TLR9 can recognise DNA molecules containing unmethylated CpG motifs found in intracellular bacteria and viruses (196). TLR9 interaction with the cytosine-phosphate-guanosine-oligodeoxynucleotides (CpG-ODNs) triggers MyD88-dependent signalling cascade that culminate in the production type I interferon and inflammatory cytokines (197).

1.6.5.1.2. Regulation of TLR signalling pathways

TLR signalling can be down-regulated at multiple levels to ensure the termination of harmful inflammatory responses. In addition, some pathogens negatively control TLR signalling by various mechanisms to evade innate responses of host defence (198, 199). Major ways of regulatory mechanisms are mainly classified into major modes of blockade of adaptor complexes formation, disruption of signal-regulated proteins, TLR trafficking and transcriptional regulatory mechanisms (200).

The modulation of TLR4-induced responses is mediated at multiple levels including negative regulation by receptor endocytosis and degradation (201), transcriptional regulation (202, 203) and regulation of intracellular signaling elements (204, 205). Some factors such as cell type and exposure circumstances affect the production of type I IFNs (206). Although macrophages and dendritic cells both express TLR4, they differentially respond to the same ligands, a fact which may account for their different roles in immune responses (207).

Multiple stimuli including DAMP molecules are known to induce the production of type I interferons (188, 189). Here, I will focus on TLR4- and TLR9-dependent mechanisms that I study in my experimental sections.

1.6.5.1.3. MIF modulates TLR4-induced pro-inflammatory responses

The regulation of TLR4-induced responses by MIF in various cell types has been reported (208). Extracellular MIF was shown to control the cell surface level of TLR4 in response to LPS through controlling TLR4 mRNA expression (209-211). However, controlling the surface TLR4 levels is not the only way in which MIF can affect TLR4 responses (212, 213). A study has shown that TLR4-induced signals can be transduced in conditions of low levels of TLR4 receptor expression observed in MIF deficiency (213, 214). Induction of TLR4 responses in peritoneal macrophages of MIF knockout mice leads to a declined level of TNF-α without affecting IL-6 production, indicating selective post TLR4 effects (214). Exogenous MIF is reported to promote LPS-induced production of IL-1β and TNF-α (215). The expression of TLR4 and LPS-stimulated responses are neutralised by anti-MIF antibodies, a finding which shows the function of extracellular MIF (216). Also, the induction of NF-κB activity by MIF has been suggested as another step of modulating the TLR4 response (217-219).

Inhibition of MIF tautomerase activity or MIF silencing by use of siRNA reduces TLR4 expression, NF-κB nuclear translocation and the production of pro-inflammatory mediators (220, 221). Therefore, MIF tautomerase function could be one of the actions responsible for its modulatory effect on TLR4-induced responses. The involvement of MIF tautomerase activity is reported to be cell-type specific. ISO-1 (a MIF tautomerase inhibitor) can have various effects on TLR4-induced responses in both human monocyte-derived macrophages (MDM) and monocytes. This was shown by a remarkable decreased production of pro-inflammatory mediators by human primary monocytes but not macrophages stimulated with LPS. In addition, expression of TLR4 on cell surface of MDMs

was not affected by ISO-1. These findings suggest that modulation of MIF tautomerase function may alter TLR4 responses but could be dependent on the type of cell involved (222).

Although TLR4 signalling mediated induction of type I interferon is well described, the role of MIF in this effect is not clear. My thesis also aims to address this experimentally.

1.6.5.2. MIF can regulate the process of tissue repair

There is growing interest in the role of danger signals in tissue repair. Aside from MIF's ability to modulate immune responses, increasing evidence suggests MIF as a mediator of tissue regeneration and wound healing. MIF-dependent tissue repair is mediated via MIF-receptor complex and subsequent cellular and molecular responses (223). However, controversy exists about the actual role of MIF in tissue repair (Figure 4). On the one hand, MIF is believed to be beneficial in the process of wound healing (224, 225); on the other hand, some data suggest opposing effects (171, 172).

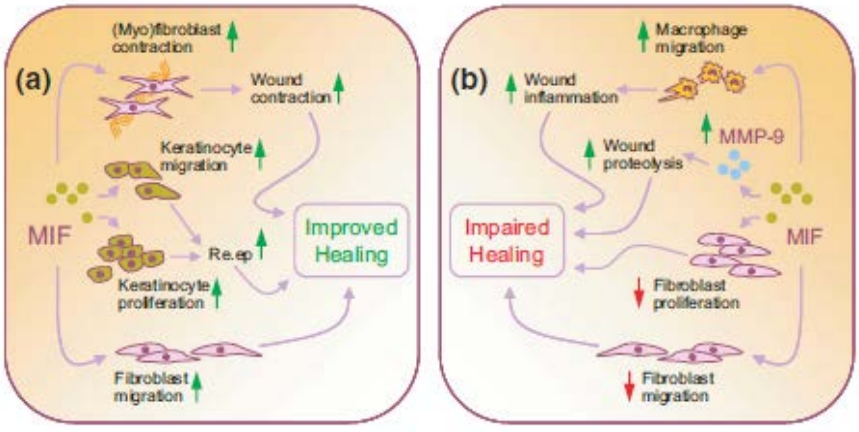


Figure 4. Opposing concepts about the role of MIF in tissue repair.
Two opposite theories including (a) advantageous and (b) unfavourable roles of MIF in wound healing presented. Re-ep; re-epithelialization. The figure is taken from Gilliver et al., 2010 (226).

1.7. MIF as a biomarker and therapeutic target

Several recent reviews suggested that MIF may serve as a clinical biomarker and novel therapeutic target for inflammatory diseases, autoimmune disorders, metabolic diseases and cancers (227-229). High MIF levels in plasma are reported in septic shock, severe sepsis and systemic inflammatory conditions (230). Although plasma levels of MIF do not explicitly discriminate the bacterial origin of infection, elevated MIF level is correlated with disease severity and early marker to predict poor outcome and mortality (231-233). In patients infected with *Neisseria meningitides*, increased MIF concentration strongly correlated with the severity of infection and fatal outcome (234). Also, higher level of MIF in cerebrospinal fluid is linked to virulent species of *Neisseria meningitis* and with disease severity (235). In addition, combination of MIF plasma level and other biomarkers is a valuable biosignature to discriminate between Tuberculosis and non-Tuberculosis pulmonary infections (236).

Furthermore, several studies pointed out a correlation between increased MIF levels in serum and other body fluids and severity of disease in some autoimmune diseases including SLE and rheumatoid arthritis (237, 238). In many cases, animal models of these diseases are improved by MIF antagonism.

1.8. Strategies for MIF inhibition

Diverse strategies have been employed to target a range of MIF physiological functions and some of them are under preclinical evaluation (239). They range from small molecule inhibitors capable of targeting the MIF tautomerase catalytic site to biologics-based inhibitors interfering with MIF production and biological functions (12). There are unique challenges and opportunities with using small molecules and biologics-based inhibitors targeting MIF function. However, small molecules have surpassed biologics as they render less immunogenicity, have more cost-effective manufacturing and are suitable for oral delivery and absorption (12).

Treatment with anti-MIF antibody and MIF antagonist was reported to diminish the severity of inflammatory responses, moderate tissue injury and improve the survival of chronic models such as obstructive pulmonary disease (COPD) and animal models such as Pneumococcal pneumonia infection (240, 241). In addition, treatment with MIF antagonist has been shown to alleviate the clinical severity of arthritis and myositis associated with Ross River virus infection in mice (242).

In cancers, MIF function contributes to multiple phases of pathogenesis including cell proliferation, angiogenesis and metastasis (73, 243). Blocking MIF by using tautomerase inhibitors, MIF siRNA or anti-MIF antibodies resulted in effective attenuation of cancer development and progression, indicating the potential application of MIF-targeted therapeutic approaches in malignancies (244-246).

1.8.1. Small molecule inhibitors

Since MIF has been recognized as a pharmacological target in many auto-inflammatory disorders, cancers and microbial infections, various types of MIF inhibitors that can target functions are under investigation (247). During recent decades, several approaches such as in silico methods and high-throughput screening (HTS) have been employed to screen and develop new drugs that interfere with the function of MIF (248).

During the past decades, several inhibitor compounds that suppress MIF tautomerase function have been discovered that could act directly via competition and allosteric or covalent binding to the tautomerase catalytic pocket (247). The MIF tautomerase active site is located adjacent to CD74 binding motif that could suggest impeding MIF signalling by inhibitors of tautomerase activity (249). Inhibition of MIF function by using covalent inhibitors of the tautomerase active site could interfere with its secretion and biological functions (248, 250).

The best-identified inhibitor of MIF tautomerase function is (*S*, *R*)-3-(4-hydroxyphenyl)-4, 5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) that is

an irreversible MIF antagonist (31). ISO-1 and some inhibitors of isoxazoline class can competitively interfere with several features of MIF activity by binding to Asn-97 residue of tautomerase catalytic site of MIF molecule (251). The effects of ISO-1 have been investigated *in vivo* in animal models of endotoxemia (252), colitis (253), melanoma (254) and colon cancer (254) and also *in vitro* glucocorticoid overriding (31), translocation of nuclear NF-κB (252, 253) and cytokine-induced death of beta cells (151) with promising outcomes. Jorgensen-3, a 1, 2, 3-triazoles derivative, acts as an agonist of MIF binding to CD74 receptor and interact with residue Asn-97 of MIF tautomerase catalytic site (255). Other examples of MIF tautomerase inhibitors are 4-iodo-6-phenylpyrimidine (4-IPP) and isothiocyanate BITC, reported to irreversibly inactivate MIF by covalent modification of N-terminus Pro-1 in tautomerase active site. These compounds can hinder several MIF biological roles, MIF secretion and binding to CD74 (80, 256, 257). Recently, the novel compound 4-(3-carboxyphenyl)-2,5-pyridinedicarboxylic acid (4-CPPC) was identified as a specific inhibitor of MIF-2 that interfere with MIF-2 interaction with CD74 (24). MIF receptor agonists and antagonists have been found to affect CD74 binding and signal transduction particularly through ERK1/2 and AMPK pathways (255). However, the administration of MIF tautomerase inhibitors as a pharmaceutical drug is currently restricted due to adverse effects such as off-target toxicity (258).

1.8.2. Biological-based inhibitors

MIF neutralising antibody treatment has been reported to effectively promote tumour regression, impede tissue healing and reduce inflammatory responses (225, 259-261). Therapeutic efficiency of a neutralising MIF antibody (Imalumab) in aggressive colorectal cancer is under clinical trial investigation in humans (262). In addition, heat shock protein 90 (HSP90) can stabilise MIF by reduction of its intracellular degradation in cancer cells (263, 264) and would be an alternative approach to target MIF activity in cancer (265).

1.9. Summary

In summary, various aspects of MIF biology suggest it as a DAMP, potentially released during cell death, and impacting on immune pathways such as type I IFN production. In my thesis, I examine these two aspects of MIF function.

Chapter 2. Materials and Methods

2.1. Chemicals and reagents

A list of all chemicals and other reagents used throughout this project and the source of their manufacture is presented in Table 7.

Table 7. List of chemicals and other reagents and the source of their manufacture.

Chemical	**Source**
Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4	Sigma-Aldrich
Bovine serum albumin (BSA)	Sigma
RPMI 1640 medium	Gibco
DMEM medium	Gibco
Fetal bovine serum (FBS)	Gibco
Penicillin/streptomycin	Gibco
NaCl	Merck
Na_2HPO_4	Merck
KH_2PO_4	Merck
NH_4Cl	Merck
$NaHCO_3$	Merck
KCl	Merck
EDTA	Merck
HEPES	Merck
$CaCl_2$	Merck
G418	Gibco
L-Glutamine	Gibco
TrypLE	Gibco

Ethanol	Merck
Nigericin	Cayman Chemical Company
Recombinant human TNF-α	ImmunoTools
BV-6	Selleckchem
Z-VAD-FMK	AdooQ Bioscience
Z-YVAD-FMK	abcam
Necrostatin-1	AdipoGen Life Sciences
Propidium iodide (PI)	Sigma
Sphero™ AccuCount Blank Particles	Spherotech Inc
Annexin V-FITC conjugate	BioLegend
Annexin V binding buffer	BioLegend
4-Iodo-6-phenylpyrimidine (4-IPP)	Sigma
Recombinant IFN type I	BEI Resources
Trypan blue solution	Sigma
Passive lysis buffer (5X)	Promega 2022-03-12
Luciferase assay reagents	Promega E1501
Human MIF ELISA kit	BioLegend
IL-1β ELISA kit	BioLegend
TNF-α ELISA kit	BioLegend
Lactate dehydrogenase (LDH) assay kit	Promega
Tetramethylbenzidine (TMB) solution	Life Technologies

2.2. Preparation of buffers and solutions

2.2.1. Phosphate-buffered saline (PBS)

137 mM NaCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, and 2.7 mM KCl were added in distilled water. The buffer was sterilized by autoclaving and stored at 4°C.

2.2.2. Red Blood Cells (RBC) lysing solution

Ammonium chloride lysis buffer (10X concentration) was prepared by adding NH_4Cl 8.02 g, $NaHCO_3$ 0.84 g and disodium EDTA 0.37 g to 100 mL of distilled water. Working solution was prepared using distilled water and stored at 4°C.

2.2.3. Propidium Iodide solution

Stock solution (1 mg/mL) of propidium Iodide (PI) was prepared in distilled water. Stock solution was protected from light and stored at 4°C.

2.2.4. Passive lysis buffer

Passive lysis buffer is formulated for rapid lysis of cells without the need for scraping adherent cells and the advantage of minimal auto-luminescence. Working solution (1X) was prepared by adding distilled water to stock buffer (5X concentration) and stored at -20°C.

2.2.5. Luciferase assay reagent

Working solution of luciferase assay reagent was prepared by adding luciferase assay buffer (10 mL) to each vial of lyophilized substrate. Working solution was protected from light and aliquots were stored at -20°C.

2.3. Media preparation

2.3.1. Complete culture media

RPMI 1640 and DMEM media were supplemented with heat-inactivated Foetal Bovine Serum (FBS, 10%, v/v), penicillin (100 U/mL), streptomycin (100 µg/ml) and L-Glutamine (2 mM). All media were stored at 4°C.

2.3.2. L-cell conditioned media

Conditioned medium obtained from L929 cells (murine fibroblast cell line) was used as an alternative to recombinant macrophage colony-stimulating factor (M-CSF) for the generation of bone marrow-derived macrophages (BMDMs). Briefly, L929 cells (5×10^5 cells) were maintained in T-175 cm^2 flasks with primary 50% confluence in 50 ml of complete RPMI 1640 media for 7 days at 37°C in a humidified atmosphere with 5% CO_2. L-cell conditioned medium was then collected, centrifuged (1200 rpm for 5 min) and passed through 0.2 µm filter. Collected media were stored at -80°C.

2.3.3. Ag8.653 conditioned media

Conditioned medium obtained from Ag8.653 cells (murine myeloma cells) was used as an alternative source of GM-CSF for the generation of murine bone marrow-derived dendritic cells (BMDCs). Briefly, Ag8.653 cells were first maintained in complete RPMI 1640 media containing G418 (1mg/mL). Then, cells (4×10^7) were cultured in T-175 cm^2 flasks containing 50 ml of complete RPMI 1640 media without G418 for 4 days. Conditioned medium was then collected, centrifuged (1200 rpm for 5 min), passed through a 0.2 µm filter and stored at -80°C. For the experiments, culture media was supplemented with 10 %, v/v of Ag8.653 conditioned media.

2.4. Animals

Female C57BL/6 Wild type (WT) and MIF-1 knockout (*Mif* $^{-/-}$) mice (8-12 weeks) were used with the approval of Monash University Ethics Committee (MMCB) and in accordance with National and institutional guidelines. *Mif*$^{-/-}$ mice were originally obtained from Professor R. Bucala, Yale School of Medicine (266, 267). All mice were housed in individually ventilated cages and were euthanized by the inhalation of carbon dioxide (CO_2).

2.5. Cell lines and culture conditions

2.5.1. Human monocyte cell line

The human monocyte THP-1 cell line has been widely used as a model for studying functions of monocytes/macrophages (268). In this study, THP-1 cells were maintained as suspension culture in complete RPMI 1640 medium at 37°C in a humidified atmosphere with 5% CO_2.

2.5.2. Immortalised mouse macrophage cell line

Mouse immortalized bone marrow-derived macrophages (iBMMs) were generated from primary bone marrow-derived macrophages from the WT and *Mif*$^{-/-}$ mice. The cells were immortalized with the CreJ2 virus and were grown as adherent cells in complete DMEM media at 37°C in humidified CO_2 incubator. For experiments, iBMM cells were detached from the flask using TrypLE solution and seeded in 96-well plates (1×10^5 cell/well) overnight (269).

2.6. Isolation and generation of mouse primary immune cells

All mouse procedures were undertaken under approval by Monash University Animal Ethics Committees.

2.6.1. Harvesting and culture of peritoneal macrophages

The peritoneal cavity is a common source for harvesting primary naive murine macrophages (270). To harvest peritoneal cavity cells (PECs), the dead mouse abdomen was firstly sprayed with ethanol (70%, v/v) and a small incision was made using a sterile scalpel. Then, abdominal skin was removed using forceps to reach the peritoneum. Next, 5 mL of pre-chilled PBS containing FBS (3%, v/v) was injected into the peritoneal cavity using a 5 mL syringe attached to a 25 gauge needle. After a gentle massage to the mouse abdomen to dislodge any attached cells, the peritoneal fluid was collected slowly. Subsequently, a plastic Pasteur pipette was used to collect the remaining fluid from the cavity. Finally, the cell suspension was collected into the tubes and centrifuged (1200 rpm, 5 min, and 4°C). The cell pellet was resuspended in complete RPMI medium and counted on a haemocytometer. Collected resident peritoneal cells were seeded in 96-well plates (3×10^5 cell/well) and incubated at 37°C in humidified incubator with 5% CO_2 overnight. Then, non-adherent cells were removed by gentle washing with medium and adherent cells were used for further treatment.

2.6.2. Isolation of bone marrow progenitor cells

To harvest bone marrow (BM) cells, muscles were cut away from the femur, knee joints were dislocated, and any remaining tissue from the femur was gently removed without any damage to the bone. Bones were placed in PBS on ice until ready to process. Then, bones were transferred to ethanol (70%, v/v) for 15 seconds, rinsed in PBS and then media. Both ends of each bone were cut off using heavy scissors and flushed gently with media using a 3 mL syringe and a 23 gauge needle. After that, the clumps of bone marrow cells were dissociated by drawing up into the syringe and gently pushing back out to obtain a single cell suspension. Harvested cells were centrifuged (1200 rpm, 5 min, and 4°C) and the pellet was resuspended in RBC lysis buffer for 5 min on ice. After that, samples were centrifuged and obtained pellet was resuspended in complete media. Viability of

cell suspension was confirmed to be more than 90% by trypan blue exclusion on a haemocytometer. Collected BM cells were divided into two aliquots to allow the generation of both macrophages and dendritic cells from each mouse.

2.6.3. Generation of bone marrow-derived macrophages

On Day 0 of BM harvesting, one part of isolated BMs was cultured in plates with low-attachment surface in complete RPMI supplemented with L-cell conditioned media (20%, v/v). The medium was renewed every 3 days (on days 3 and 6) to allow differentiation of BMs to bone marrow-derived macrophages (BMDMs). On Day 7, BMDMs were detached from the plate using a cell scraper, seeded in 96-well plate (1×10^5 cell/well) and incubated at 37°C in a humidified incubator with 5% CO_2 overnight. Using plates with low-attachment surface allows easier and better detachment of BMDMs. The myeloid progenitor cells that were cultured *in vitro* show relatively stable differentiation into BMDMs, suggesting better homogeneity than peritoneal macrophages (271).

2.6.4. Generation of bone marrow-derived dendritic cells

BM cells were seeded onto the culture flasks on day 0 in complete RPMI medium supplemented with Ag8.653 conditioned media (10%, v/v). Media was renewed on days 3, 6, 8 and 10 to allow differentiation of BM cells into immature bone marrow-derived dendritic cells (BMDCs). On Day 11, BMDCs were collected and seeded in 96-well plate (1×10^5 cell/well) (272).

2.7. Cell death induction

A textbook chapter authored during my candidacy outlines these methods in additional detail and is attached as appendix 1.

2.7.1. Induction of apoptosis and primary necrosis

Staurosporine and ethanol were employed to induce apoptotic and necrotic forms of cell death, respectively (273, 274). For this purpose, THP-1 cells were firstly challenged overnight with/without LPS (100 ng/mL) and then exposed to staurosporine (1 μM) or ethanol (%10, v/v) for a time course of 1-6 hrs.

2.7.2. Induction of pyroptosis

THP-1 cells were firstly challenged overnight with/without LPS (100 ng/mL). Then, LPS-stimulated THP-1 monocytes were exposed to nigericin (10 μM) for 3 h to induce pyroptotic cell death (275).

2.7.3. Induction of necroptosis

THP-1 cells were treated with a combination of recombinant human TNF-α (30 ng/mL), SMAC mimetic BV-6, (1 μM) and general caspase inhibitor (Z-VAD-FMK, 25 μM) for 24 h for induction of necroptotic cell death (276).

2.8. Cell death inhibition

These experimental details are also outlined in the published textbook chapter in Appendix 1.

Different types of cell death inhibitors were used in separate experimental groups for the following reasons:

- As positive control to verify the occurrence of the desired form of cell death
- To define cell death form leading to MIF release

2.8.1. Inhibition of pyroptosis

THP-1 cells in were pre-treated with pan caspase inhibitor (Z-VAD-FMK, 50 μM, 1h) or caspase-1 inhibitor (Z-YVAD-FMK, 25 μM, 1h) to hinder the occurrence of pyroptosis induced by nigericin in LPS-stimulated cells.

2.8.2. Inhibition of necroptosis

THP-1 cells were pre-treated with necrostatin-1 (30 μM, 1h) to block the occurrence of necroptotic cell death induced by a combination of TNF-α, SMAC mimetic BV-6, and general caspase inhibitor.

2.9. Assays to detect cell death

See also Appendix 1.

2.9.1. Propidium Iodide (PI) staining

The pattern of cell death induced by ethanol was detected using PI staining and SPHEROTM AccuCount particles to improve the precision of cell counting. For this purpose, counting particles were added to each well (1:10 dilution in FACS buffer, v/v) and the plate was then centrifuged (1200 rpm, 5 min, and 4°C). Next, pellets were washed with PBS and incubated in FACS buffer containing PI solution (5 μL) for 5 min at room temperature in the dark. Finally, samples (10 000 events) were assessed within 1 h using a flow cytometer and data were analysed by FlowJo v10 software. Cells positive for PI indicated the occurrence of necrotic cell death. The absolute cell count was calculated using the following equation: (A/B) x (C/D) = Number of cells per μL. Where:

A = number of events for the sample

B = number of events for the particles

C = number of particles per 50 μL

D = volume of test sample initially used in μL

2.9.2. Lactate dehydrogenase release assay

Pyroptotic and necroptotic forms of cell death were quantified by detection of lactate dehydrogenase (LDH) released due to the loss of cell membrane integrity. This enzymatic assay measures the released levels of LDH using a colorimetric-based method. The amount of formed product is proportional to the number of

necrotic cells. After the treatment, samples were centrifuged (1200 rpm, 5 min, and 4°C) and supernatants were collected. Next, equal amounts of supernatant from each sample was mixed with CytoTox reagent and incubated for 30 min at room temperature in darkness. Finally, equal amounts of stop solution was added to each well and the absorbance (490 nm) of product read using a microplate reader within 1 h. The following controls were included in the assay:

- No cell control, to exclude background readings of culture media
- Maximum LDH release control: lysis solution added to a separate group of untreated cells 45 min prior to supernatant collection to obtain total cellular LDH

The value for all samples were subtracted from background value and the corrected values were used to calculate cytotoxicity percentage.

2.9.3. SYTOX™ Green Nucleic Acid Stain

SYTOX® Green nucleic acid stain is a green-fluorescent nuclear and chromosome counterstain that allows quick determination of cell viability using flow cytometry (277). Briefly, harvested cells were exposed to 5 nM of SYTOX® Green solution (5 min, dark). The fluorescence emission was detected using a fluorescent reader.

2.9.4. Annexin V-FITC and PI dual staining

The pattern of cell death induced by staurosporine or blocked with pan caspase inhibitor was identified by dual labelling with Annexin V-FITC and PI. For these experiments, THP-1 cells were harvested from each well and washed with PBS. Then, cells were resuspended in staining buffer containing Annexin V-FITC (5 µL) in binding buffer (100 µL) and incubated for 15 min at room temperature in dark. After that, PI staining solution (5 µL) was added to each sample and incubated for a further 5 min at room temperature in the dark. Finally, samples (10 000 events) were assessed within 1h using a flow cytometer and data obtained were analysed by FlowJo v10 software. Cells positive for Annexin V-FITC and

negative for PI were considered as early apoptosis. Double positive cells were considered late apoptosis/necrotic forms of cell death.

2.10. Detection of cytokine release using ELISA

2.10.1. Measurement of human MIF release

Cell supernatants were collected and kept at -80 ˚C. The level of MIF in the supernatants was measured using ELISA kit (BioLegend, Inc) as the manufacturer's instruction. It is unclear from the manufacturer's instructions whether this ELISA kit can distinguish between MIF-1 and MIF-2.

2.10.1.1. Preparation of required buffers and solutions

- Coating buffer: 0.1 M Sodium Carbonate, 7.13 g $NaHCO_3$ and 1.59 g Na_2CO_3 were added to 1.0 L of distilled water and pH was adjusted to 9.5 with 10 N NaOH. Coating buffer was freshly prepared or used within 7 days of preparation and stored at 2-8°C.

- Reagent diluent: BSA (1%, v/v) was mixed with PBS and pH was adjusted to 7.2-7.4. Reagent diluent was freshly prepared.

- **Wash buffer:** PBS containing Tween-20 (0.05%, v/v) was prepared and stored at 2-8°C.

- **Stop solution:** 2 N H_2SO_4 was prepared and used as stop solution.

- Standards: Top standard (2000 pg/mL) was prepared from the stock standard using reagent diluent and vortexed to mix well. Then, serial dilutions of six tubes of standards at final concentrations of 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL and 31.25 pg/mL were prepared. Reagent diluent alone was used as blank to adjust for background absorbance.

2.10.1.2. Assay procedure and calculation of data

Human MIF (pg/ml) released by THP-1 cells in culture supernatants was measured using ELISA. In the first step, 100 µL of human MIF capture antibody (1:200 in coating buffer) was added to each well and incubated overnight at 4°C. Then, plate was aspirated and washed three times with wash buffer (≥ 300 µL/well). After the last wash, plates were inverted and blotted on absorbent paper to remove any residual wash buffer. After that, plates were blocked with assay reagent (≥ 200 µL/well) at room temperature for 1 h, followed by washing. Next, 100 µL of each standard and sample was added into the appropriate well and incubated at room temperature for 2 h. The plates were then aspirated and washed. In the next step, 100 µL of diluted detection antibody (1:200 in reagent diluent, v/v) was added to each well and incubated at room temperature for 1 h. Plate was then aspirated and washed. Afterwards, 100 µL of diluted Streptavidin-HRP A enzyme reagent (1:250 in reagent diluent, v/v) was added to each well and incubated at room temperature for 20 min. Contents were then aspirated and plate washed. In the final wash step, plate was soaked in wash buffer for 30 seconds to 1 min for each wash. Finally, TMB substrate solution (100 µL) was added to each well and incubated at room temperature for 30 min in darkness, followed by stopping the reaction by adding stop solution (50 µL) to each well. Finally, the plate was read on a Fluostar OPTIMA instrument (BMG LABTECH) at 450 nm within 30 min of stopping reaction and wavelength correction at 540 nm. The blank absorbance was then subtracted from the value of each standard and sample. The standard curve was plotted using Optima Control Software (BMG LABTECH) and was employed to calculate the concentration of human MIF (pg/ml) in samples.

2.10.2. Measurement of human IL-1β secretion

Cell supernatants were collected and kept at -80 ˚C. The level of IL-β in the supernatants was measured using ELISA kit (BioLegend, Inc) as the manufacturer's instruction.

2.10.2.1. Preparation of required buffers and solutions

- Coating buffer: 0.1 M sodium carbonate, 7.13 g $NaHCO_3$ and 1.59 g Na_2CO_3 were added to 1.0 L of distilled water and pH was adjusted to 9.5 with 10 N NaOH. Coating buffer was freshly prepared or used within 7 days of preparation and stored at 2-8°C.

- Assay diluent: FBS (10%, v/v) was mixed with PBS and pH adjusted to 7.0.

- **Wash buffer:** Tween-20 (0.05%, v/v) was added to PBS and used fresh or within 3 days of preparation (stored at 2-8°C).

- **Stop solution:** 1 M H_3PO_4 or 2 N H_2SO_4 was prepared and used as stop solution.

- Standards preparation: Top standard concentration of human IL-1β protein (2000 pg/mL) was prepared from the stock standard in reagent diluent and was vortexed to mix. Then, serial dilutions of six tubes of human IL-1β standards (31.25-100 pg/mL) were prepared. Reagent diluent alone was used as blank to adjust for the background absorbance.

2.10.2.2. Assay procedure and calculation of data

Level of human IL-1β (pg/ml) in culture supernatants was measured using ELISA. Firstly, 100 µL of diluted human IL-1β capture antibody (1:200 in coating buffer) was added to each well and incubated overnight at 4°C. Then, plates were aspirated and washed three times with wash buffer (≥ 300 µL/well). After the last wash, plates were inverted and blotted on absorbent paper to remove any residual buffer. Then, plates were blocked with assay diluent (≥ 200 µL/well) at room temperature for 1 h. Next, plates were aspirated and washed. After that, 100 µL

of each standard or sample was added into appropriate well and incubated at room temperature for 2 h. Plates were then aspirated and washed. Afterwards, 100 µL of diluted detection antibody (1:200 in assay diluent, v/v) was added to each well and incubated at room temperature for 1 h. Plates were then aspirated and washed. Subsequently, 100 µL of diluted Avidin-HRP enzyme reagent (1:1000 in assay diluent, v/v) was added to each well for 30 min at room temperature. Plates were then aspirated and washed. In the final wash step, plates were soaked in wash buffer for 30 seconds to 1 min for each wash. Finally, TMB substrate solution (100 µL) was added to each well and incubated at room temperature for 30 min in darkness, followed by adding stop solution (50 µL) to each well. The plates were then read on a Fluostar OPTIMA instrument (BMG LABTECH) at 450 nm within 30 min of stopping reaction and using wavelength 540 nm. The blank absorbance was subtracted from the value of each standard and sample. The standard curve was plotted using Optima Control Software (BMG LABTECH) and was employed to calculate the concentration of human IL-1β level (pg/ml) in each sample.

2.10.3. Measurement of mouse TNF-α secretion using ELISA

Cell supernatants were collected and kept at -80 ˚C. The level of TNF-α in the supernatants was measured using ELISA kit (BioLegend, Inc) as the manufacturer's instruction.

2.10.3.1. Preparation of required buffers and solutions

- Coating buffer: 0.1 M sodium carbonate, 7.13 g $NaHCO_3$ and 1.59 g Na_2CO_3 were added to 1.0 L of distilled water and pH was adjusted to 9.5 with 10 N NaOH. Coating buffer was freshly prepared or used within 7 days of preparation and stored at 2-8°C.

- **Assay diluent:** FBS was mixed with PBS (10%, v/v) and pH was adjusted to 7.0. Assay diluent was freshly prepared and stored at 2-8°C.
- **Wash buffer:** Tween-20 (0.05%, v/v) was mixed with PBS and used fresh or within 3 days of preparation (stored at 2-8°C).
- **Stop solution:** 2 N H_2SO_4 was prepared and used as stop solution.
- **Standard preparation:** Top standard of mouse TNF-α protein (1000 pg/mL) was prepared from the stock standard and vortexed to mix. Then, serial dilution of six tubes of mouse TNF-α protein standards (15.6-500 pg/mL) were prepared in assay diluent. Assay diluent alone was used as blank.

2.10.3.2. Assay procedure and data calculation

The levels of mouse TNF-α (pg/ml) in supernatants were measured using ELISA. Firstly, 100 µL of diluted capture antibody (1:250 in coating buffer) was added to each well and incubated overnight at 4°C. Plates were then aspirated and washed three times with wash buffer (≥ 300 µL/well). After last wash, the plates were inverted and blotted on absorbent paper to remove any residual buffer and, the plates were blocked with assay reagent (≥ 200 µL/well) at room temperature for 1 h. The plates were then aspirated and washed. After that, 100 µL of each standard or sample was added into appropriate wells and incubated at room temperature for 2 h. The plates were then aspirated and washed. Afterwards, 100 µL of diluted detection antibody (1:250 in assay diluent, v/v) was added to each well and incubated at room temperature for 1 h. Subsequently, the plates were aspirated and washed. Then, 100 µL of diluted SAv-HRP enzyme reagent (1:250 in assay diluent, v/v) was added to each well at room temperature for 1 h. The plates were then aspirated and washed. In the final wash step, the plates were soaked in wash buffer for 30 sec for each wash. Finally, TMB substrate solution (100 µL) was added to each well and incubated at room temperature for 30 min in the dark, followed by adding stop solution (50 µL) to each well. The plates were read on a Fluostar OPTIMA instrument (BMG LABTECH) at 450 nm

within 30 min of stopping reaction and wavelength correction at 540 nm. The blank absorbance was subtracted from the value of each standard and sample. The standard curve was plotted using Optima Control Software (BMG LABTECH) and was employed to calculate the concentration of TNF-α (pg/ml) level in each sample.

2.11. Induction of type I interferon production

Cells were pre-treated with/without an inhibitor of MIF tautomerase activity (4-IPP, 50 μM, and 1h) and were then stimulated with agonists for TLR4 (LPS, 100 ng/ml) or TLR9 (CpG ODNs; 0.05 and 0.1 μM). The incubation period for most experiments were 2, 6 and 24 hrs.

2.12. Detection of type I interferon using luciferase bioassay

The level of mouse interferon type I (IU/mL) in culture supernatant was measured based on a luciferase bioassay system. Stimulation of an ISRE-luciferase reporter cell line (LL171) with interferon induces expression of luciferin that can be determined using a luminescence assay (278, 279). Briefly, LL171 cells were seeded (2×10^4 cells/well), and then exposed to each sample supernatant or serial dilutions of standard (50-0.781 IU/mL). After 6 h, media was removed and LL171 cells were washed and lysed in passive lysis buffer (1X). Equal volume of each cell lysate was mixed with luciferase assay reagent and luciferase signal measured using a luminescence reader Fluostar OPTIMA (BMG LABTECH). The standard curve was plotted using Microsoft Excel and employed to calculate the concentration of mouse interferon type I (IU/mL) in each sample.

2.13. Statistical analysis

Data analysis was performed by Graph Pad Prism 7 using One-way ANOVA and Tukey's multiple comparison test. *P* values less than 0.05 were considered statistically significant.

Chapter 3. MIF Is Released by Necrotic Cells like a DAMP Molecule

3.1. Introduction

As outlined in chapter 1, migration inhibitory factor (MIF) is an evolutionarily conserved non-glycosylated protein (4) which exists preformed in multiple cell types including monocytes and macrophages and is expressed in a wide range of other tissues (11). MIF is generally considered as a multi-functional protein that can act as a cytokine, enzyme and hormone (280-282). MIF acts in both an autocrine and a paracrine manner to regulate innate and adaptive responses (283) and amplifies the production and release of inflammatory mediators (166, 167, 282). MIF also exerts a role in tissue repair and wound healing (223, 284).

MIF is constitutively released by cells *in vitro* as a result of *de novo* synthesis or release from preformed cytosolic stores (47). Induction of MIF release has been described by inflammatory stimuli such as lipopolysaccharide (LPS), tumor necrosis factor (TNF)-α and also several hormones (3, 47, 53, 54). However, some evidence suggests that MIF release is an outcome of tissue damage, raising the possibility of considering MIF as a damage-associated molecular pattern molecule (DAMP) (87, 146-148). DAMPs are released by cells undergoing life-threatening stress (285), and exert effects on innate and adaptive immune responses, and promote tissue healing (286) after inflammation, both sterile and infection-associated (287-289). Whether the release of MIF to the extracellular space reflects a DAMP-like process has not been previously examined.

Cell death is considered in two main forms, apoptotic and necrotic death. Whereas apoptosis is a normal programmed process for cell clearance, necrosis is triggered by external factors or disease. Many studies have reported that by using different doses of a particular stimulus, apoptotic and necrotic death types can be induced sequentially in an individual cell population (102, 290, 291). Necrosis is a non-programmed form of cell death which can be caused by toxic substances or physiological damage such as oxidative stress. The phenomenon of apoptotic necrosis is seen in cells during late apoptotic stages in cells that have not been phagocytosed. This is particularly pertinent to *in vitro* experiments, where apoptosis might be induced, but phagocytosis of dying cells does not occur, as the whole population is undergoing apoptosis (292, 293).

Necrosis can occur in a variety of contexts, depending on the stimulus, and different terms have employed to describe various forms of these processes. Pyroptosis is generally mediated by inflammasome complexes activating caspase-1, such that using caspase-1 inhibitors can distinguish pyroptosis from other necrotic cell death forms (119, 294-296). Necroptosis is initiated through the activity of receptor-interacting protein 1 (RIP1), for example, induced by TNF-α binding to TNFR1 or experimentally induced by endogenous proteins called second mitochondria-derived activator of caspases (SMAC) or artificial SMAC-mimetics in combination with caspase inhibitors. Necroptosis is inhibited by suppression of RIP1 kinase activity with necrostatin-1 (276, 297-301).

As monocyte/macrophages are a major source of MIF (302), the studies designed in this chapter aimed to examine whether MIF release is associated with induced cell death in monocyte/macrophages. For this purpose, we induced well-described forms of cell death and measured MIF release.

Methods used in this chapter are described in chapter 2 and the published book chapter (Appendix 1).

3.2. Results

3.2.1. MIF is not released by THP-1 cells in response to inflammatory stimuli or glucocorticoid

Cells were treated with LPS (TLR4 ligand), TNF-α (a pro-inflammatory cytokine) and the glucocorticoid, dexamethasone. As shown in Figures 5A, B and C, none of the tested stimuli induced significant MIF release in THP-1 monocytes. As shown in Figures 5D, E and F, none of the tested modulators induced death in THP-1 monocytes.

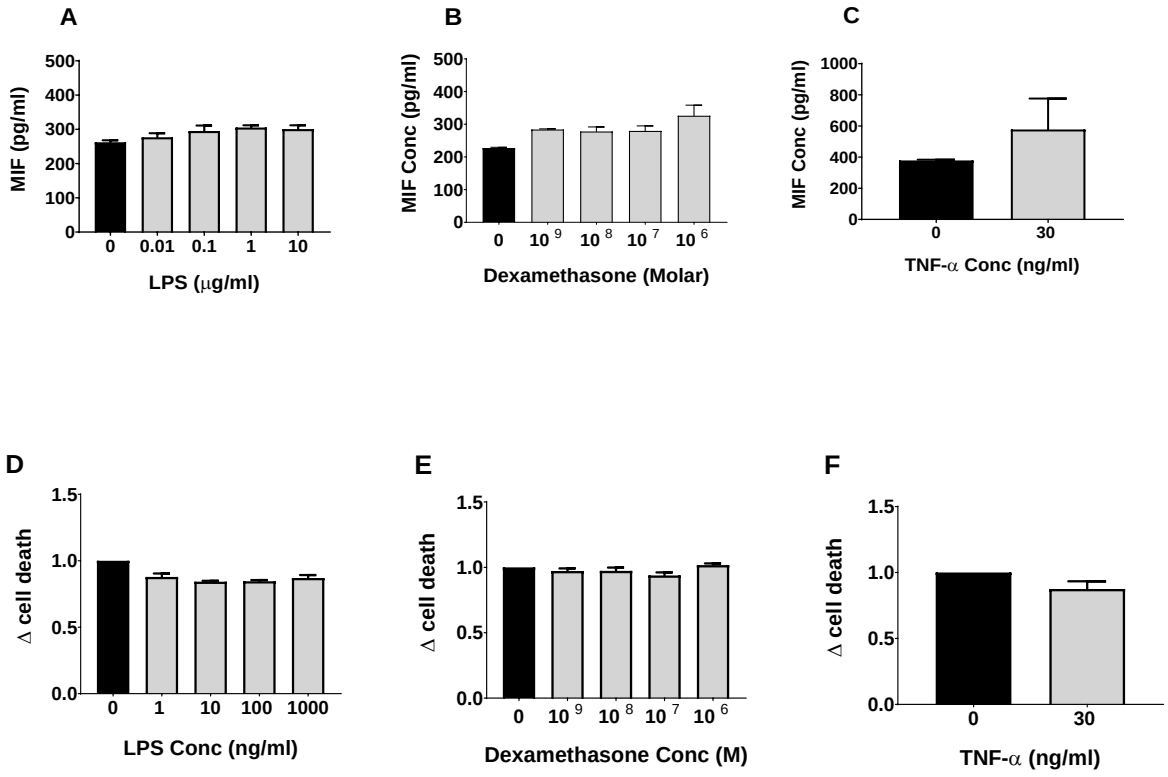


Figure 5. Effect of various modulators on MIF release.
THP-1 monocytes were treated with stimuli including bacterial lipopolysaccharide (LPS, 0.01 - 10 µg/ml), dexamethasone (10^{-6} -10^{-9} M) and TNF-α (30 ng/ml) overnight. The released level of MIF was measured in culture supernatants using ELISA (A-C). Cell death was detected using SYTOX™ Green Nucleic Acid Stain (D-F). Data are represented as mean ± SEM of triplicate technical and independent experimental replicates.

3.2.2. MIF is released by THP-1 cells during pyroptosis and necroptosis

Recent studies have demonstrated that MIF is required for NLRP3 inflammasome-dependent IL-1 and IL-18 release by human and mouse monocytes/macrophages (280, 303). Moreover, inhibition or loss of MIF abrogates caspase-1-dependent pyroptosis (280). Here, I investigated whether MIF is released by THP-1 cells following activation of the NLRP3 inflammasome and induction of pyroptosis. Cells were primed with LPS overnight, then treated with the NLPR3 activating pore-forming toxin nigericin for 1-3 hours. In addition, cells were treated with either the caspase-1 inhibitor Y-VAD-FMK or the pan-caspase inhibitor Z-VAD-FMK.

Following priming with LPS, treatment of cells with nigericin induced significant cell death after 1 hour, increasing after 2 and 3 hours. This was partially inhibited by caspase-1 inhibition at 1 and 2 hours, suggesting at least some of the cell death is due to pyroptosis, particularly at the earlier time point (Fig 6A-C). The pan-caspase inhibitor, on the other hand, had a much more pronounced inhibitory effect on cell death at each time point (Fig 6A-C). Thus, the data suggest that pyroptosis is a significant component of the cell death response to nigericin at early time points, but after that death proceeds via other caspase-dependent pathways. Moreover, in the absence of LPS priming, nigericin induced cell death that was largely independent of caspase-1 activation but was abrogated with the pan-caspase inhibitor (Fig 6A-C).

As expected, release of IL-1 only occurred in response to nigericin after LPS priming and its release was significantly reduced with caspase-1 inhibition (Fig 6D-F). In contrast, significant release of MIF was seen in response to nigericin regardless of whether the cells were primed with LPS or not, and was inhibited by treatment with both the caspase-1 inhibitor and the pan-caspase inhibitor (Fig 6D-F). These data suggest that MIF is released regardless of the mode of cell death; be it dependent on caspase-1 or other caspases. However, it is interesting to note that the caspase-1 inhibitor had a greater effect on MIF release than it did on cell death, possibly suggesting a more direct connection between caspase-1 and MIF release.

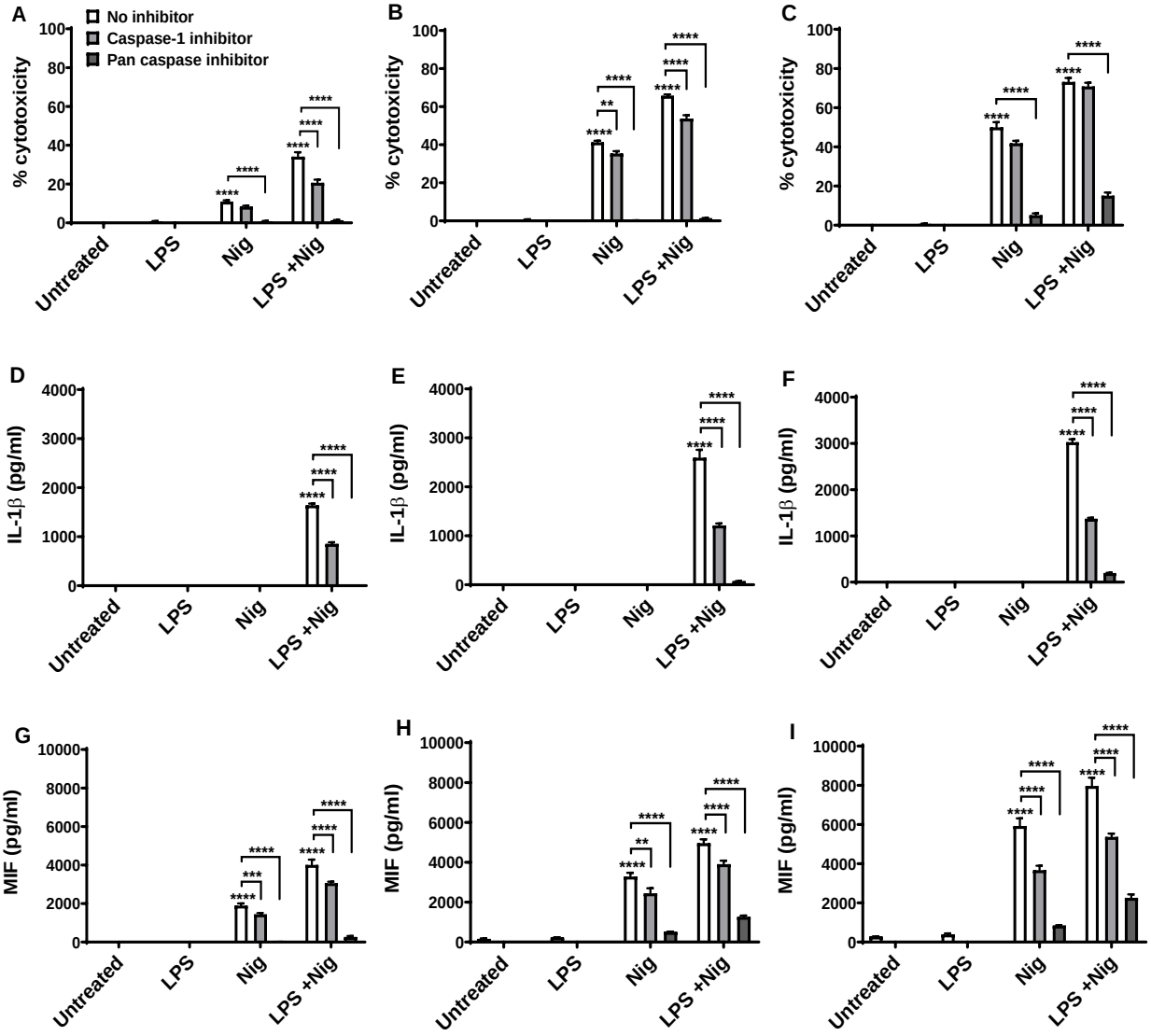


Figure 6. Effect of pyroptosis on MIF release.

Pyroptosis was induced using nigericin in LPS-stimulated monocytes for 1-3 hours. (A-C) Cell death was detected using LDH release assay. The level of IL-1β (D-F) and MIF (G-I) in the supernatant was measured using ELISA. The effects of pan caspase and caspase-1 inhibitor were also examined. Panels A, D, G reflect 1h, panels B, E, H reflect 2h and panels C, F, I reflect 3h time points. Data are represented as mean ± SEM of triplicate technical and independent experimental replicates. **P < 0.01, and ****P < 0.0001.

To determine whether MIF is released by THP-1 cells following induction of necroptosis, cells were treated with rhTNF, BV-6 and Z-VAD-FMK for 6 hours to induce necroptotic cell death. Cell death occurrence was detected by LDH

release and secreted MIF level was measured in culture supernatants by ELISA. As shown in Figures 7A and B, cytotoxicity in necroptosis-induced cells compared to untreated cells (media only), accompanied by a significant increase in MIF release. Treatment of cells prior to necroptosis induction with a specific inhibitor, necrostatin-1, suppressed both cytotoxicity and MIF release. Together, these data confirm that MIF is released by cells undergoing both pyroptotic and necroptotic cell death.

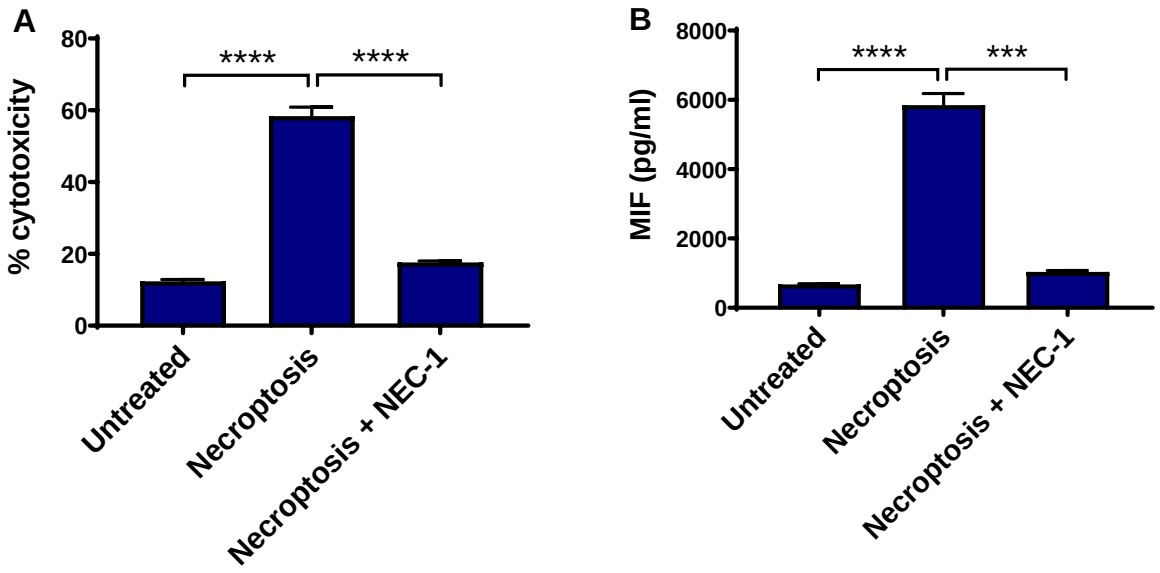


Figure 7. Effect of necroptosis on MIF release.
Induction of necroptosis using a combination of BV-6, rhTNF-α and Z-VAD-FMK for 18 hours led to necroptosis. Necroptosis cell death was detected using LDH release assay. The effect of necroptosis on MIF release was assessed using ELISA. These effects were remarkably inhibited by using necrostatin-1 (NEC-1). Data are represented as mean ± SEM of triplicate technical and independent experimental replicates. ***$P < 0.001$.

3.2.3. MIF is released by necrotic, but not apoptotic THP-1 cells

In order to induce primary necrosis, THP-1 monocytes were exposed to ethanol (10%, V/V) for a period of 1-6 h. Necrosis was detected using PI staining and

flow cytometry analysis. The released level of MIF in culture supernatants was measured using ELISA. The results are shown as the percentage of viable cells relative to untreated cells.

As presented in Figure 8A, ethanol exposure induced necrotic cell death in THP-1 monocytes, demonstrated as a remarkable reduction of cell viability from the first hour of treatment compared to untreated cells. Results obtained from ELISA showed a remarkable MIF release by THP-1 monocytes following the first hour of ethanol exposure (Figure 8B).

In order to clarify the cell death form responsible for MIF release, THP-1 monocytes were treated with a well-defined apoptosis inducer (staurosporine, 10^{-6} M) for a period of 1-6 hours. The proportion of cells undergoing apoptotic and necrotic death was determined by Annexin V-FITC and PI dual staining using flow cytometry, and MIF released was assessed by ELISA at the indicated time points during 1-6 hours of incubation.

MIF appeared in the supernatant simultaneously with the significant increase in double Annexin positive and PI positive population, indicating release by necrotic but not early apoptotic cells (Fig 8). These findings suggest that MIF release only follow the kinetics of necrosis and not early apoptosis in THP-1 monocytes.

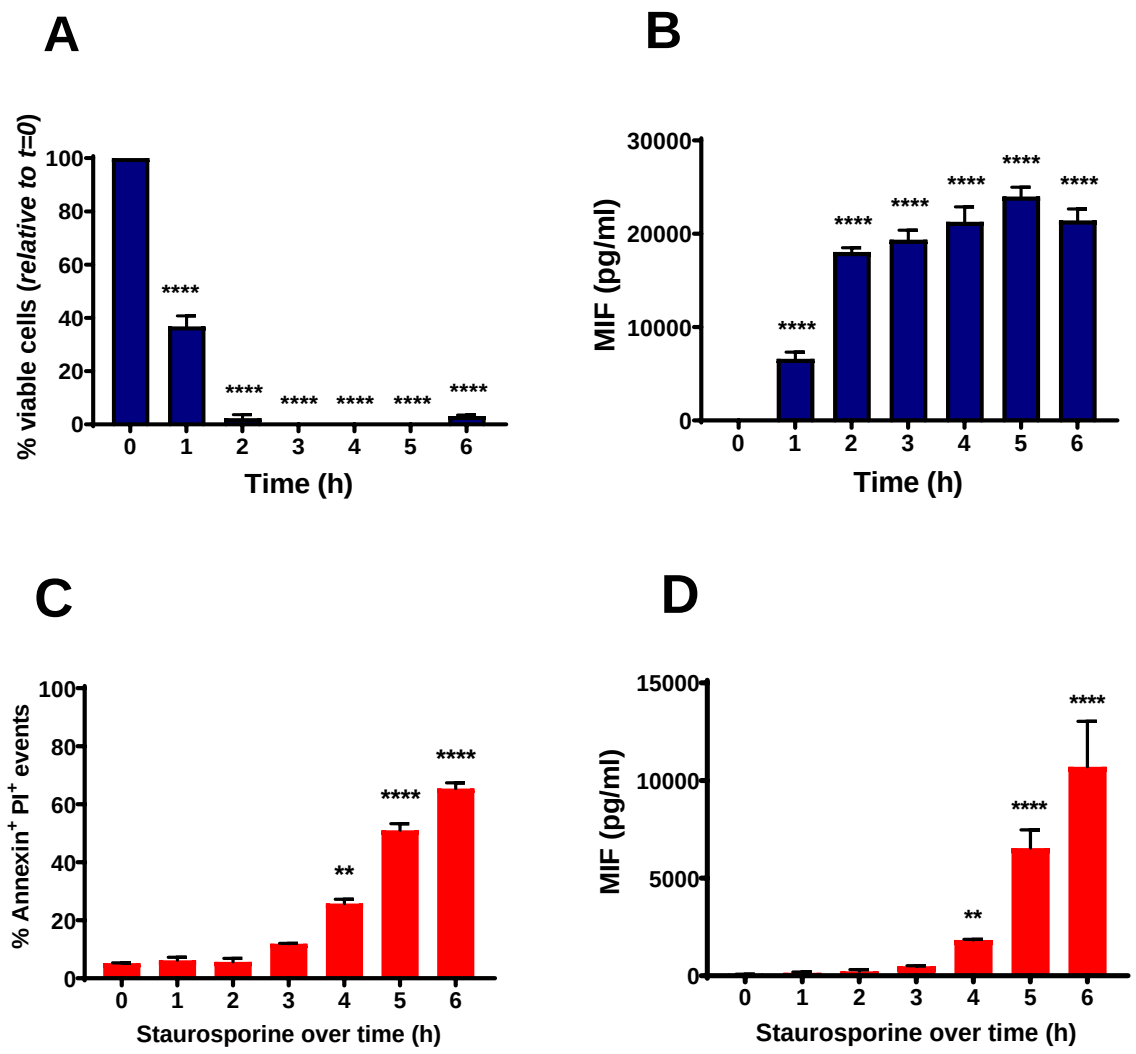


Figure 8. Effect of ethanol on cell death induction and MIF release.
THP-1 monocytes were treated with ethanol (10%, v/v) or staurosporine (10^{-6} M) for a period of 1-6 hours. Cell death induced by (A) ethanol and (C) staurosporine was assessed using flow cytometry. MIF concentration in culture supernatants obtained from treatment with (B) ethanol and (D) staurosporine was quantified by ELISA. Data are represented as mean ± SD of triplicate independent experimental replicates. ****$P < 0.001$, one-way ANOVA with a correction provided by the Tukey's multiple comparisons test.

3.3. Discussion

MIF is reported to be constitutively secreted by monocytes and macrophages even in the absence of stimulation (47, 304). Several available studies have highlighted a remarkable MIF release as the consequence of damage in various tissues (87, 147) and also by neutrophils undergoing secondary necrosis *in vitro* (152). However, any association between MIF release and cell death has not been reported in monocytes or macrophages.

The main properties of a DAMP is to be released by cells under stress or by dying cells, and to elicit inflammatory responses (84). As MIF has been widely reported to play various inflammatory effects, it potentially meets the criteria of being considered as a DAMP. To examine this hypothesis, I induced different necrotic forms of cell death in THP-1 monocytes and measured MIF release, to find any association between cell death and MIF release.

Firstly, I treated monocytes with well-defined monocyte activation modulators, followed by measuring the level of MIF released. The selected macrophage modulators failed to induce a noticeable MIF release. This calls into some published findings suggesting LPS induces MIF release by macrophages.

In comparison with some previous studies reporting significant MIF release induced by LPS, here the level of MIF released by stimulation of THP-1 monocytes with a wide range of LPS concentrations (0.01 - 10 µg/ml) overnight did not show any significant changes compared with unstimulated cells.

One explanation might be the methodological differences between this study and others in regards to LPS incubation time, LPS concentration and the type and source of tested cells. Here, I treated human THP-1 monocytes with LPS overnight. However, some previous studies suggested maximum MIF levels are observed 9 to 12h of LPS stimulation in murine RAW 264.7 macrophage cell line, followed by degradation after its peak level without affecting cell viability (302). More evidences suggest that the peak of MIF secretion by THP-1 cells following LPS treatment can be observed between 2 and 6h without inducing cell

death or cell stress (51, 53). In accordance with the results obtained here, LPS (10 µg/ml) reduced the viability of bovine mammary epithelial cells (BMEC) to around 50% after 24h of LPS treatment (305). In addition, LPS (10 µg/ml) induced about 40% cell death in murine macrophages after 48h of treatment (306). In bone marrow-derived macrophages (BMDM), LPS (100 ng/ml) has been demonstrated to induce early apoptosis at about 6 hours of treatment (307).

In exploring the effect of cell death on MIF release, I examined whether cell death can affect the level of MIF released by monocytes. Various cell death types including apoptosis, primary necrosis, pyroptosis and necroptosis were induced in THP-1 monocytes. Assessing the type of death induced in monocytes and measuring the level of MIF released revealed a strong correlation of MIF release with all forms of necrotic cell death but not with early apoptosis.

MIF was significantly released following necrotic forms of cell death including primary necrosis, NLRP3-dependent pyroptosis and RIP1-dependent necroptosis. Therefore, I can conclude that MIF possesses the first condition of being a DAMP. Overall, results presented in the current chapter propose that the release of MIF is highly significant following various necrotic forms of cell death. Considering the pro-inflammatory function of MIF, its release by dying cells would introduce MIF as a novel DAMP. This study importantly demonstrated that necrosis can be considered a significant method of MIF release by macrophages *in vitro*.

Chapter 4. MIF Differently Regulates TLR-induced Interferon Production in Macrophages and Dendritic Cells

4.1. Introduction

Type I interferons are a family of cytokines produced by macrophages and dendritic cells upon toll-like receptor (TLR) activation which can modulate innate and adaptive immune responses. Macrophages and dendritic cells differentially respond to the same stimuli which accounts for their dissimilar roles in immune responses (207). Type I interferon signalling is tightly regulated and dysregulation could lead to tissue damage and autoimmunity (296, 308).

Both TLR4 and TLR9 are expressed by macrophages and dendritic cells and are activated following ligand binding. Although other TLRs are also important, bacterial lipopolysaccharide (LPS) can induce the TLR4 signalling pathway, leading to the production of pro-inflammatory mediators including type I interferon (190, 309).

The regulation of TLR4-induced pro-inflammatory responses by MIF has been reported in several contexts, including modulation of host responses against gram-negative bacteria by macrophages, production of pro-inflammatory mediators, TLR4 receptor expression and NF-κB activation (208-211, 214-219). MIF tautomerase function is suggested to be one of the activities responsible for MIF modulatory effect on TLR4-induced inflammatory responses, depending on the type of cell involved (220-222). The role of MIF in the regulation of TLR4-induced IFN production has not been examined. In addition, no evidence is available regarding whether MIF is involved in TLR9-induced IFN type I production.

This part of my project aimed to evaluate the possible role for endogenous MIF in type I interferon production by different cell types. I used mouse macrophages of different sources (iBMMs, PECs and BMDMs) and bone-marrow derived dendritic cells (BMDCs) to examine MIF effects on IFN type I produced by

different sources. In addition, the effects of genetic MIF deletion and the suppression of its tautomerase enzymatic activity were compared. MIF regulation of type I IFN produced in response to stimulation of different TLRs including TLR4 and TLR9 (in some experiments) was examined.

4.2. Results

4.2.1. MIF and type I interferon production by macrophages

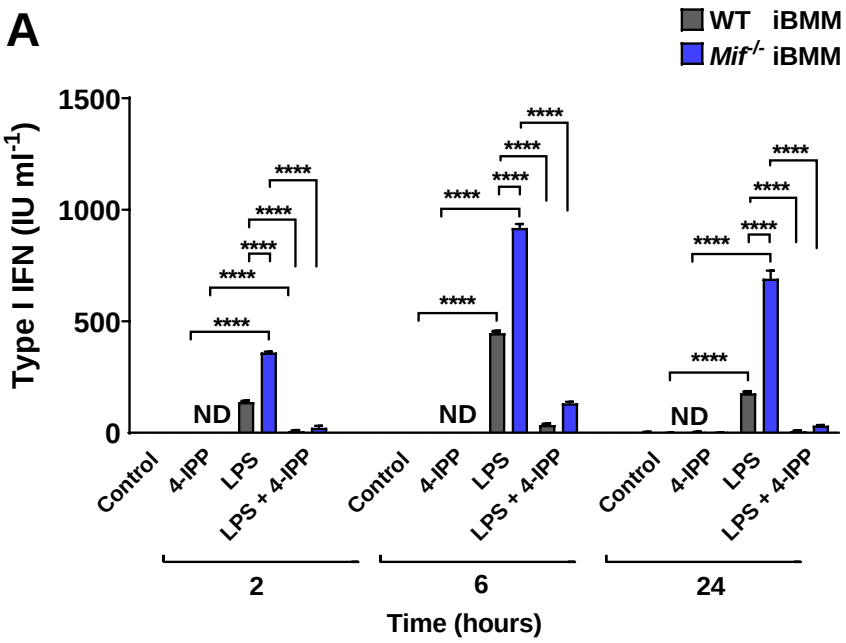
I planned formal confirmation of preliminary data (not shown) across multiple representative immortalized and primary macrophage cell types. Mean data from three independent experiments each in biological triplicate represent the formal experiments. Murine WT and *Mif*$^{-/-}$ macrophages of different sources including iBMMs, PECs and BMDMs derived from wild-type (WT) and MIF gene deleted (*Mif*$^{-/-}$) mice were treated with/without the agonist for TLR4 (LPS; 100 ng/ml) for various time points (2, 6 and 24h). Also, WT and *Mif*$^{-/-}$ cells in separate groups were pre-treated with/without an inhibitor of MIF tautomerase activity (4-IPP; 50 μM). Cell culture supernatants were then examined for secreted level of type I IFN using a luciferase luciferase reporter bioassay which detected total type I interferon activity. Levels of secreted TNF-α were measured using ELISA.

As shown in Figure 9A, the addition of LPS to WT and *Mif* $^{-/-}$ iBMM cells induced the release of type I IFN into the culture medium. LPS-induced type I IFN secretion by *Mif* $^{-/-}$ iBMMs was significantly higher than their WT counterparts. In contrast, type I IFN secretion in response to LPS was significantly reduced by inhibition of MIF tautomerase activity in WT macrophages, with similar effects in *Mif* $^{-/-}$ cells.

To confirm this, further experiments were performed using bone marrow-derived macrophages (BMDMs). Data again showed no inhibitory effect of MIF deletion on type I IFN, and indeed showed significant increases under LPS stimulation (Figure 9C). This was further confirmed in peritoneal macrophages (PECs), where *Mif* $^{-/-}$ cells demonstrated an increase in type I IFN release compared to WT

cells, as seen in iBMM and BMDMs, suggesting a suppressive effect of endogenous MIF on type I IFN production (Figure 9B). However, inhibition of tautomerase action of MIF-1 by the addition of 4-IPP in both WT and *Mif* $^{-/-}$ cells reduced the level of type I IFN released in LPS- stimulated cells (Figure 9B).

These findings would suggest that endogenous MIF has a possible down-regulatory role for LPS-induced type I IFN secretion in macrophages, distinct from the effect of suppression of MIF tautomerase activity which caused a remarkable reduction of type I IFN production by LPS-stimulated macrophages. Overall, type I IFN production is enhanced in *Mif* $^{-/-}$ iBMMs, PECs and BMDMs by LPS and is inhibited by tautomerase inhibitor.



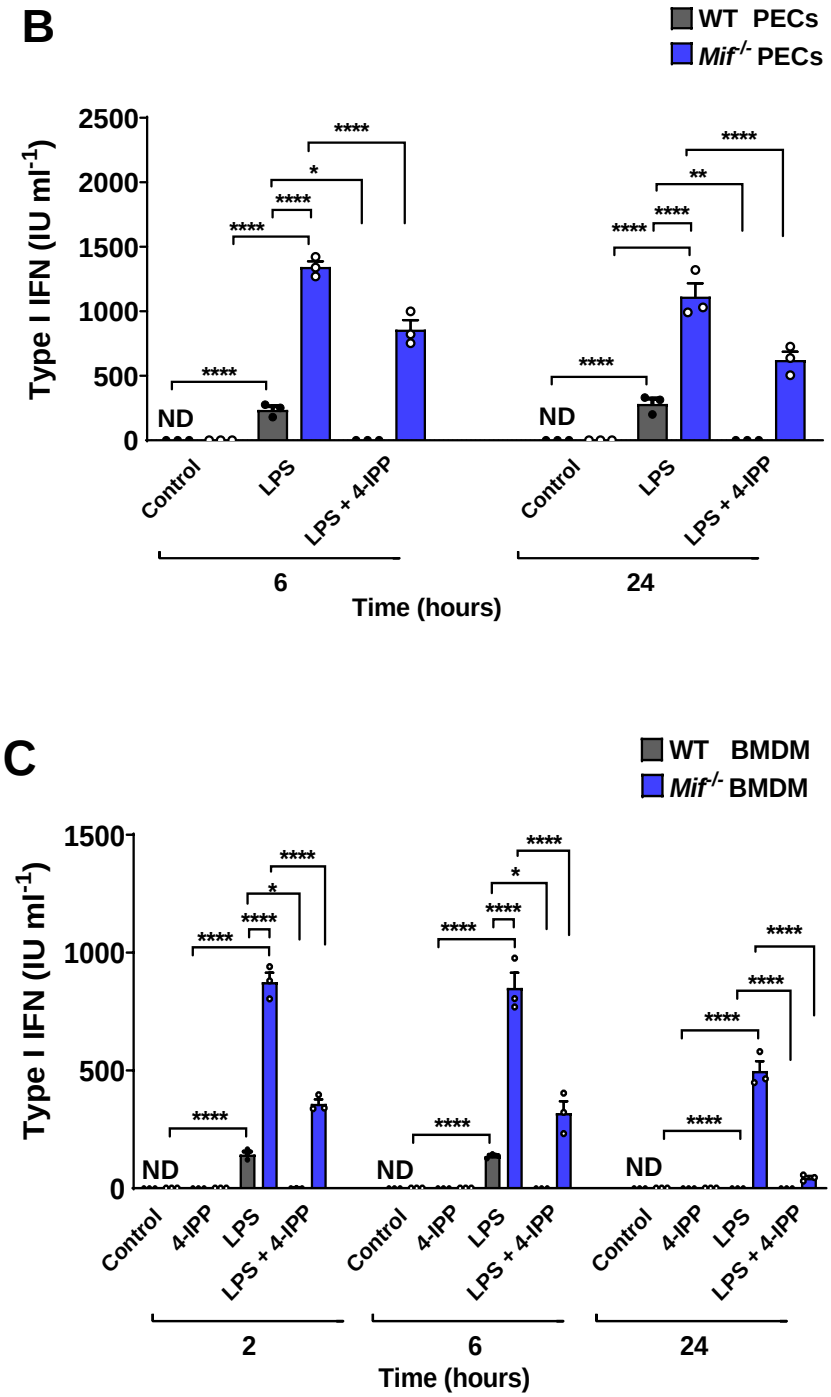
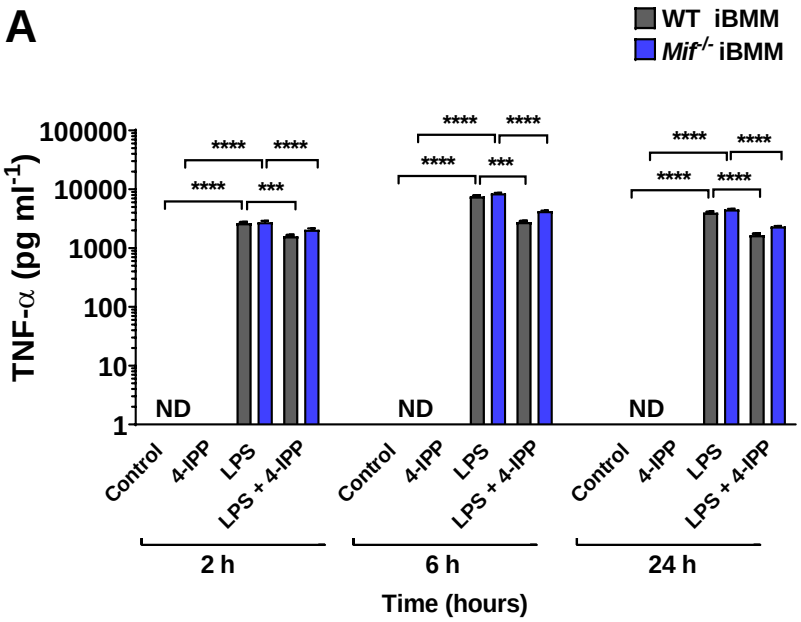


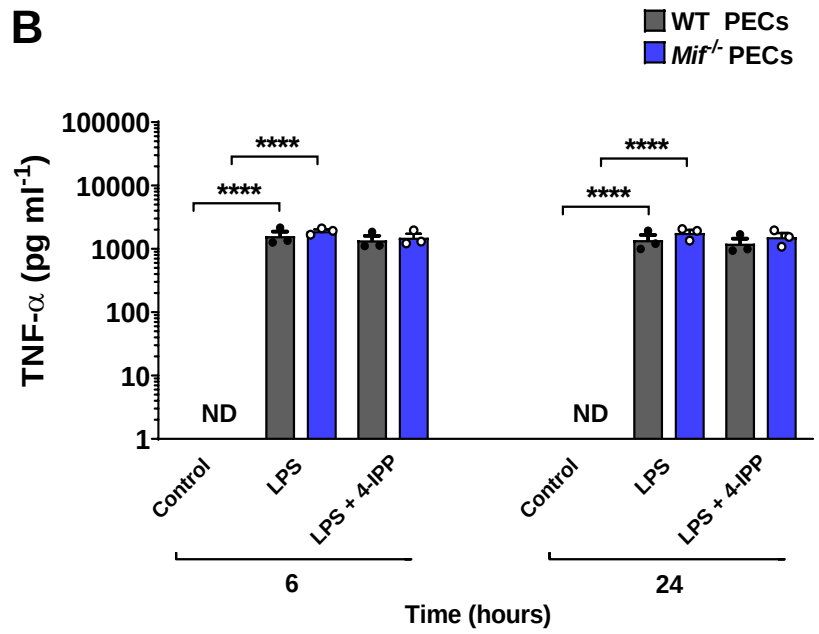
Figure 9. Type I interferon produced by WT and *Mif* $^{-/-}$ macrophages.
Murine WT and *Mif* $^{-/-}$ macrophages including (A) iBMMs, (B) PECs and (C) BMDMs were pre-treated with/without MIF inhibitor (4-IPP, 50 μM) and then stimulated with/without LPS (100 ng/ml) for 2, 6 or 24 h. Levels of type I interferon in cell culture supernatants was measured using luciferase bioassay. Data are expressed as mean ± SEM, n = 3 mice per each group (triplicate experimental replicates). iBMM cells were tested in four independent experimental replicates in technical quadruplicate, data are expressed as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.005, or ****P < 0.001, one-way ANOVA with a correction by Tukey's multiple comparisons test. ND; non-detectable.

4.2.2. MIF regulates the production of TNF-α by macrophages

To assess whether MIF plays a role in the secretion of pro-inflammatory cytokines like TNF-α following TLR4 activation, both WT and *Mif* $^{-/-}$ macrophages were exposed to LPS. In separate groups, cells were pre-treated with/without MIF tautomerase suppressor (4-IPP) before LPS challenge. Levels of secreted TNF-α were measured using ELISA.

As shown in Figures 10A-C, the addition of LPS to WT and *Mif* $^{-/-}$ cells induced the secretion of TNF-α into the culture medium from iBMMs, PECs and BMDMs at all tested time points. *Mif* gene deletion in LPS-stimulated macrophages showed no significant effect on TNF-α secretion compared with their WT counterparts. However, inhibition of MIF tautomerase function by 4-IPP modestly suppressed TNF-α secretion in LPS-stimulated WT and *Mif* $^{-/-}$ iBMM and BMDM macrophages but not PECs (Figure 10B).





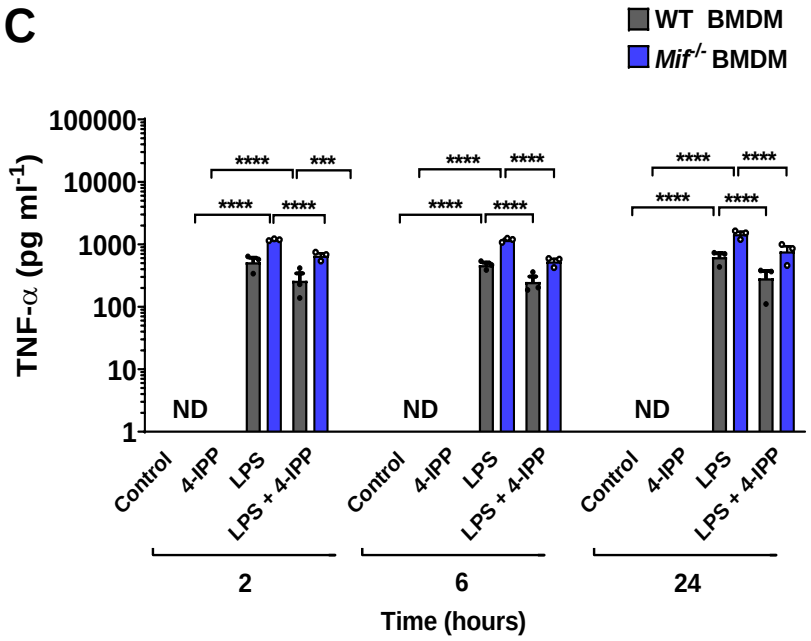


Figure 10. Levels of TNF-α secreted by WT and *Mif* $^{-/-}$ cells. Murine WT and *Mif* $^{-/-}$ cells including (A) iBMMs, (B) PECs and (C) BMDMs were pre-treated with/without MIF inhibitor (4-IPP, 50 μM) and then stimulated with/without LPS (100 ng/ml) for 2, 6 or 24 hours. TNF-α in cell culture supernatants was measured using ELISA. Data are expressed as mean ± SEM, $n = 3$ mice per each group (triplicate experimental replicates). iBMM cells were tested four times (four experimental replicates) and four replicates in each separate experiment (four technical replicates). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, or $^{****}P < 0.001$, one-way ANOVA with correction by Tukey's multiple comparisons test. ND; non-detectable.

4.2.3. MIF and type I interferon in primary dendritic cells – preliminary data

Next, preliminary experiments were undertaken to examine whether the production of type I IFN followed by TLR stimulation is affected by endogenous MIF function in dendritic cells. Bone marrow-derived dendritic cells (BMDCs) generated from wild-type (WT) and MIF gene deleted (*Mif*$^{-/-}$) mice were treated with/without agonists for either TLR4 (LPS; 100 ng/ml) or TLR9 (CpG ODNs; 0.1 μM) for various time points (2, 6 and 24h). In separate groups, WT BMDCs were treated with/without an inhibitor of MIF tautomerase activity (ISO-1; 100 μM) before TLR4/TLR9 activation. Murine type I IFN was measured using luciferase bioassay in cell culture supernatants.

Data show that the activation of TLR4 in both WT and *Mif*$^{-/-}$ BMDCs led to type I IFN production after 6 hours of stimulation in comparison with unstimulated cells (Figure 11A). The production of type I IFN in response to TLR4 activation was significantly lower in *Mif*$^{-/-}$ BMDCs compared to WT BMDCs. However, inhibition of MIF tautomerase activity using ISO-1 in WT BMDCs showed no effect on type I IFN production.

As demonstrated in Figure 11B, type I IFN was also produced upon TLR9 stimulation after 24 hours in both WT and *Mif*$^{-/-}$ BMDCs (Figure 11B). However, MIF gene deficiency or the suppression of MIF tautomerase activity failed to affect TLR9-induced type I IFN production by BMDCs at this time point.

These preliminary data suggest that MIF is required for TLR4-induced type I IFN production in dendritic cells at early time points. These experiments encouraged development of a formal study of this question in further experiments, data from which follows.

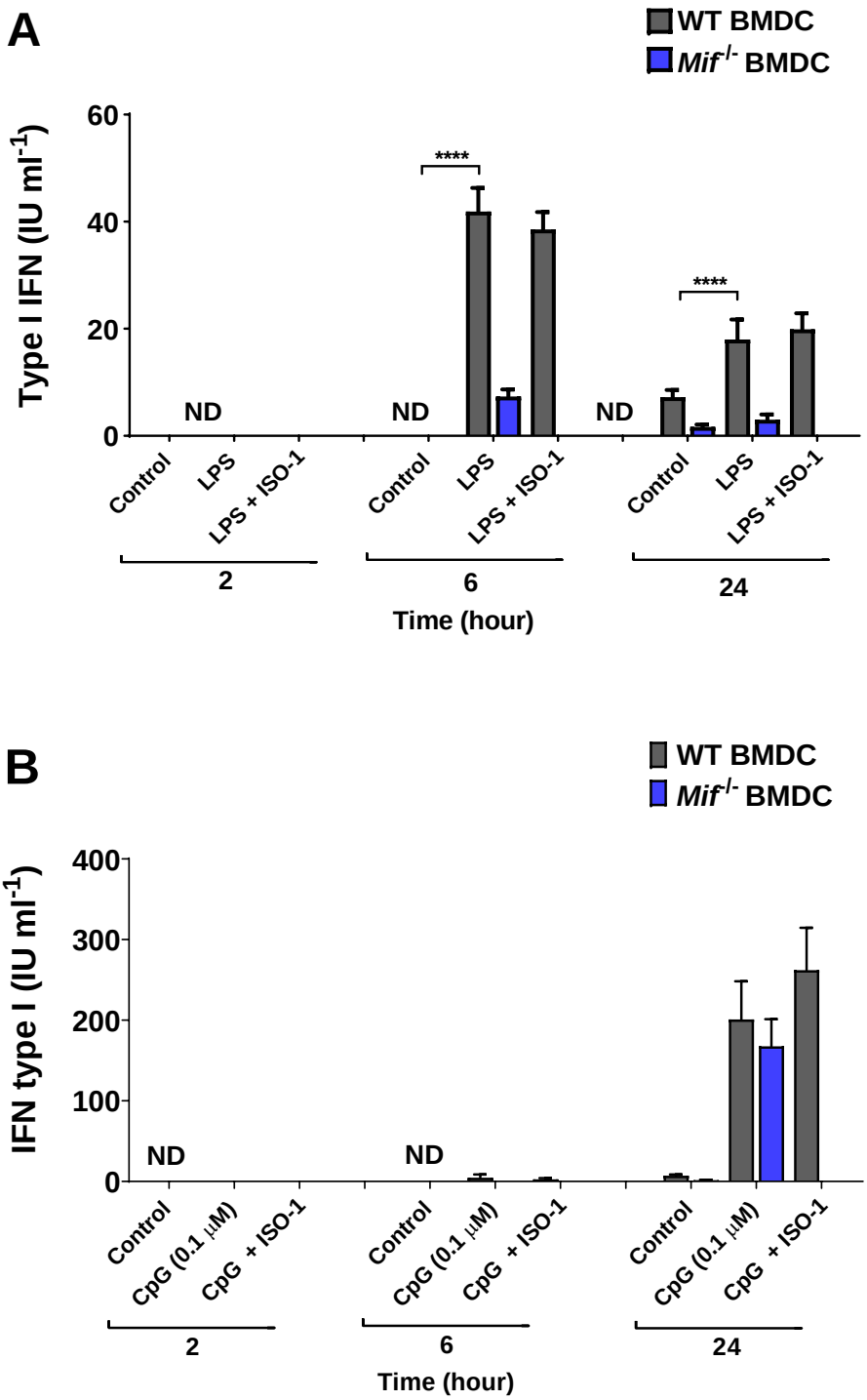


Figure 11. Type I IFN production by dendritic cells upon TLR4 or TLR9 stimulation. Bone marrow-derived dendritic cells (BMDCs) generated from WT and *Mif* $^{-/-}$ mice were pre-treated with/without MIF inhibitor (ISO-1; 100 μM) and then stimulated with/without TLR4 agonist (LPS; 100 ng/ml) or TLR9 agonist (CpG ODNs; 0.1 μM) for 2, 6 or 24 h. The levels of IFN type I produced upon either TLR4 (A) or TLR9 (B) activation was measured using luciferase bioassay. Data represents the mean ± S.E.M. of three mice. *$P < 0.05$, **$P < 0.01$, ***$P < 0.005$, or ****$P < 0.001$, one-way ANOVA with a correction by Tukey's multiple comparisons test. ND; non-detectable.

Next, I investigated the role of MIF in TLR4-induced TNF-α responses in dendritic cells. WT and *Mif*$^{-/-}$ BMDCs were treated with/without TLR4 agonist (LPS; 100 ng/ml) for various time points (2, 6 and 24h). In separate groups, WT cells were pre-treated with inhibitor of MIF tautomerase activity (ISO-1; 100 μM). The secreted level of TNF-α in culture supernatants was detected using ELISA. Data show TNF-α production in response to TLR4 stimulation in both WT and *Mif*$^{-/-}$ BMDCs in comparison with unstimulated cells (Figure 12). *Mif*$^{-/-}$ BMDCs showed reduced TLR4-induced TNF-α production only at 6 hours. However, suppression of MIF tautomerase activity significantly declined TNF-α release by LPS stimulated dendritic cells at 6 hours in comparison with LPS alone. These preliminary data do not make clear the requirement of MIF for TNF-α production by dendritic cells. These experiments encouraged development of a formal study of this question in replicated experiments, data from which follows.

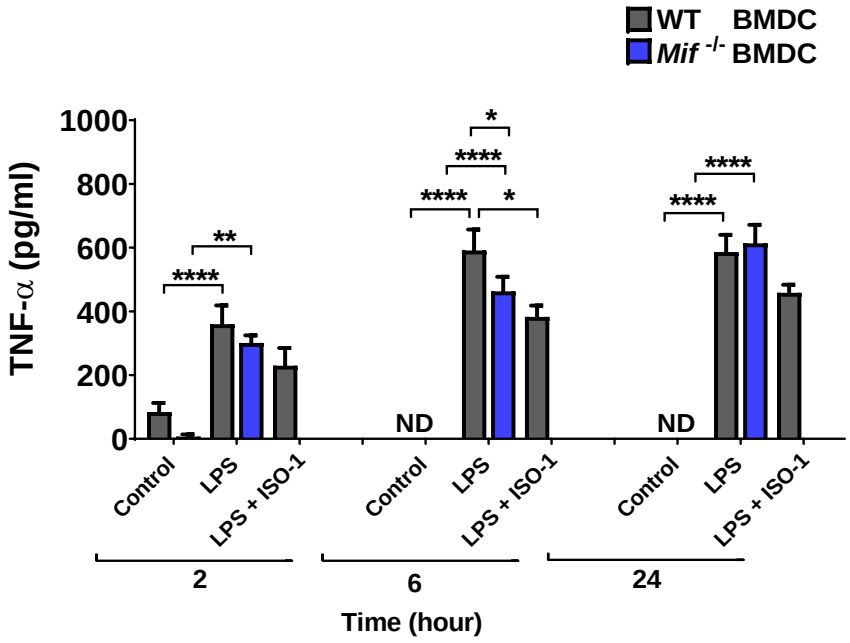


Figure 12. TNF-α production by dendritic cells upon TLR4 stimulation.
Bone marrow-derived dendritic cells (BMDCs) from WT mice were treated with/without MIF inhibitor (ISO-1; 100 μM) and then both WT and *Mif* $^{-/-}$ cells were stimulated with/without TLR4 agonist (LPS; 100 ng/ml) for 2, 6 or 24 h. The levels of TNF-α produced was measured using ELISA. Data represents the mean ± S.E.M. of three mice. *P < 0.05, **P < 0.01, or ****P < 0.001, one-way ANOVA with a correction by Tukey's multiple comparisons test. ND; non-detectable.

4.2.4. MIF is required for type I interferon production by dendritic cells

I next undertook experiments to formally examine whether MIF imparts a role in type I interferon secretion in response to TLR4 agonist (LPS, 100 ng/ml) activation by primary dendritic cells. As shown in Figure 13A, the addition of LPS to WT and *Mif* $^{-/-}$ cells induced the release of type I IFN from BMDCs at all tested time points. However, *Mif*$^{-/-}$ BMDCs secreted significantly less type I IFN compared with their WT counterparts.

As shown in Figures 13B, the addition of LPS to WT and *Mif* $^{-/-}$ cells also induced significantly the secretion of TNF-α by BMDCs. Primary bone marrow-derived dendritic cells (BMDCs) from *Mif* $^{-/-}$ mice produced less TNF-α compared with their WT counterparts after 24h of treatment, indicating a possible role for MIF on LPS-induced TNF-α production in primary dendritic cells. The suppression of MIF tautomerase activity using 4-IPP caused a remarkable reduction in LPS-stimulated type I interferon and TNF-α secretion by both WT and *Mif* $^{-/-}$ BMDCs. These findings suggest that MIF is required for LPS-stimulated secretion of type I interferon by primary dendritic cells and that its tautomerase activity has a role to play.

Overall, LPS or CpG stimulation does not enhance type I interferon production in BMDCs from *Mif* $^{-/-}$ mice compared to WT mice. Furthermore, while tautemerase inhibition by ISO-1 did not inhibit (or marginally inhibit) the interferon production from WT cells, 4-IPP inhibits the interferon production in both WT and *Mif* $^{-/-}$ BMDCs.

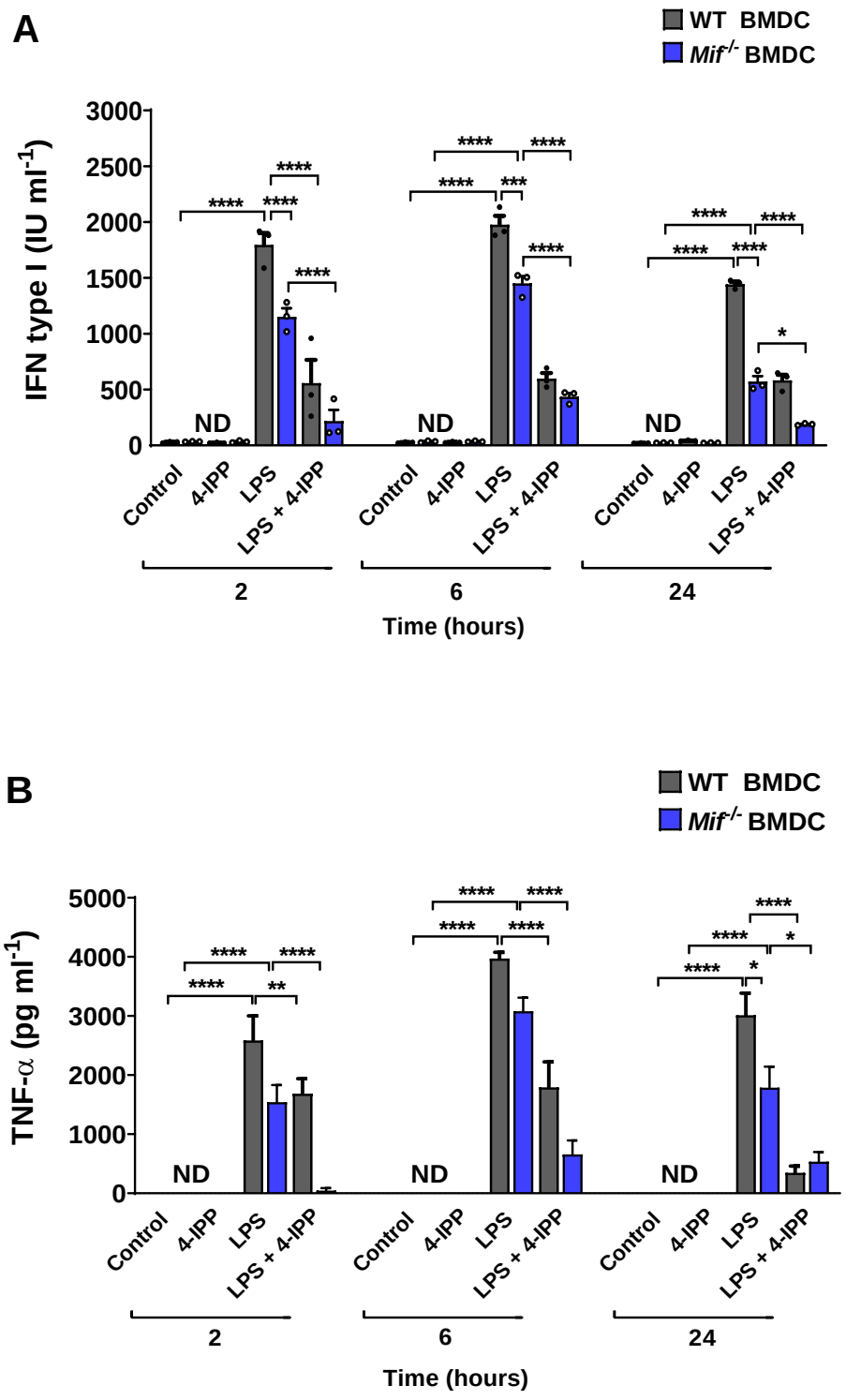


Figure 13. Levels of type I IFN and TNF-α production by WT and *Mif* $^{-/-}$ dendritic cells. Murine WT and *Mif* $^{-/-}$ dendritic cells (BMDCs) were pre-treated with/without MIF inhibitor (4-IPP, 50 μM) and then stimulated with/without LPS (100 ng/ml) for 2, 6 or 24 h. Secreted level of type I interferon in cell culture supernatants was measured using luciferase bioassay. Data are expressed as mean ± SEM, n = 3 mice per each group (triplicate independent replicates). *P < 0.05, **P < 0.01, ***P < 0.005, or ****P < 0.001, one-way ANOVA with correction by Tukey's multiple comparisons test. ND; non-detectable.

4.3. Discussion

Type I interferons (IFNs) are produced by macrophages and dendritic cells as one of the main elements of host immune defences against microbial invasion. They impart both beneficial and detrimental roles in various immune and autoimmune inflammatory disorders (190, 207, 309). The production of type I IFN is triggered by various receptors including toll-like receptors (TLRs) in response to pattern-associated molecular pattern (PAMP) and danger-associated molecular pattern (DAMP) recognition (310). The production of type I IFNs is tightly regulated and over-production is associated with uncontrolled immune reactions and impaired host immune defence in some chronic auto-inflammatory conditions such as SLE (296, 308, 311, 312).

MIF in both intracellular and extracellular forms can regulate immune responses through the effect on the production of several mediators and cell interactions (217). MIF possesses multiple biological functions in autoimmune diseases, inflammatory disorders and cancers through receptor-mediated and endocytic pathways (71). Although regulation of various pro-inflammatory mediators by MIF has been reported (266, 313), its role in the production of type I IFNs by macrophages and dendritic cells has not been examined.

MIF has been shown to contribute to pathophysiology of several TLR4 related diseases. In type 1 diabetes, oxidative stress and inflammatory reactions is linked with activation of TLR4 pathway (314, 315). In an experimental type 1 diabetes mellitus model, MIF deficiency was associated with reduction of serum levels of pro-inflammatory cytokines and impaired activation of splenic and pancreatic macrophages and dendritic cells (316). A lower expression of TLR4 and co-stimulatory molecules, and decreased induction of lymphocyte proliferation was shown in MIF-deficient cells (317). This study confirmed that MIF may be linked with the development and progression of type 1 diabetes mellitus (318). Although enhanced expression of IFN-α and IFN-β by islet cells and infiltrating macrophages and dendritic cells was described to exacerbate the destruction of

β-cell, no study demonstrated any direct link between MIF and type I IFN in this context (319, 320).

This chapter of my thesis aimed to investigate MIF involvement in the production of TLR-induced type I IFNs by murine macrophages and dendritic cells. I also sought to investigate whether the pattern of type I IFN production between macrophages and dendritic cells differs in response to TLR4 activation. Moreover, the possible role of the tautomerase enzymatic activity of MIF was studied by using well-defined inhibitor. My findings in this chapter show that macrophages demonstrated enhanced type I IFN production in the absence of MIF compared to WT cells. This observation was consistent across all macrophage types with the same pattern demonstrated in immortalized and primary macrophages.

The discrepancy between the levels of IFN detected in BMDCs stimulated with LPS in different experiments (data depicted in Figure 11 versus Figure 13) is likely due to differences in the purity, source and serotypes of LPS used across the two different laboratories in which these experiments were conducted. Regardless, the results consistently show a role for MIF regulating IFN production in BMDCs.

In addition, enhanced type I IFN production in *Mif*$^{-/-}$ mice which lack MIF-1 (but do express MIF-2), suggests that MIF-1 acts as an endogenous negative regulator of LPS-induced type I IFN in examined macrophages. However, blockade of tautomearse activity by a potent antagonist (4-IPP) that binds covalently to enzymatic active site of both MIF-1 and MIF-2 molecules also caused a significant reduction in LPS-induced type I IFNs productionsuggesting a possible regulatory role for intracellular MIF-1 and MIF-2 in of type I IFN production in macrophages through their enzymatic tautomerase function. Furthermore, this is further shown when LPS-induced production of pro-inflammatory cytokine (TNF-α) by iBMMs and BMDMs from *Mif*$^{-/-}$ mice was unaffected, yet reduced when MIF-1 and MIF-2 tautomerase activity was blocked.

The results of this study suggest that MIF-1 and maybe MIF-2 molecules can influence IFN induction following TLR4 induction; however, more experiments are required to confirm this.

In line with the results described above, the regulation of TLR4-induced macrophage pro-inflammatory responses by MIF has been reported in several settings, including modulation of host responses against gram-negative bacteria (LPS) by macrophages, production of pro-inflammatory mediators, TLR4 receptor expression, and NF-κB activation (208-211, 214-219). In terms of tautomerase activity, some studies suggested tautomerase function to be one of the activities responsible for MIF modulatory effect on TLR4-induced inflammatory responses, depending on the type of involved cell (220-222). However, any role for MIF on type I IFN production has not been reported.

In investigating a possible role for MIF in dendritic cells, my results suggest that MIF may be a positive regulator of type I IFN production in these cells since *Mif*$^{-/-}$ BMDCs showed reduced IFN production compared to those derived from WT mice. In addition, dual inhibition of MIF-1 and MIF-2 tautomerase activity using 4-IPP in WT and *Mif*$^{-/-}$ BMDCs inhibited IFN production further in LPS-stimulated type I IFNs secretion. Cultured BMDCs are more similar than other primary macrophages to pDC that are the dominant source of type I IFN production *in vivo* (ref for this here). My duration of this study did not allow me to undertake studies in pDCs, which would be an obvious next step and important to fully understand the role of MIF in type I interferon production.

With regards to IFN production, the results obtained in this chapter indicate that MIF molecules exerts opposite regulatory roles in macrophages and dendritic cells upon the activation of TLR4 pathway. In addition, tautomerase enzymatic activity was identified as a possible mechanism by which MIF may regulate type I IFN production in both macrophages and dendritic cells.

In this chapter, I also investigated the role of MIF in regulating TNF-α production by LPS in macrophages (PECs and BMDMs) and in BMDCs. Unlike

macrophages, TNF-α induced by LPS in bone marrow dendritic cells was noticeably reduced in *Mif* $^{-/-}$ cells compared to WT cells suggesting that MIF might be required for LPS-stimulated secretion of TNF-α in BMDCs and that its tautomerase activity may also have a role to play. The effects of 4-IPP in *Mif* $^{-/-}$ cells suggests a possible role for MIF-2. MIF-1 and tautomearse activity is required for IFN and TNF by BMDCs, and MIF-2 is probably involved as well. Briefly, we demonstrated that MIF-1 deficiency and MIF-1/MIF-2 tautomerase inhibition reduced the ability for type I IFN and pro-inflammatory TNF-α cytokine production in dendritic cells in response to TLR4 stimulation. It is possible that both MIF-1 and MIF-2 influences on dendritic cell activation; however, further investigations are essential to establish this.

My results with regards to the effect of MIF tautomerase inhibitor, are in agreement with other studies in the literature. A study confirmed requirement of tautomerase activity for MIF autocrine and paracrine actions in macrophage inhibition (321). In addition, inhibition of MIF-1 tautomerase activity using ISO-1 is considered a potential therapeutic strategy in several TLR-mediated inflammatory conditions (247). For example, enhanced MIF expression during viral infections such as H5N1 influenza virus pneumonia and Respiratory syncytial virus (RSV) is essential for viral clearance and production of inflammatory cytokines induced by TLR4 activation such as TNF-α. In these studies, ISO-1 suppressed TNF-α secretion by infected macrophages (222, 322-324). In comparison, the compound 4-IPP covalently binds within the tautomerase enzymatic sites of both MIF-1 and MIF-2 and block their signalling pathways possibly through the inhibition of the NF-κB (325-328).

The main finding of this chapter is that MIF modulates TLR4-induced responses in macrophages and dendritic cells. It is widely known that controlling TLR4-mediated inflammatory responses is crucial to restore the homeostasis and prevent development of auto-inflammatory diseases and septic shock (200). Several pieces of evidence shows that negative regulation of TLR4 signalling

pathway occurs at multiple levels via intracellular regulatory checkpoints (329). In this regards, several intracellular molecules such as chaperones and adaptor proteins identified that negatively regulate TLR4 signalling (329). In addition, various natural and synthetic compounds are presented as TLR4 antagonists and TLR4-induced type I IFN production at drug development stage (330-333). Results of this chapter are in line with the previously published studies suggesting that intracellular MIF might play a direct role by interacting with one or several regulatory molecules in TLR signalling pathway as a mechanism to down-regulate the production of both type I IFN and TNF-α in macrophages and dendritic cells.

The regulation of TLR4-induced responses by MIF in various cell types including dendritic cells and macrophages of various sources has been reported (208). Extracellular MIF was shown to control the cell surface level of TLR4 in response to LPS through controlling TLR4 mRNA expression (209-211). Induction of TLR4 responses in peritoneal macrophages of MIF knockout mice leads to a declined level of TNF-α, indicating selective post TLR4 effects (214). Also, the induction of NF-κB activity by MIF has been suggested as another step of modulating the TLR4 response (217-219). Inhibition of MIF tautomerase activity reduces TLR4 expression, NF-κB nuclear translocation and the production of pro-inflammatory mediators (220, 221). Therefore, MIF tautomerase function could be one of the actions responsible for its modulatory effect on TLR4-induced responses. There are some hypotheses that might partly explain the opposite results obtained here on macrophages versus dendritic cells. Firstly, MIF-1 is shown to be expressed on both murine macrophages (334) and dendritic cells (335) but MIF-2 is only reported in murine dendritic cells (336). Another explanation could be difference between macrophages and dendritic cells in terms of MIF expression and type I IFN and TNF-α production upon TLR4 activation (337).

It is known that MIF is involved in host resistance to several parasitic infections. In mouse models of infection with *Toxoplasma gondii* and *Trypanosoma cruzi*, MIF-deficient DCs displayed weakened resistance to the parasites. In the absence of endogenous MIF, impaired maturation of DCs was associated with a weaker production of IL-12 and pro-inflammatory mediators, and reduced expression of costimulatory molecules (338, 339). These studies confirmed the requirement of endogenous MIF for early maturation of DCs and host protection against parasitic infections. It is known that one of the main effects of MIF on the immune system is the upregulation of TLR4 expression (212). Activation of TLR4 pathway and induction of type I IFN response in dendritic cells following by recognition of PAMPs derived from *T. gondii* and *T. cruzi* play critical role in conferring protection during the infection (340-345). In addition, MIF has been shown to the important in the pathogenesis of TLR4-related viral infections caused by influenza virus (346), dengue virus (347, 348), Ebola virus (349), and HIV (350). It has been shown that maturation of conventional BMDCs by TLR4 stimulation is associated with significant up-regulation of MIF, type I IFN (mainly IFN-β) and TNF-α production (335, 351, 352). These findings suggest that modulation of MIF tautomerase function may alter TLR4 responses but could be dependent on the type of cell involved (222). Although TLR4 signalling mediated induction of type I interferon is well described, the role of MIF in this effect is not clear. This chapter of my thesis partly addressed this experimentally.

Taken together, the present study for the first time demonstrates that intracellular MIF-1 and possibly MIF-2 molecules regulate the production of type I IFN upon TLR4 stimulation. In addition, MIF molecules have opposite effects on primary macrophages and dendritic cells. Moreover, a potential role for tautomerase enzymatic activity of MIF is suggested. These findings reveal MIF as an important regulator of type I IFN production and pro-inflammatory response in macrophages and dendritic cells. Although more detailed experiments are necessary to explain the exact mechanism involved in this effect, there is no doubt

that MIF-1/MIF-2 and the tautomearse function represent potential target for TLR related diseases.

Conclusion

In summary, the present study for the first time suggests that MIF-1 gene deletion was consistently associated with increased IFN release in response to LPS, suggesting that MIF-1 acts as an endogenous inhibitor of LPS induced type I interferon release. Future studies should examine MIF effects in pDCs, as well as examine the potential role of MIF-2 that is suggested by the opposing effects of MIF deficiencies and tautomerase inhibition in macrophages and the effects of tautomerase inhibition in cells deficient in MIF-1. Some evidence of a role for MIF in supporting TNF-α production was found.

Chapter 5. Summary Discussion

MIF as a multifunctional molecule can control inflammation, direct innate and adaptive immune responses, and orchestrate tissue regeneration and healing (12) which are the main properties of characterised DAMP molecules (165). Importantly, MIF exerts physiological functions inside cells and modulates immune responses and tissue healing upon active secretion during inflammatory conditions (12, 144). Several studies have demonstrated significant release of MIF in conditions associated with tissue damage and injury (87, 145-151). In addition, a DAMP-like passive release of MIF by neutrophils undergoing necrotic cell death has been shown (152, 153). Although considerable indirect evidence is available, to our knowledge, ours is the first study to clearly show that MIF possesses characteristics of a DAMP molecule. In the first part of this project, I aimed to study whether MIF is released like a DAMP molecule. In the next step, I examined whether MIF regulates TLR dependent responses, which may reflect DAMP like activity.

The results obtained in this study showed that MIF was passively released like a DAMP molecule by monocytes undergoing necrotic (primary necrosis, pyroptosis and necroptosis) but not apoptotic cell death. This finding identifies MIF as a candidate DAMP molecule. It has been proven that many DAMP molecules are secreted in extracellular vesicles (EVs) as an important route to mediate cell-to-cell communications in physiological and pathological conditions (52, 55). In this regard, MIF has been shown to be found in EVs in response to particular signals and thus transfer regulatory signals (47, 58, 59). For example, MIF is co-expressed with well-defined biomarkers in the exosomes secreted from lung cancer cells that could improve the specificity of lung cancer diagnosis (60). In addition, MIF is secreted in exosomes by adipocytes in response to adipokines that affect insulin resistance (61, 62). These findings could provide a link between two parts of my thesis that has not been investigated here. Following release or secretion, MIF impart a wide range of biological functions (87, 145-151). In

conclusion, my study demonstrates that MIF meets several criteria for it to be considered a DAMP, as it is released during necrotic cell death and is active in inflammation.

In an attempt to reveal a novel innate immune immunomodulatory role for MIF, we also showed a divergent role for endogenous MIF in the regulation of type I interferon in macrophages and dendritic cells. Moreover, my research work showed the involvement of MIF in IFN release varies according to cell type, with a role provisionally demonstrated in dendritic cells, but also that a role for MIF-2 cannot be excluded as demonstrated by the use of tautomerase inhibitors. Future studies should address these remaining knowledge gaps. Overall, my findings expand the current knowledge and understanding of the mode of release and biological function of MIF in immune cells.

Future directions

Future studies are required to investigate the mechanism behind the role of MIF in type I IFN production. Also, I suggest performing the same experiments on pDC as the dominant source of IFN-α *in vivo*. Identification of intracellular transcriptome profiles of relevant inflammatory genes is recommended to detect differential gene expression following cellular stimuli or damage in wild-type and *Mif*$^{-/-}$ cells. Also, study of the stimulation of other toll-like receptors like TLR7 and TLR9 may provide more information to the field. In addition, using other approaches to inhibit MIF tautomerase activity, and studying the effect of MIF-2 such as through the use of MIF-2 deficient, or MIF-1/MIF-2 double deficient cells, could be worthy. Furthermore, these findings should prompt future *in vivo* experiments in autoimmune and infectious animal models.

Appendix 1. Assays for measuring cell death to detect macrophage migration inhibitory factor (MIF) release

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Abstract

Cell death is a vital process for maintaining tissue homeostasis and removing potentially harmful cells. Cell death can be both programmed and non-programmed and is commonly divided into two main forms termed apoptotic and necrotic death modes. In this chapter cell death is classified into apoptosis, primary necrosis, pyroptosis and necroptosis. This chapter outlines the measurement of these different types of cell death and the relationship of measuring MIF release in these assays.

Key words MIF, Apoptosis, Necrosis, pyroptosis, necroptosis, Flow cytometry, Colorimetric assay, ELISA

1. Introduction

Macrophage migration inhibitory factor (MIF) is an immunoregulatory cytokine and pleiotropic inflammatory mediator that is constitutively produced by a variety of cells, including monocytes and macrophages (5, 12, 353). In contrast to many cytokines, MIF is constitutively expressed and stored in intracellular cytoplasmic pools, and therefore does not require *de novo* protein synthesis before secretion. As a result, MIF can be rapidly released in response to stimuli, such as microbial products, pro-inflammatory mediators, proliferative signals, hypoxia and stress (302, 354). Importantly, MIF is implicated in the pathogenesis of sepsis (355), inflammatory and autoimmune diseases such as rheumatoid arthritis (238, 356) and systemic lupus erythematosus (237, 357). Thus suggesting MIF-directed therapies might offer new treatment opportunities for human diseases in the future (247). Multiple clinical studies have also pointed to the potential of MIF as a biomarker in the context of inflammatory diseases, including systemic infections and sepsis, autoimmune diseases, cancer, and metabolic disorders (358).

The mechanism by which MIF is secreted/released has not been fully established, although in a recent study MIF was found to be released by neutrophils during secondary necrosis, but not in response to microbial stimulators (153, 359). Cell death is commonly divided into two main forms termed apoptotic and necrotic death modes. Apoptosis is a programmed non-lytic mode of cell death that is tightly regulated through extrinsic and intrinsic major signalling pathways mediated by caspases (360), and apoptotic cells are regularly cleared by phagocytosis without triggering inflammation (361-364).

Here, we describe techniques for inducing and measuring different forms of cell death in cells of the monocyte/macrophage lineage. In particular, we cover techniques for examining apoptosis, necrosis, pyroptosis and necroptosis (Table 1).

Pyroptosis and necroptosis are forms of non-apoptotic cell death, where the cell swells and membrane rupture releases cellular contents (365). Pyroptosis is a form of programmed necrosis which is caspase-dependent and can be activated by microbial pathogens (366). Detecting the caspase-1 dependency using caspase-1 inhibitor can be used to distinguish pyroptosis from other necrotic cell death forms as outlined below in this methods chapter, and additionally so is the measurement of IL-1β via ELISA (89, 90, 294, 295, 367-369).

Necroptosis is initiated through several internal and external ligand-receptor interactions. Similar to pyroptosis, it can result in organ swelling, membrane rupture and release of cell contents. For necroptosis to occur Kinase activity of receptor-interacting protein 1 (RIP1) is required and is induced by TNF-α by binding to its receptor (TNFR1), which causes the recruitment of the TNF-receptor-associated death domain (TRADD), RIPK1 and ubiquitin E3 ligases to form a complex (370). It is then the deubiquitination of RIPK1, which causes its disassociation from this complex and a formation of a new complex termed complexIIb (the necrosome). Caspase-8 then needs to be inhibited for necroptosis to occur. Necroptosis can be experimentally induced by endogenous protein called second mitochondria-derived activator of caspases (SMAC) or artificial SMAC-mimetics in combination with caspase inhibitors. Necroptosis can be inhibited by suppression of RIP1 kinase activity with necrostatin-1, which can be done experimentally as outlined in the methods below (276, 297-301, 371). The detection of pyroptosis and necroptosis is via the lactate dehydrogenase (LDH) assay. This assay measures the release of this enzyme upon cell death due to plasma membrane damage, with LDH activity proportional to cell lysis.

This chapter outlines the measurement of various forms of cell death and how measuring the level of MIF is a tool which can be correlated with necrotic cell death regardless of the necrosis form and involved pathways. Blocking necrosis is also associated with suppression of MIF release. In summary, measuring MIF release/level can be considered as a biomarker of necrotic cell death and tissue

injury and is a beneficial additional measurement to be performed in cell death assays.

Table 1. Overview of multiple modalities of induced cell death and methodology.

Cell death induced	Inducer/s	Intermediate signalling	Inhibitor/s	Detection methods	References
Necrosis	Ethanol	None	Pan caspase inhibitor (Z-VAD-FMK)	Flow cytometry assay: quantification of PI staining.	(22)
Pyroptosis	LPS + Nigericin	Caspase-1 dependent, NLRP3 inflammasome activation	Pan caspase inhibitor (Z-VAD-FMK) Caspase-1 inhibitor (Z-YVAD-FMK)	Florescence-based detection of LDH activity MIF and IL-1β ELISA	(30)
Necroptosis	TNF-α + Z-VAD-FMK + BV-6	Caspase independent, RIP1 kinase	Necrostatin-1	Florescence-based detection of LDH activity MIF ELISA	(32)

2. Materials

2.1. Inducing apoptosis with staurosporine

1. Staurosporine (1 µM)
2. Pan caspase inhibitor, Z-VAD-FMK (50 µM),

2.2. Inducing necrosis with ethanol

1. Ethanol 10%, v/v

2.3. Inducing pyroptosis with nigericin

1. Nigericin (10 µM)
2. Lipopolysaccharide (100 ng/ml)
3. Caspase-1 inhibitor, Z-YVAD-FMK (25 µM)

2.4. Inducing necroptosis

1. SMAC mimetic BV-6 (IAP antagonist, 1 µM, *see* **Note 1**)
2. Necrostatin-1 (30 µM, *see* **Note 2**)
3. Recombinant human TNF-α (30 ng/ml)

3.5 Colorimetric-based Lactate dehydrogenase (LDH) release assay

1. LDH assay kit (*see* **Note 4**)

Cell Line: Human monocyte THP-1 leukemic cells (ATCC TIB-202, *see* **Note 5**)

Complete media: 500 mL RPMI 1640 media supplemented with 10% Heat-inactivated Foetal Bovine Serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin and 2 mM L-Glutamine

Tissue culture consumables: T75 vented flasks, 6 well plates and 96 well plates.

3. Methods

All procedures are carried out at room temperature unless otherwise specified. All procedures should be carried out in a class II biological hazard cabinet to maintain a sterile environment unless otherwise specified.

3.1. Inducing necrosis with ethanol

1. Seed THP-1 cells (1×10^5 cells/well) in round bottom 96-well plate in 160 µl in complete media (*see* **Note 6**).
2. Keep a set of triplicate wells as media alone control wells for the assay. These wells complete the same experimental procedure outline lined below with the exception that media is added to the wells at each step instead of reagents.
3. Add 50 µM Z-VAD-FMK to triplicate wells and incubate at 37°C in CO_2 incubator for 30 min.
4. Add ethanol 10%, v/v in triplicate wells. Incubate for 1-6 hours at 37°C in a CO_2 incubator.
5. Centrifuge the cells at 1200 rpm for 5 minutes. Harvest the cell pellets for flow cytometry based cell death assay (section 3.5). If required, supernatants

can be collected and stored at -20/-80°C for analysis of secreted factors (e.g. by ELISA).

3.2. Inducing pyroptosis with nigericin

1. Seed THP-1 cells (1×10^5 cells/well) in flat bottom 96-well plate in 160 µl in complete media.
2. Keep a set of triplicate wells as media alone control wells for the assay. These wells complete the same experimental procedure outline below with the exception that media is added to the wells at each step instead of reagents.
3. Add LPS (20 µl, final concentration of 100 ng/ml) in a total to a 200 µl volume to triplicate wells and incubate overnight (16-18 h) at 37°C in a CO_2 incubator.
4. Centrifuge and remove the media. Add 20 µl of 10x concentrate of caspase-1 inhibitor (25 µM, Z-YVAD-FMK) and incubate for 30 minutes at 37°C in a CO_2 incubator.
5. Add 20 µl of nigericin (10 µM) to LPS stimulated wells in triplicate and create a time course with hourly intervals for 1 to 3 hours at 37°C in a CO_2 incubator. Create a separate plate for each time point.
6. At each hour time point. Centrifuge the cells at 1200rpm for 5 minutes and collect 180 µl of the supernatant for MIF, IL-1β (*see* **Note 8**) cytokine assays (as per ELISA protocol) and measurement of cell death by LDH assay (section 3.6) (372). Supernatants can be stored at -20/-80°C until assays are performed.

3.3. Inducing necroptosis

1. Seed THP-1 cells (5×10^5 cells/well) in round bottom 96-well plate in 160 µl in complete media.

2. Keep a set of triplicate wells as media alone control wells for the assay. These wells complete the same experimental procedure outline lined below with the exception that media is added to the wells at each step instead of reagents.
3. Add 20 µl of Necrostatin-1 (30 µM) in triplicate wells to create a necroptosis induced group.
4. To these wells, add 20 µl of a mixed combination of recombinant human TNF-α (30 ng/ml, final concentration) and SMAC mimetic BV-6 (IAP antagonist, 1 µM, final concentration) for 18 hours.
5. Centrifuge the cells at 1200 rpm for 5 minutes and collect 180 µl the supernatant for MIF ELISA assay (as per ELISA protocol). Cell death was detected by LDH release assay (section 3.6). Supernatants can be stored at -20/-80°C until assays are performed.

3.4. Colorimetric-based Lactate dehydrogenase (LDH) release assay

Vehicle-Only Cells Control: Untreated cells serve as a control for the use of the solvent delivery vehicle in the assay. Add the same solvent used to deliver the test compounds to the vehicle control wells.

1. Carry out protocols for inducing pyroptosis (section 3.3) and necroptosis (section 3.4) as per methods above. In addition, for the LDH assay include the following controls; a no cell control (this will determine background readings of the culture media), an untreated cell control and a maximum LDH release control (add 10 ul of 10x lysis solution per 100 ul of untreated cells, 45 minutes prior to adding CytoTox 96R reagent) to allow calculation of % cytotoxicity.
2. Forty-five minutes prior to harvesting supernatants (from the pyroptosis and necroptosis experiments sections 3.3 and 3.4 above), induce cell lysis in the set of Maximum LDH Release Control wells by adding 10 µl of Lysis Solution (10X) for every 100 µl of cells. Total cellular LDH is measured by lysis of cells.

3. Spin at 1200 rpm for 5 minutes.
4. Collect 100 µl of the supernatant for LDH release assay.
5. Add 50 µl of the supernatant all wells using a multichannel pipette to a fresh 96-well flat-bottom (enzymatic assay) plate.
6. Add 50 µl of CytoTox 96R Reagent to each well of the enzymatic assay plate containing the samples.
7. Cover the plate with foil to protect it from light and incubate for 30 minutes at room temperature.
8. Add 50 µl of Stop Solution to each well.
9. If any large bubbles are present pop these with sterile tip.
10. Read the absorbance at 490nm, using a microplate reader, within 1 hour of adding the Stop Solution.
11. For calculation of results, subtract the average absorbance value for the Culture Medium Background from all absorbance values.
12. Subtract the average absorbance values for the Volume Correction Control from the absorbance values obtained for the Target Cell Maximum LDH Release Control. Use the corrected values obtained in the following formula to calculate percent cytotoxicity.
13. Results are presented as cytotoxicity percentage.

3.5. Propidium Iodide (PI) staining

The pattern of cell death induced by ethanol was detected using PI staining and SPHEROTM AccuCount particles to improve the precision of cell counting. For this purpose, counting particles were added to each well (1:10 dilution in FACS buffer, v/v) and the plate was then centrifuged (1200 rpm, 5 min, and 4°C). Next, pellets were washed with PBS and incubated in FACS buffer containing PI solution (5 µL) for 5 min at room temperature in the dark. Finally, samples (10 000 events) were assessed within 1 h using a flow cytometer and data were analysed by FlowJo v10 software. Cells positive for PI indicated the occurrence

of necrotic cell death. The absolute cell count was calculated using the following equation: (A/B) x (C/D) = Number of cells per μL. Where:

A = number of events for the sample

B = number of events for the particles

C = number of particles per 50 μL

D = volume of test sample initially used in μL

4. Notes

1. Use of SMAC mimetic BV-6 (IAP antagonist) is to reduce polyubiquitination of RIP1 and increases sensitivity to cell death.
2. Necrostatin-1 is a specific inhibitor of RIP1 kinase inducing necroptosis.
3. Counting beads for use in flow cytometry are available from a range of scientific companies for purchase.
4. LDH assay kits are commercially available to quantify cell viability via the release of LDH. It is an enzymatic assay allowing the quantification of released LDH to be measured via a colorimetric assay. The amount of colour formed is proportional to the number of lysed cells.
5. Beware of cell line variability for the induction of different forms of cell death. Some cell lines may have varying caspases and some may lack function. Perform an initial dose response curve to ensure the appropriate concentration of compound for your cell line is used.
6. To perform these assays, THP-1 cells can be seeded at a range of 1 to 5×10^5 cells/well, keeping cell number consistent between wells in a single experiment.
7. For pyroptosis assays, release of IL-1β can be used as a specific readout for inflammasome activation.
8. The use of counting beads can assist the standardization between cell death assays and determination of absolute cell count via the equation in the methods section 3.5.

9. PI staining alone can be performed to indicate late apoptosis/necrosis cell death. PI is a membrane impairment dye which is largely excluded from viable cells and binds to double stranded DNA.

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