Analysis of the effects of Macrophage Migration Inhibitory Factor (MIF) on the activation of Toll-like receptor 4 (TLR4)

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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of my own investigation except where a reference has been made to published literature or acknowledgement is made for unpublished research.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 UEL, I have not been a registered or enrolled for another award of this university or of any other academic or professional institution.

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Abstract

Macrophage Migration Inhibitory Factor (MIF) was one of the first cytokines to be discovered over 50 years ago and has been characterised in a wide range of mammalian and parasite species. This unique protein possesses the characteristics of a cytokine, chemokine and hormone along with two distinct enzymatic activities. MIF has been shown to be involved in innate and adaptive immunity by modulating Toll-like receptor 4 (TLR4) intermediates such as MAPK and downstream pathways in inflammation and in cell growth and differentiation, thus playing a key role in chronic inflammation and tumorigenesis. In this study, the role of parasitic and mammalian MIF in TLR4 signalling was investigated. *Mus musculus* MIF1 (MmMIF1) cDNA was isolated by gene cloning and recombinant fusion MmMIF1 was purified alongside *Trichinella spiralis* MIF1 (TsMIF1) by affinity and ion exchange chromatography. The effects of MIF on TLR4 signalling were examined by analysing the downstream activation of NFκB and AP-1 via a transcriptional reporter using HEK-Blue™-hTLR4 cells as a model. It was found that upon co-administration of TsMIF1 with LPS, NFκB activation was significantly reduced when compared to LPS treatment alone while MmMIF1 caused no significant changes in the activation levels of TLR4. Since no changes were observed in the NFκB and AP-1 activation levels upon co-administration of MIF in the Tumour necrosis factor receptor 1 (TNFR1) pathway, this downstream effect of TsMIF1 can be said to occur within the TLR4 cascade upstream of Transforming growth factor beta-activated kinase 1 (TAK1).

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ALP	Alkaline Phosphatase
AP-1	Activator protein 1
APC	Antigen-presenting cell
AU	Arbitrary unit
BCA	Bicinchoninic acid
Bp	Base pair
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
COX-2	Cyclooxygenase 2
D-DT	D-Dopachrome Tautomerase
DMEM	Dulbecco’s Modified Eagle’s Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DPBS	Dulbecco's phosphate-buffered saline

EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Fetal Bovine Serum
GALT	Gut-associated lymphoid tissue
GI	Gastro-intestinal
HRP	Horseradish Peroxidase
IELs	Intraepithelial lymphocytes
Ig	Immunoglobulin
ILC	Innate Lymphoid cell
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JAB1	JUN-activation domain-binding protein 1
JNK	JUN N-terminal kinase
LB	Luria-Bertani medium
LBP	LPS-binding protein
LAL	Limulus Amebocyte Lysate
LPS	Lipopolysaccharide
LRRs	Leucine-rich repeats
MAPK	Mitogen- activated protein kinase
M cells	Microfold cells
MIF	Macrophage Migration Inhibitory Factor
MmMIF1	*Mus musculus* MIF1
NFκB	Nuclear factor-κB
NK	Natural Killer
NLR	Nucleotide-binding oligomerization domain like receptor

NOD	Nucleotide-binding oligomerization domain
NMWL	Nominal molecular weight limit
OD	Optical Density
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pNPP	p-Nitrophenyl phosphate
PMB	Polymyxin B sulfate
PRRs	Pattern Recognition Receptors
RBC	Red blood cells
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEAP	Secreted alkaline phosphatase
TAK1	TGFβ activated Kinase
Taq	Thermus aquaticus
TNF	Tumour-necrosis factor
TNFR1	TNF α receptor 1
TIR	Toll- IL-1 receptor
TLR	Toll like Receptor
TsMIF1	*Trichinella spiralis* MIF1

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1.0 Introduction

1.1 Innate Immunity and Pathogen Recognition

The human body is constantly exposed to the external environment in the form of air, water and food that facilitate the entry of harmful factors such as hazardous chemicals, toxins, radiation and pathogenic microorganisms. The skin and the mucosal tissues of the respiratory tract, gastrointestinal (GI) tract and urogenital tract are often the point of entry for most pathogens. In addition, these surfaces are also occupied by harmless and beneficial microorganisms called commensal bacteria that live in symbiosis with the host. In order to tackle such an exposure and owing to the need of managing both commensal and harmful bacterial populations, our bodies have evolved to protect itself by developing the innate immunity and adaptive immune responses acting at both body surfaces and within immune hubs such as the peripheral lymph nodes (Murphy and Weaver 2017). The cells that assist in these processes are immune cells such as lymphocytes, macrophages, dendritic cells and different repertoires of cells that trigger effector mechanisms tailored to offer the most effective protection to these sites. For instance, within the mucosal epithelial cells there are intraepithelial lymphocytes (IELs) present (Fig.1.1) which are mostly T cells that maintain homeostasis and serve as a hub for barrier functions such as antigen interaction (Cheroutre, 2004), cytotoxic activity (Cheroutre *et al.,*2011) and regulation of both innate and adaptive responses (Sheridan and Lefrançois, 2010). To illustrate this with an example, the state of the immune system including the repertoire of cells and response during infection in the GI tract is shown in Fig. 1.1. When the body encounters a pathogen, the innate immune response is first triggered to detect and destroy the pathogen. Pattern Recognition Receptors (PRRs) such as Toll like Receptors (TLR) are present on the

surface of dendritic cells, macrophages and other immune cells that are capable of recognising molecules that are unique to bacteria, fungi and viruses. These molecules are called Pathogen Associated Molecular Patterns (PAMPs). Examples of PAMPs include lipoteichoic acid derived from the cell wall of Gram-positive bacteria, lipopolysaccharide (LPS) derived from the outer membrane of Gram-negative bacteria, zymosan derived from the cell wall of fungi and single or double-stranded RNA encoded within viral genomes. Another type of an innate immune sensory complex, comprised of Nucleotide-binding oligomerization domain (NOD) and other variable domains, are collectively called NOD like receptors (NLRs) which are present within the cytoplasm of host cells. The NOD proteins are important in the detection of PAMPs derived from intracellular pathogens and in the activation of signalling cascades (Murphy and Weaver 2017).

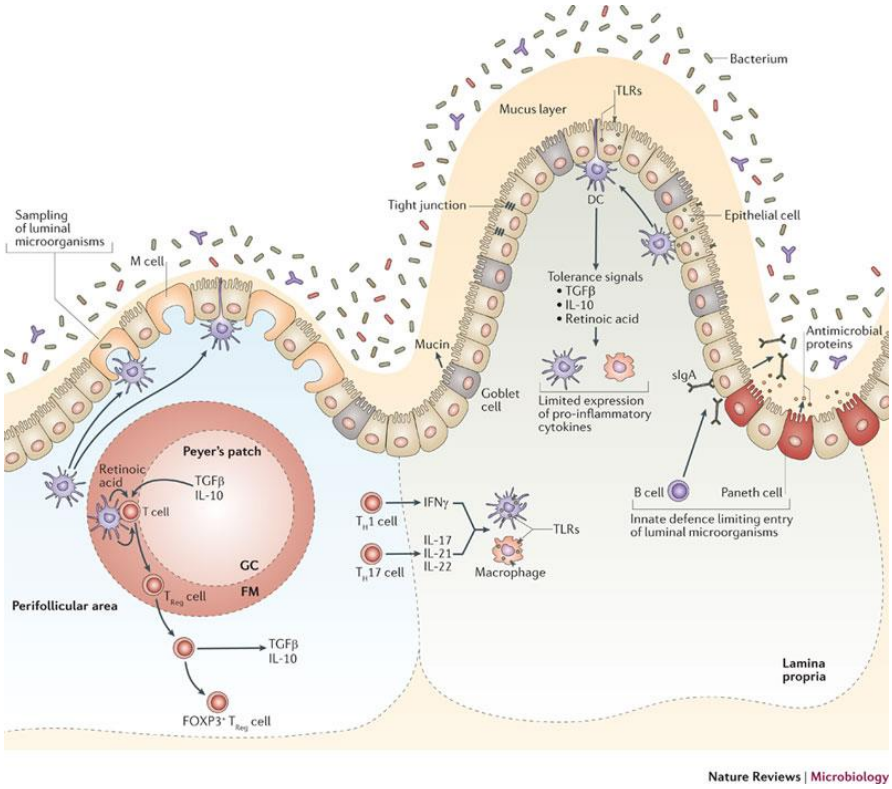

Figure 1.1: Example of the immune system within the GI tract where immune cells are integrated into mucosal surfaces and secondary lymphoid organs ready to respond to pathogens. This figure adapted from Bron *et al.,* 2012 shows the different layers of the gut and the immune cells they contain. Here, an infected state with bacterium is shown that involves various molecules such as antimicrobial peptides, antigens and PAMPs from the pathogen. The figure shows the outermost mucosal barrier followed by the epithelium that contains the TLRs at the surface. The epithelial layer also consists of the intraepithelial lymphocytes (IELs) that deliver the antigens to the Peyer's patches. M cells in the underlying membrane then transport the antigens to dendritic cells and macrophages to further activate T cells and B cells leading to an active inflammatory response.

1.1.1 Toll-like receptor (TLR) 4

Toll-like receptors (TLRs) are transmembrane proteins that consist of 18-25 copies of leucine-rich repeats (LRR) in the extracellular region (Fig. 1.2). The number of TLR-like genes expressed in different organisms varies with 10 being found in humans and 12 in mice. Each TLR has distinct ligands and is responsible for specific recognition of a single PAMP or set of PAMPs. PAMPs play a key role in activating the immune system and initiating the recruitment of immune cells. In mammals, TLRs are widely expressed in different innate and adaptive immune cells such as macrophages, dendritic cells, and B cells but are also expressed in other cells found at body surfaces such as epithelial and stromal cells. The activation of TLR receptors aids in stimulation of responses against pathogens (Murphy and Weaver 2017, p.88). Signalling is activated only upon dimerisation of TLRs or conformational changes in the dimer that are induced by binding of a ligand. All mammalian TLR proteins have a TIR (Toll- IL-1 receptor) domain in their cytoplasmic tail. The TIR domains interact with other TIR- type domains which are usually present in accessory signalling molecules involved in subsequent steps of TLR signal transduction (Murphy and Weaver 2017).

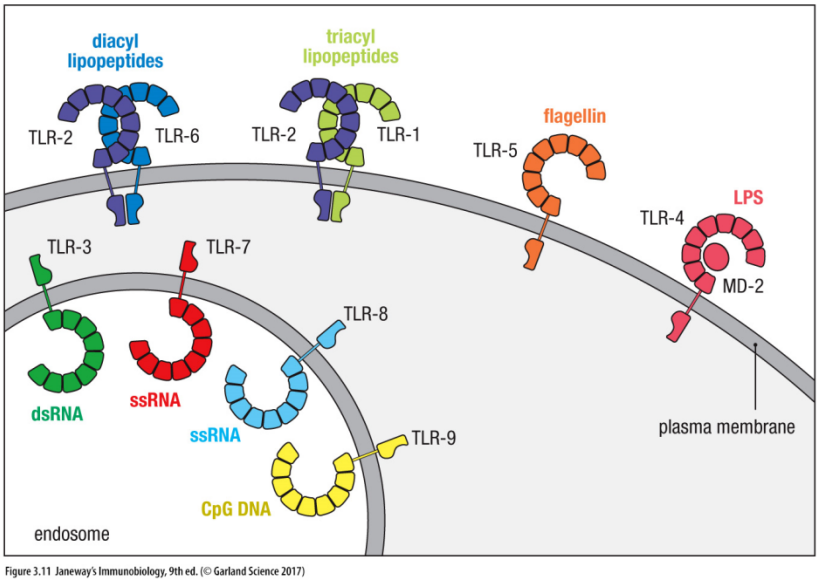

Figure 1.2: A representation of mammalian TLRs. This figure from Murphy and Weaver, 2017 shows mammalian TLRs that recognise a wide variety of distinct PAMPs and are found in different cellular locations. The figure shows nine of the TLRs expressed in humans and mice. The cellular localization and PAMP recognized by each receptor is also shown. TLRs are transmembrane proteins with 18-25 copies of leucine-rich repeats (LRR). For ease of representation, this figure displays only 9 LRRs which are illustrated using the small box-like structures. Some TLRs are localized within the cell membranes of many cells and are capable of detecting extracellular microbial PAMPs while others are found within endosomes which contain materials sampled from either the extracellular or intracellular compartments. While it is predicted that all TLRs form dimers, only those that form heterodimers are shown in dimeric form here.

Toll-like Receptor 4 (TLR4) is one TLR that is most widely expressed by many cells. LPS, a significant component of the outer membrane of Gram-negative bacteria is one of the best characterised PAMPs which is recognised by the TLR4 receptor. Recognition of LPS by TLR4 results in the activation of a series of signalling pathways that leads to activation of transcription factors such as Nuclear factor-κB (NFκB) and Activator protein 1 (AP-1). Activation of these factors leads to the recruitment of active macrophages and other immune cells along with release of cytokines, chemokines, other chemical mediators and in some cases effector molecules. TLR4 activation

requires three other accessory molecules which are involved in the binding and sequestration of LPS. These include LPS-binding protein (LBP), myeloid differentiation factor 2 (MD-2) and cluster of differentiation 14 (CD14). LBP binds directly to the extracellular LPS and assists in the interaction with CD14 (Tobias *et al.,* 1986, Wright *et al.,* 1989). CD14 further facilitates the association of LPS with the MD-2/TLR4 complex for LPS recognition (Wright *et al.,* 1990). TLR4 is incapable of binding to LPS independently hence, MD-2 is required for both- the trafficking of TLR4 receptor to the cell surface and recognition of LPS (Murphy and Weaver, 2017 p. 92). Together, the binding of LPS to this hetero-tetrameric complex initiates the activation of a series of signalling cascades (Gioannini and Weiss, 2007; Miyaki, 2007; Lu et al., 2008). A brief outline of the TLR4 activation pathway adapted from Miyaki, (2007) and Fitzgerald et al., (2004) is shown in Fig.1.3. After activation, the TIR domain of TLRs interacts with other TIR domains, and those domains are of adaptor molecules that are responsible for initiation of intracellular signalling. For mammalian TLRs, there are four adapters that are used: MyD88, TIRAP, TRIF and TRAM. Although most TLRs interact with MyD88 only or the MyD88/TIRAP pair, other TLRs like TLR3 use only TRIF; TLR2 heterodimers (TLR2/1 and TLR2/6) use the MyD88/TIRAP or TRIF/TRAM. TLR4 particularly uses both MyD88/TIRAP and TRIF/TRAM for signalling and gives rise to two types of signalling routes- MyD88- dependent and MyD88- independent pathways. Transduction of signals through these pathways activates a range of transcription factors such as NFκB and members of the activator protein 1 (AP-1) family like c-Jun. AP-1 proteins are activated through the mitogen- activated protein kinases (MAPK) pathway and will be discussed further later. The intracellular responses generated as a result of these cascades leads to the production of inflammatory cytokines, chemotactic factors and antimicrobial peptides. It also promotes maturation of antigen

presenting cells (Lu et al., 2008). At the same time, the host is unresponsive to LPS from commensals that reside in the epithelial surfaces such as the GI tract since gastrointestinal epithelial cells do not express TLR4 at the surface of the lumen under normal condition (Backhed and Hornef, 2003). To protect the host from opportunistic infections at submucosal surfaces by commensals during an invasion due to injury for example, TLR4 expression occurs at those surfaces. Other studies have also demonstrated that differences in the Lipid A moiety of LPS distinguish commensals from pathogens and commensal LPS either antagonise TLR4 or simply do not trigger a response (Munford and Varley, 2006).

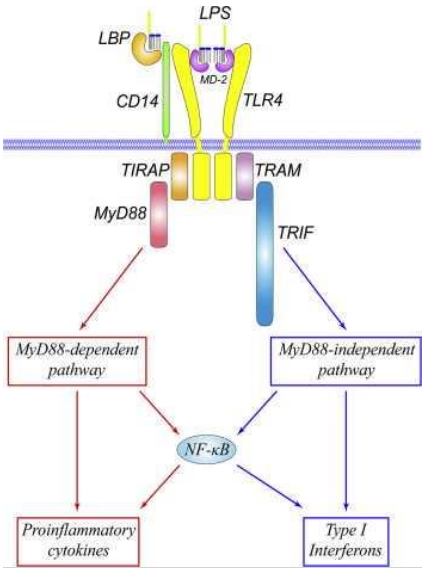

Figure 1.3: Two routes of TLR4 signalling after LPS administration. This figure adapted from Miyaki, (2007) and Fitzgerald et al., (2004) shows a brief overview of the intermediate steps in TLR4 signalling pathway occurring after TLR4 expressing cells are treated with LPS. LBP, CD14 and MD-2 are essential cofactors in the activation of the TLR4 receptor complex. After activation of TLR4, the transduction pathway bifurcates into MyD88 dependent and MyD88 independent signalling intermediates that result in the promotion of pro-inflammatory cytokines and type I interferons.

1.1.2 Adaptive immune response and dysregulation of the immune system

If the innate immune response fails to control and eliminate a foreign microbe or if a previously encountered pathogen is detected, the adaptive immune system is activated that ultimately results in the release of cytokines by innate immune cells that promote and polarize the adaptive immune responses into routes which encourage distinct immune outcomes. These outcomes are determined by the coordination of three major Innate Lymphoid cell (ILC) responses- ILC1, ILC2, ILC3 and T cell effector modules- T_H1, T_H2, T_H17 that collectively classify as Type 1, Type 2 and Type3 responses respectively. Type 1 response is directed towards intracellular pathogens that lead to the action of T_H1 cells, ILC1s, macrophage activation, recruitment of Natural Killer (NK) cells and release of IFN-γ. Type 2 responses are mainly targeted towards elimination of parasitic organisms that involve the activity of ILC2s, T_H2 cells, eosinophils, basophils and mast cells leading to antibody production, antigen-specific activation and recognition. Type 3 responses are triggered against extracellular bacterial and fungal infections which are characterised by the activity of ILC3s, T_H17 cells that opsonises IgG and recruits neutrophils (Murphy and Weaver, 2017, p. 27, p.451, Calandra and Roger, 2003). At the same time, the immune system is capable of distinguishing between pathogenic and non-pathogenic encounters which are mediated by regulatory T cells (Tregs). Tregs maintain self- tolerance thus preventing autoimmune diseases (Sagakuchi *et al.,* 1995, Wang *et al.,* 2007, Piao *et al.,* 2008, Wang *et al.,* 2008). They also maintain tolerance to: antigens derived from food (Chen *et al.,* 1994); foetus by carrying mother (Aluvihare *et al.,* 2004); and commensal microbiota thus preventing their elimination (Dembic, 2008). During these processes, complex sets of secreted immunoregulatory molecules and co-stimulatory molecules

are released from the cells to the surfaces. Most of these molecules are small proteins called cytokines and play a fundamental role in regulation of immune responses and coordination of humoral and cellular arms of the immune system (Calandra and Roger, 2003).

In most circumstances these activities result in clearance or control of the potential pathogens within a minutes or hours of entry into the body without disruption of homeostasis or the development of overt symptoms (Calandra and Roger, 2003). However in the rare instances when disruption of the homeostasis and function of the immune machinery occurs, undesirable or dysregulated immune responses can develop into loss of barrier function (Mankertz and Schulzke, 2007). Loss of barrier function can lead to systemic infections or pathogen induced inflammation and diarrhoeal disease which are a major cause of deaths in the world. For example, *Salmonella* is a most commonly occurring food borne pathogen that has been associated with the highest impact on disability in human health (Kirk *et al*., 2015; Scallan *et al*., 2015) and the most number of deaths (Havelaar *et al*., 2015). There are a large variety of bacterial, viral and protozoal pathogens whose infections can cause these effects. In addition there are groups of pathogens such as parasitic nematodes which establish long-term associations with the tissues of mucosal surfaces such as the GI tract. While they generally do not cause lethal infections they induce insidious pathological states that either have long term effects on adolescent development or adult disability (Stepek *et al.,* 2006). A dysregulation of one or more components of the immune system can lead to development of a range of outcomes including immunodeficiency disorders, chronic inflammation and in some instances cancer (Murphy and Weaver 2017, p. 533, Wen *et al.*, 2008, Lee *et al.*, 2010, Berer *et al.*,

2011, Wu *et al.*, 2010). Chronic inflammatory states are now recognized as a key preceding factor to the development of malignancies (Brey *et al.*, 2012) and within the GI tract the dysregulated inflammatory responses have been linked to progression of gastric and colon cancer (Bucala and Donnely, 2007, Gordon-Weeks *et al.,* 2015). Within the innate immune system, defects in the signalling cascades underlying PRR activation can give rise to chronic inflammation or immunodeficiency. Recently, several master regulators of PRR activation including a protein called Macrophage Migration Inhibitory Factor (MIF) which is a pleotropic cytokine, chemokine and hormone have been linked to the development of both chronic inflammation and cancer (de Jong, 2001, Murakami *et al.,* 2001).

1.2 Macrophage Migration Inhibitory Factor

Macrophage Migration Inhibitory Factor (MIF) is one of the first cytokines to be discovered about 5 decades ago. Produced by T-lymphocytes, it was shown to inhibit the random migration of macrophages (David, 1966, Bloom and Bennet, 1966). It is pleotropic in nature and has chemokine and hormone like characteristics. The MIF gene (Fig. 1.4) is located at Chromosome 22 in the human genome and Chromosome 10 in the mouse genome. This gene is found to be highly conserved within vertebrates. Many MIF homologues have also been found across the eukaryotes including species of plants and parasitic organisms (Calandra and Roger, 2003, Bernhagen *et al.,* 1994). The presence of MIF and its homologues across various species indicate that it has a significant conserved biological role (Calandra and Roger, 2003). Although T lymphocytes were initially thought to be the main source of MIF, it was eventually found that other cells such as macrophages, neutrophils, eosinophils, mast cells, basophils, blood dendritic cells and B cells also express and secrete MIF (Baugh and Bucala, 2002, Lue *et al.,* 2002)

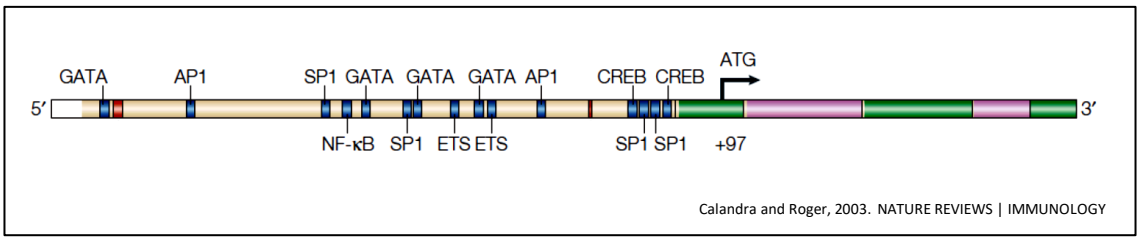

Figure 1.4: A schematic representation of the human MIF gene. The MIF gene comprises of three exons (highlighted in green) and two introns (shown in pink). The regulatory region at the 5' end consists of several consensus DNA-binding regions such as that of transcription factors like activator protein- 1 (AP-1), nuclear factor κB (NF-κB), ETS and GATA. However, how these DNA-binding sites interact with other proteins and regulate biological activities is not known.

The MIF protein has a molecular weight of 12.5 kDa and is secreted despite the fact it lacks the N-terminal endoplasmic reticulum (ER) targeting sequence. It is constitutively expressed in a wide variety of cells (Bernhagen *et al.,* 1994). One of the unique characteristics of mammalian MIFs is that they possess two distinct enzymatic activities, acting as both a tautomerase and an oxidoreductase. Structure function studies have shown that these enzymatic activities require the N-terminal Proline (P2 in all MIFs) for tautomerase activity and the CXXC motif (Cys-56 and Cys-59 in mammalian MIF1) motif at the C-terminus for oxidoreductase activity (Rosengren *et al.,* 1997, Kleeman *et al.,* 1998). While most identified MIFs have the P2 and are predicted to have tautomerase activity many MIF-like proteins including some parasite derived MIFs lack the CXXC motif and thus would not be expected to exhibit oxidoreductase activity (Fig. 1.5). In mammals a second MIF-like protein D-Dopachrome Tautomerase (D-DT)/MIF2 has been identified (Sugimoto et al., 1999, Merck *et al.,* 2012) which possess the tautomerase activity but lacks one of the CX domain (Cys-59), thus having an incomplete CXXC domain and no detectable oxidoreductase activity (Fig. 1.5).

Figure 1.5: Conservation of MIF1 and D-DT (MIF2) across mammalian and parasitic nematode species. Protein sequences of mammalian and parasite MIF1 and D-DT (MIF2) taken from NCBI (gene) and aligned using the multiple sequence alignment tool Clustal Omega. Conserved amino acids between the species are marked with an asterix (*). Amino acids highlighted in yellow shows the tautomerase active site that is conserved across all species for both MIF1 and MIF2 while the oxidoreductase active site (CXXC) is shown in green that is conserved only between human and murine MIF1. Blue highlighted amino acids are conserved Cys59 between human and murine D-DT.

The crystal structure of human MIF was characterised using X-ray Crystallography by Sun *et al.,* (1996). As seen in Fig. 1.6, MIF is a homotrimer and contains one β sheet with two additional β strand and two anti-parallel α-helices. The trimeric structure of MIF is arranged to form an inner, hydrophobic pore that is crucial for the maintenance of its enzymatic activities. However in one study, it was found that under certain physiological conditions, MIF can also exist in monomeric and dimeric forms (Sun *et.al.*, 1996) but how this affects its biological functions is not known. Structure and function studies have established that the C-terminus of MIF contains the motif responsible for chemokine receptor binding which results in the promotion of inflammatory processes by acting as a chemoattractant (Kraemer et al., 2011) and for binding to CD74 which is a putative receptor of MIF (Leng *et al.,* 2003).

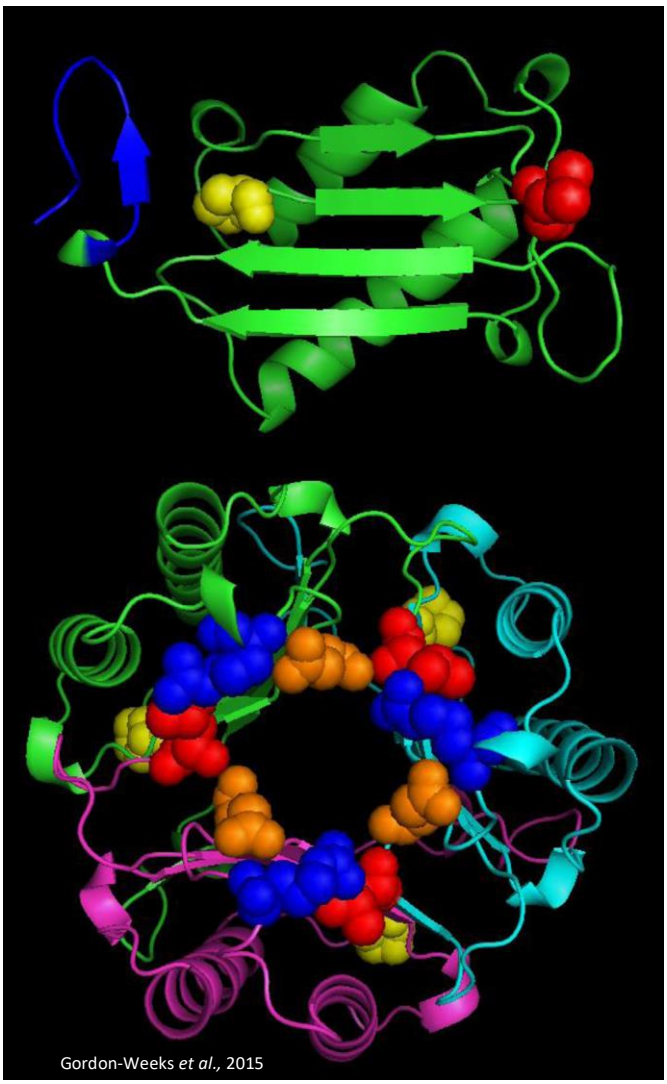

Figure 1.6: The three dimensional structure of human Macrophage Migration Inhibitory Factor (MIF). The image adapted from Gordon-Weeks et al., 2015 shows the tertiary protein structure of MIF in their monomeric and trimeric forms (top, PDB ref: 2WKF and bottom, PDB ref: 1MIF respectively). The oxidoreductase catalytic site can be seen in blue in the monomer while the CXCR2 motif- a chemokine receptor of MIF is shown in red in both the monomer and trimer. Proline 1 is displayed in yellow for both the monomer and trimer which confers the tautomerase activity to MIF. The trimeric structure shows the amino acids Aspartic acid-44 (orange) and Arginine-11 (blue) that are important for the chemoattractant properties of MIF. Individual monomers in the trimeric form are represented by cyan, purple and green.

In terms of biological functions and immunological activities, MIF was initially identified because of its chemoattractant properties for various leucocyte subsets. Later it was found that when mice are injected with endotoxin (LPS), MIF is released from the anterior pituitary gland and adrenal cortex like a hormone, antagonising glucocorticoid activity and promoting systemic endotoxemia (Xia et al., 2005, Calandra and Roger, 2003). The adrenal cortex significantly contributes to the systemic levels of MIF present maintaining both basal concentration and elevating levels of MIF post LPS administration (Bernhagen *et al.,* 1993). Cells such as macrophages and monocytes can also secrete large quantities of MIF when treated with LPS (Calandra et al., 1994) which is linked to control of local inflammatory responses. However, the basis for how MIF exerts its pro-inflammatory activities remained a mystery until relatively recently which will be discussed in detail further below.

1.3 Role of MIF in Innate Immunity

1.3.1 Influence of MIF in inflammation

Studies have shown evidence of MIF being directly or indirectly involved in the production of a large number of pro-inflammatory molecules in a wide variety of cell types. These include cytokines such as IL-1β, IFN-γ, tumour-necrosis factor (TNF), IL-2, IL-6, IL-8 and macrophage inflammatory protein 2 (Calandra *et al.,* 1994, Calandra *et al.,* 1995, Bacher *et al.,* 1996, Donnelly *et al.,* 1997, Makita *et al.,* 1998), nitric oxide (NO) (Bozza *et al.,* 1999, Bernhagen *et al.,* 1994), as well as Cyclooxygenase 2 (COX2) and other elements of the arachidonic acid pathway (Mitchell *et al.,* 1999, Mitchell *et al.,* 2002). Thus, MIF seems to have a crucial role in the inflammatory response after exposure to pathogenic molecules, other pro-inflammatory cytokines and antigen-specific stimulation. Since MIF is released not only as a cytokine but also as a hormone from the anterior pituitary gland, MIF levels can systemically aggravate an inflammatory response. For example, in one study, mortality in patients with sepsis was correlated with high MIF levels (Chuang *et al.,* 2014, Bozza *et al.,* 2004, Chuang *et al.,* 2007). Studies in mice showed that inhibition of MIF protected the mice from lethal toxaemia post LPS administration (Bernhagen et al., 1994). These studies show that inhibition of MIF could control inflammatory responses and thus can have therapeutic potential in some inflammatory diseases. With such observations, an unexpected finding was that glucocorticoid hormones promoted MIF secretion rather than inhibiting it (Calandra *et al.,* 1995). MIF acts as an antagonist to glucocorticoids thus inhibiting its anti-inflammatory function and the effects of other immunosuppressive cytokines (Santos *et al.,* 2001, Bacher *et al.,* 1996). In *in vitro* studies, it was found that MIF counter-regulated glucocorticoid impedance of IL-8, IL-6, IL-1 and TNF production

in peripheral blood mononuclear cells (Calandra *et al.,* 1995). This behaviour was confirmed in mice experiments related to endotoxaemia and antigen-induced arthritis (Leech *et al.,* 2000). It was also found in various studies that along with glucocorticoids, levels of circulating MIF increased during inflammation and infection (Calandra *et al.,* 2000, Calandra *et al.,* 1995, Beishuizen *et al.,* 2001). MIF's involvement with TLR4 and intermediates such as the mitogen- activated protein kinases (MAPK) that are crucial mediators of pro-inflammation has been evidenced and are elaborated in further detail below.

1.3.2 Modulatory activities of MIF in TLR4 signalling and the MAPK Pathway

MIF is constitutively expressed in many cells including macrophages, which is unusual for other cytokines which are known to modulate pro-inflammatory immune reactions. A number of studies have shown that MIF can modulate TLR4 signalling in immune cells (Roger et al., 2001, Sivaram et al., 2012) however the exact mechanism underlying this activity has not been fully elucidated. Studies with macrophages lacking the MIF protein revealed that those cells had reduced response to LPS but a normal response to other stimuli. The hyporesponsiveness observed in MIF deficient cells was subsequently shown to arise from the downregulation of TLR4 receptor which subsequently lead to lower downstream pro-inflammatory cytokine production (Roger et al., 2001, Roger et al., 2003). In the study by Calandra and Roger (2003), MIF was shown to upregulate TLR4 expression by controlling the activity of the ETS transcription factors which are important for the transcription of TLR4 gene in mice. Thus, it was thought that MIF assists the cells that are in the forefront of the pathogen defence system such as macrophages and allows rapid detection of endotoxin carrying bacteria. This was validated by another finding where MIF- deficient mice were unable

to inhibit the growth of *Salmonella typhimurium*- an intracellular pathogen (Koebernick et al., 2002). Studies carried out later reported that there was reduced secretion of pro- inflammatory molecules such as IL-1β, TNF and Prostaglandin E2 (PGE_2) in the survival of MIF-deficient macrophages over wild type macrophages post LPS treatment (Mitchell *et al.,* 2002). This provides an explanation to the constitutive expression of MIF by tissues and cells associated with external surfaces.

However, in other studies of MIF which were not focused on TLR4 activity, MIF has been shown to modulate the activity of key signalling intermediates downstream of TRAF6 and TAK1 (Fig. 1.7) such as extracellular signal-regulated kinase (ERK) 1 and ERK2 of the MAPK family. Experiments using quiescent fibroblasts showed that MIF caused rapid activation of ERK1/ERK2 and was able sustain the activated and phosphorylated state in these kinases for up to 24 hours (Mitchell *et al.,* 1999). Whether this also impacts on the activity of TLR4 in cells along with the transcriptional control of TLR4 expression has not been determined. Mitogen-activated protein kinase (MAPK) pathways consist of conserved kinase molecules that regulate growth, differentiation, apoptosis and migration of cells (Dhillon et al., 2007). The triggering of ERK1/ERK2 by MIF was shown to be dependent on Protein kinase A and has been linked to increased enzyme activity of cytoplasmic phospholipase A2 (PLA2). This is fundamental because PLA2 acts as a crucial mediator for the initiation of the pro-inflammatory cascade leading to production of arachidonic acid followed by prostaglandins and leukotrienes. Glucocorticoids target PLA2 to diminish the inflammatory state and induce immunosuppressive effects. Thus, MIF may be able to antagonise glucocorticoid action via PLA2 through the ERK1/ERK2 pathway (Mitchell *et al.,* 1999). There is also evidence of MIF inhibiting JUN-activation domain-binding

protein 1 (JAB1), a co-activator of AP-1 and a postulated intracellular receptor of MIF (Kleeman *et al.,* 2000) that causes phosphorylation of JUN through activation of the JUN N-terminal kinase (JNK). AP-1 is a transcription factor that is known to be involved in cell survival and proliferation (Kleeman *et al.,* 2000). This is a puzzling finding considering that it seems to contradict observations in other cell types where MIF has been shown to promote cell survival and proliferation.

MIF has been shown to have high-affinity binding for CD74 (Leng *et al.,* 2003) which may be involved in the cell kinetics and stimulation of pathways. Although the exact mechanism of how the signalling cascade proceeds is not known, it was observed that activation of the MAPK pathway by MIF was dependent on binding to CD74 and required a complete CD44 molecule (Leng *et al.,* 2003 Shi *et al.,* 2006). Studies provide evidence of elevated levels of MIF in tumour cell lines and primary tumours (Mitchell *et al.,* 2000). With findings on various activities of MIF in different pathways, remain several unanswered questions about how MIF may control aspects of TLR4 activation and inflammation. Figure 1.7 shows the three main receptors- TLR4, CD74 and TNF α receptor 1 (TNFR1) whose signal transduction cascades commonly intersect at the MAPK and lead to activation of transcription factors NFκB and AP-1 that initiate production of pro-inflammatory mediators and growth factors. This is important because MIF has displayed modulatory action on MAPK that was measured either by TLR4 receptor expression levels or production of pro-inflammatory molecules. Studying the effects of MIF within these pathways may help identify MIF interactions and lead to understanding of mechanisms through which MIF extends its behaviour.

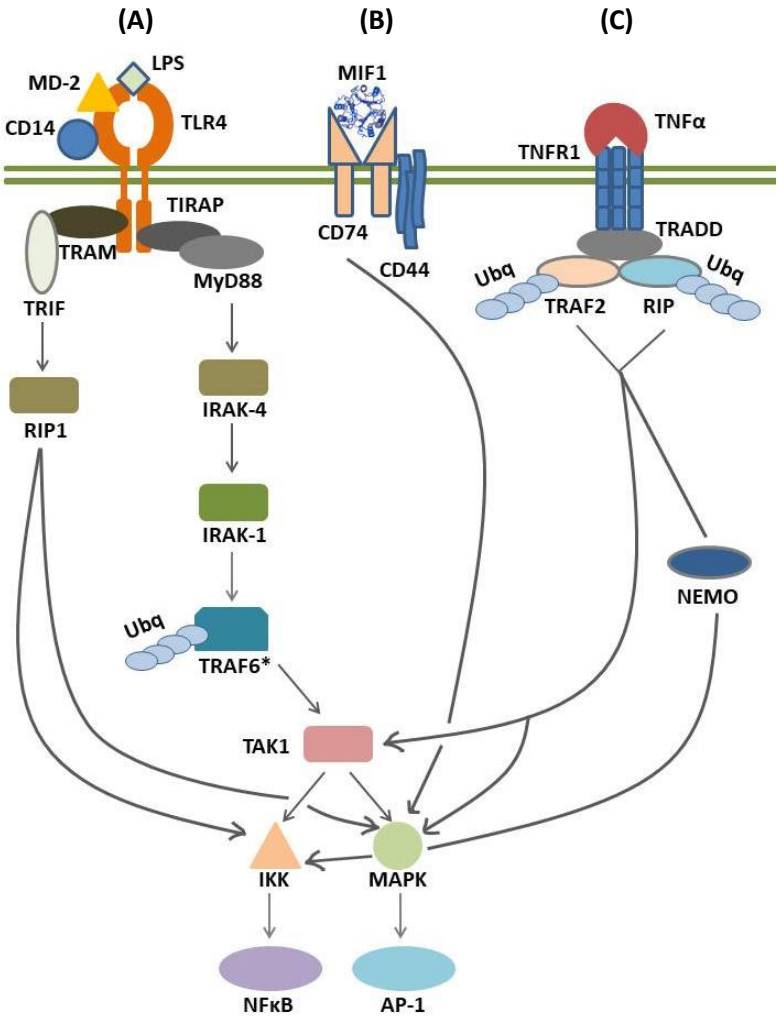

Figure 1.7: Potential intersection points of MIF signalling. This figure outlines the transduction pathway of three receptors TLR4 (A), CD74 (B) and TNF α receptor 1 (TNFR1) (C). MAPKs are one of the proteins that are activated when these receptors are triggered with their ligands as shown ultimately leading to the triggering of NFκB and AP-1 that produce pro-inflammatory molecules. MIF has shown to modulate ERK1/ERK2 of the MAPK and bind strongly to CD74.

1.3.3 Pathogen derived MIF homologues

MIF has been characterised from various parasites including the nematodes *Trichinella spiralis* (Pennock *et al.,* 1998, Tan *et al.,* 2001), *Brugia malayi* (Zang *et al.,* 2002), malarial parasite *Plasmodium falciparum* (Cordery *et al.,* 2007) and trypanosomatid *Leishmania major* (Richardson *et al.,* 2009). It has been shown that these parasites secrete MIF in the infected host and modulate the activities of the host immune system. For example, *Plasmodium falciparum* MIF was found to be secreted in the RBCs of the infected host and suppress activation of TLR4 (Cordery *et al.,* 2007). Another parasite, *Trichinella spiralis*, secretes a MIF homologue called TsMIF1 which shares properties similar to that of human MIF1 such as the tautomerase activity (Fig. 1.5), monocyte migration inhibition and chemotactic ability (Tan *et al.,* 2001). *T.spiralis* larvae have been shown to secrete higher amounts of MIF than that present in the human plasma, which may affect the host's monocyte migration and chemotactic actions (Tan *et al.,* 2001). This is particularly important because newborn larvae (NBL) produced after *T.spiralis* infection invade the skeletal muscles and transform them into nurse cells (Despommier, 1975, Despommier, 1993), causing drastic tissue damage and impairment of functions. These nurse cells are a niche for this parasite to reside stably for long periods of time (Despommier, 1993). Thus, investigating the effects of TsMIF1 will further our understanding on the influence of this parasite derived MIF homologue and its contribution to immune dysregulation during *T.spiralis* infections.

2.0 Aims

The aim of this research was to expand our understanding of how MIFs may modulate the elements of TLR4 signalling. It has been known that human and murine MIF1 can upregulate the expression of TLR4 after LPS treatment. However, it has also been shown in other studies which are not focussed on TLR4 activity that MIF1 was capable of activating ERK1/ERK2 of the MAPK pathway. Hence, it is possible that MIF1 controls both the abundance of the TLR4 receptor and activation of TLR signalling components. In addition, in spite of having the distinct enzymatic activities of MIF1 well characterised, their contribution (if any) to the modulation of TLR signalling remains unexplored.

The aim of this work was to further characterise the modulation of TLR4 activation by MIF1, specifically focusing on determining if it modulates signalling intermediates down-stream of the TLR4 receptor. To exclude the previously characterised effects of MIF on TLR4 transcription from our studies we will utilised a cell system where TLR4 expression is not controlled by its endogenous receptor. Unfortunately, while recombinant human MIF1 is commercially available, testing within our and other laboratories have shown that it lacks both of its known enzymatic activities and shows very modest activity in cell chemotaxis assays. This has lead us to conclude it is not suitable for use in our studies. Therefore, the specific aims of this study were:

1. To design and construct bacterial expression vectors that produce recombinant *Mus musculus* MIF1 and obtain purified and endotoxin-free protein.
2. To validate the commercially available HEK 293 blue hTLR4 cell system (Invivogen). This is a HEK cell line that stably expresses all the functional components of the TLR4 receptor system. This includes TLR4, MD-2 and CD14 along with a NFκB and AP-1 inducible reporter controlling the expression of the reporter, secreted alkaline phosphatase (SEAP). Wild-type HEK cells are not responsive to LPS, do not express TLR4, MD-2 or CD14, have minimal SEAP activity but do express the other downstream components of the TLR signal transduction pathway.
3. To analyse the activity of enzymatically active recombinant murine MIF1 protein using HEK-Blue™ hTLR4 to examine if MIF1 modulates TLR4 activation. As part of this study a MIF1 homologue derived from the parasite *Trichinella spiralis* (TsMIF1) which lacks the oxidoreductase activity found in murine MIF1, will be use as a comparator.

3.0 Materials and Methods

3.1 Bacterial and Mammalian Cell culture

The TOP10 *Escherichia coli* strain was used to perform all transformations in the cloning procedures. The BL21 Codon plus *E. coli* (Weiner et al., 1994, Statgene) strain was used for efficient expression of MIF1, The *E.coli* strain has been engineered to translate GC-rich open- reading frames by the inclusion of extra copies of tRNA genes (argU and proL) for recognition of arginine and proline codons. All bacterial cultures were grown and maintained in one of the following media along with appropriate antibiotics: LB broth (40% tryptone, 20% yeast extract, 40% NaCl) , LB agar (24% tryptone, 12% yeast extract, 24% NaCl, 40% agar) and 2 X YT broth (52% tryptone, 32% yeast extract, 16% NaCl).

The cell lines used for all TLR4 activity assays were HEK-Blue™ hTLR4 which are engineered HEK 293 cells transfected with TLR4, MD-2 and CD14 along with nuclear factor κB (NFκB) and activator protein 1 (AP-1) inducible reporter plasmid pNiFty2-SEAP that produces secreted alkaline phosphatase (SEAP). HEK293 wild type (WT) cells stably transfected with pNiFty2-SEAP (HEK293 pNifty2-SEAP) were used as a control for all experiments. All HEK cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing high glucose (Sigma Aldrich) at 5% CO_2. The DMEM was further supplemented with 10% FBS, 1% Penicillin/Streptomycin. Selection of HEK-Blue™ Selection of HEK-Blue™ hTLR4 (Invivogen) was carried out using the antibiotics blasticidin, hygromycin and Zeocin™ at concentrations that have not been disclosed by the manufacturer (HEK-Blue™Selection). Selection of HEK293 cells transfected with pNifty2-SEAP was maintained by culture in 100 µg/ml zeocin containing media. HEK-

Blue™ hTLR4 and HEK293 pNifty2-SEAP were used between Passage 1 and Passage 20 (P1-P20).

3.2 Plasmids, Media and Reagents

Plasmids used for incorporation of the DNA insert and facilitating expression were pGEM®-T Easy Vector (Promega) and pET29b (Novagen). pGEM®-T Easy Vector carried the ampicillin resistant gene along with multiple cloning site within the α-peptide coding region of the β-galactosidase enzyme. Thus TOP10 transformants were selected using media containing ampicillin for selection of pGEM®-T Easy Vector and X-gal + Isopropyl β-D-1-thiogalactopyranoside (IPTG) for blue-white selection. The expression vector pET29b carried the kanamycin resistant gene which was used for selection of transformants. Cloning of murine MIF1 was performed so a His-tag comprised of six histidine amino acids was added in-frame to the C-terminus and this was used for subsequent protein isolation. The reporter plasmid pNifty2-SEAP was transfected into wild-type HEK293WT in order to use it as a SEAP assay reporter control. All reagents were used from Sigma-Aldrich, Thermo Fisher Scientific and Fisher Scientific UK unless otherwise stated.

3.3 Cloning

The cDNA encoding the sequence for murine MIF1 (MmMIF1) was previously obtained via reverse transcriptase PCR (RT-PCR) (Isolate II RNA Mini Kit, Bioline) and amplified with primers as described in Table 1 using Polymerase Chain Reaction (PCR) (Phusion® High-Fidelity DNA Polymerase, unit assay conditions: 25 mM TAPS-HCl (pH 9.3 @ 25°C), 50 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol, 200 µM dNTPs including [3H]-dTTP and 400 µg/ml activated Calf Thymus DNA). Primers were designed to include the

restriction sites Nde1 and Xho1 on the 5' end and 3'end respectively, allowing the construction of an in-frame fusion of the MmMIF1 cDNA to the His-tag in the expression plasmid pET29b. The amplified MmMIF1 DNA sequence was poly A-tailed and then incorporated into the shuttle vector pGEM®-T Easy (Promega) by TA cloning and then ligating at 4°C overnight using T4 DNA ligase (New England Biolabs (NEB)). The plasmid-insert sequence was transformed into chemically competent *E.coli* (TOP10) using the standard $CaCl_2$ method (See section 3.3.1), screened using PCR and confirmed by sequencing (University of Dundee, https://www.dnaseq.co.uk/). Colonies with confirmed MmMIF1 inserts in shuttle vector pGEM®-T Easy plasmid were picked and the plasmid was isolated using Monarch® Plasmid Miniprep Kit from NEB. Both the MmMIF1 insert and the expression plasmid pET29b (Novagen) were subjected to sequential restriction enzyme digestion with Xho1 and Nde1 (NEB) respectively to create compatible ends. After each digestion reaction, samples were checked on 1.5% agarose gel, excised to extract DNA and purified using Monarch® DNA Gel Extraction Kit from NEB. The purified insert and plasmid were ligated using T4 DNA ligase (NEB) at 16°C overnight and transformed into chemically competent *E.coli* (TOP10) the next day. All reactions were carried out as per the manufacturer's instruction. Successful MmMIF1-pET29b transformants were checked by PCR and validated by sequencing where the presence of 6x His epitope tag on the C-terminal was also confirmed. All PCR reactions for screening were carried out using *Taq* DNA Polymerase with Standard *Taq* Buffer (NEB), 2mM dNTPs, 10µM of each primer, 3µg of DNA template and set up as per the manufacturer's instruction. All PCR products, ligation reactions and restriction digest reactions were checked using electrophoresis on 1.5% agarose gel containing 0.01% SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). Samples were stained with 6X Purple Gel Loading Dye (NEB) and loaded on to the gel. The size of DNA

bands were checked using Quick-Load® Purple 2-Log DNA Ladder (NEB). Colonies successfully harbouring the MmMIF1-pET29b-6x His were picked and the plasmid was transformed into BL21 Codon plus *E. coli* bacterial expression system to further carry out protein expression. Fig. 3.1 shows a schematic layout of MIF1 in pET29b with the six histidines.

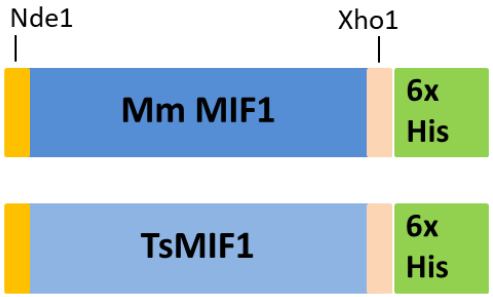

Figure 3.1**: A schematic representation of fusion MIF cDNA for *Homo sapiens* (Hs), *Mus musculus* (Mm) and *Trichinella spiralis* (Ts).**

Table 3.1: **A list of the sequence of primers used in the cloning procedure**

Sr. No.	Primer name and sequence	Use of sequence for corresponding plasmid/MIF
1.	Forward primer: pET29b.F2 5'- GTGATGCCGGCCACGATGCGTCC- 3' Reverse primer: pET29b.R2 5'- ATCCGGATATAGTTCCTCCTTTC- 3'	To screen pET29b plasmid and MIF1 fusion sequence.
2.	Forward primer: T7up 5'- TAATACGACTCACTATAGGG- 3' Reverse primer: T7down 5'-GCTAGTTATTGCTCAGCGG- 3'	Commercially available primer sequences for pET29b plasmid.
3.	Forward primer: Mm_MIF1 Fw 5'-CATATGCCTATGTTCATCGTGAACACC- 3' Reverse primer: Mm_MIF1 Rv 5'-CTCGAGAGCGAAGGTGGAACCGTTCCAGCC- 3'	To amplify and screen the murine MIF1 DNA sequence
4.	Forward primer: M13R 5'-AGCGGATAACAATTTCACACAGGA- 3' Reverse primer: M13L 5'-CGCCAGGGTTTTCCCAGTCACGAC- 3'	Primer sequences for pGEMT shuttle vector.

3.3.1 Escherichia coli Transformation

Cells were made chemically competent using a protocol adapted from Inoue *et al.,* 1990. In a microcentrifuge tube, 10 µL of DNA was used per transformation experiment which was added to 100 µL of *E.coli* and placed on ice for 15 minutes. The transformation mixture was then heated exactly at 42^{o} C for 45 seconds and promptly allowed to recover on ice for 2-5 minutes. The reaction mixture was subjected to addition of 250 µL of LB or 2 X YT broth and incubated at 37^{o}C for an hour. The transformations were then plated onto the relevant antibiotic containing agar plates.

3.4 MmMIF1 expression

3.4.1 Induction of proteins

pET29b containing the MmMIF1 insert was transformed into BL21 Codon plus *E. coli* and used for subsequent protein expression and purification. Previously cloned and purified *Trichinella spiralis* (*T.spiralis*) MIF1 (Guiliano, unpublished) (TsMIF1) was used alongside MmMIF1 to compare the effects of mammalian MIF1 vs parasite MIF1 in the bioassays. Expression and production of soluble protein in BL21 Codon plus cells was tested using two methods which were induction with 50µM-1mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) or culture in an auto-induction system with Overnight Express™ Instant TB Medium (Merck Millipore). If protein production was induced with IPTG, 5mL of the cell culture was first set up to grow overnight (o/n) with appropriate antibiotics. The next day, o/n culture was diluted 1:50 and added to fresh 25mL 2xYT media. Cells were allowed to grow until the optical density$_{600}$ (OD_{600}) reached 0.6-0.7. 50µM-1mM of IPTG was added and left for cells to carry out protein production for 4-5 hours. Cells were then centrifuged, pellets collected and frozen for subsequent processing and analysis. If the expression of MIF1 was induced by culturing in Overnight Express™ Instant TB Medium, large cultures were set up for an overnight incubation. The next day, cultures were spun down to obtain pellets which were either frozen or used immediately for processing and analysis. For both the methods, samples were collected before and after induction and normalised for analysis.

3.4.2 SDS-PAGE and Western blot

Presence of MIF proteins was visualised by Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and coomassie blue staining. For initial analysis of total protein, OD normalised cell pellets were resuspended in 1X Laemmli buffer (10% 2 β-Mercaptoethanol, 90% loading mix) and briefly sonicated to lyse the cells. The lysed samples were then loaded onto a 15% polyacrylamide gel (3% stacking gel- 30% polyacrylamide mix, 1M Tris pH 6.8, 10% SDS, 10% APS, TEMED; 15% resolving gel- 30% polyacrylamide mix, 1.5M Tris pH 8.8, 10% SDS, 10% APS, TEMED) and allowed to run by electrophoresis in 1X Tris/Glycine/SDS buffer (BioRad). The gel was later stained with Coomassie blue (50 % methanol, 10% acetic acid 40% H2O) and visualised on a Chemidoc (BioRad). For analysis of soluble and insoluble MIF protein, the cell pellets were resuspended in 1XPBS or 1XHBSS and sonicated to lyse the cells. Samples were centrifuged and the supernatant was collected which contained the soluble fraction of the protein while the cell pellets contained the insoluble fraction. 4X Laemmli buffer was added to the soluble fraction for a final concentration of 1X Laemmli buffer while the insoluble fraction was treated with 1X Laemmli buffer. The samples were loaded on to 15% polyacrylamide gel and allowed to separate by size by electrophoresis. The gels were stained with Coomassie overnight, destained the next day with destaining solution (40% methanol, 10% acetic acid, 50% H_2O) and visualised on Chemidoc. Protein ladder, Precision Plus Protein™ Dual Color Standards (BioRad) was used as a protein marker.

To confirm the presence of the His-tagged MmMIF1, western blot analysis was performed using a HRP conjugated anti-his tag antibody (Monoclonal Anti-polyHistidine, A7058 Sigma Aldrich). SDS-PAGE was conducted with proteins as

previously described and proteins from the gel were transferred onto a nitrocellulose membrane through a semi-dry transfer (Trans-Blot® Turbo™ Transfer System, Bio-Rad). After transfer, the membrane was blocked with 5% milk in 1 X PBS to prevent non-specific binding of the antibody. The membrane was washed with 1x TBST (0.01M Tris, 0.14M NaCl, 0.05% Tween 20, pH 8.0) and incubated overnight (o/n) with the anti-his antibody that was added at a concentration of 1:3000 to 5% milk in PBS. On the following day, the membrane was washed three times with 1xTBST and visualized with Pierce™ ECL Western Blotting Substrate (1:1 Thermo Fisher Scientific) on a Chemidoc analyser (BioRad).

3.5 Isolation and purification of MmMIF1

3.5.1 Isolation of recombinant MmMIF1 by Ni+ affinity chromatography

The His tag within the recombinant MmMIF1 protein was used for initial purification via Ni+ affinity chromatography. For small scale purification, His SpinTrap columns (GE Healthcare) were used whereas for large scale protein purification, 1 mL Protino® Ni-NTA Column (Macherey- Nagel) was used on the ÄKTAprime plus (GE Healthcare). Proteins were induced with 50µM IPTG and pellets were collected after 4-5 hours. The following day, cell pellets were resuspended in binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.4), mechanically lysed via sonication and centrifuged to separate the soluble and insoluble material. The supernatants containing soluble protein were collected for purification either with His SpinTrap column (small scale for testing) or the ÄKTAprime plus (large scale). For The His SpinTrap columns, micro-centrifugation was carried out at 7000 x g. The mini-columns were first centrifuged to remove the storage liquid and equilibrated using 600µL of binding buffer. Post equilibration, 600 µL of sample was applied to the column and centrifuged for 1 minute. The column was washed using binding buffer and eluted using 200 µL elution buffer (20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4). The step was repeated resulting in two 200 µL fractions of purified protein samples which was measured (Absorbance 260/Absorbance 280) using Nano drop 1000 (Thermo Scientific).

For the ÄKTAprime plus, the same binding and elution buffers were used. Soluble fractions were resuspended in binding buffer to inject into the system. The system was programmed to run a 60 minute cycle and the breakpoints can be seen in table 3.2. The other key parameters of the machine were: 1 mL/min flow rate or 0.5 mL/min

during injection of sample; 5 mL collection fraction when the slope of the UV peak was ≤ 500 mAu (milli arbitrary unit). These breakpoints allowed washing and elution of the sample efficiently and eluted fractions were collected in the fraction collector that contained isolated MmMIF1 protein.

Table 3.2: **The breakpoints of the programme used on the ÄKTAprime plus for the isolation of MmMIF1 using Ni+ affinity chromatography**

Sr. No	Time (in minutes)	Action	Flow position	Concentration of elution buffer (in %)
1.	0	Washing	Load	0
2.	8	Washing	Load	0
3.	10	Washing	Inject	0
4.	20	Washing	Inject	0
5.	30	Washing	Load	0
6.	30.1	Elution	Load	100
7.	40	Elution	Load	100
8.	40.1	Elution	Load	100
9.	50	Washing	Load	0
10.	60	Washing	Load	0

3.5.2 Buffer exchange and removal of excess salts

To obtain MmMIF1 protein free from salts and imidazole, Amicon Ultra-15 (Merck, UFC900308) with a nominal molecular weight limit (NMWL)/ pore size of 3kDa was used. The NMWL allowed retention of the high molecular weight MmMIF1 protein (12.5 kDa) in the membrane bed whilst removing the lower molecular weight contaminants including salts and imidazole. Briefly, 15mL of sample was added to the filter and centrifuged at 4000 x g for 30 minutes. After multiple runs, the concentrated sample was washed three times with 50 mM of Tris buffer, pH 8.0 and resuspended in a final volume of 2.4 mL of the same buffer. This buffer exchange was necessary for the subsequent anion exchange chromatography.

3.5.3 Removal of residual endotoxin by anion exchange chromatography

Residual endotoxin is one of the most common contaminants of recombinant proteins produced in bacterial systems. Removal of endotoxin was essential and crucial for the experiments conducted within this study since effect of the MIF protein was being examined after co-administration with LPS. Residual endotoxin was removed from purified MmMF1 by anion exchange chromatography (Pierce Strong Anion Exchange Spin Column, Mini, 90010). The buffer in which the protein was resuspended after Amicon filtration conferred a neutral charge to the protein thus effectively allowing the capture of negatively charged endotoxin by the positively charged resin contained within the column. Briefly, the column was washed with 50mM Tris, pH 8.0 buffer and centrifuged between 5000 to 7000 rpm. 400 µL of sample was loaded onto the column and centrifuged at the same speed. The flow through in the tube contained MmMIF1 protein which was collected and pooled together after each column run.

3.6 Bicinchoninic acid (BCA) assay

For accurate quantification of purified MIF protein, Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) was used. Protein standard BSA ranging from 2000 µg – 25 µg was prepared and used to generate a standard curve. Samples were diluted as necessary to fit into the standard curve. The microplate procedure was used where 190 µL of working reagent was added to 10 µL of each sample was added per well, shaken to mix and incubated for 30 minutes at 37^{o}C. The working reagent was prepare using Reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1M sodium hydroxide) and Reagent B (4% cupric sulfate) in a 50:1 ratio. Post incubation of the samples, the absorbance was measured at OD_{562} using a multi-mode microplate reader (BioTek, Synergy™ HTX).

3.7 Tautomerase enzyme assay

To validate the presence of enzymatically active MmMIF1 at different stages of purification, the tautomerase assay adapted from Kudrin *et al.*, 2006 was used. Briefly, 20µL of 10mM L-dopa methyl ester and 20µL of 20 mM sodium periodate was added to 160µL of Buffer A (Potassium phosphate, 0.2% Tween 80). 4-6 µL of MIF was added along with 20 ng/mL of TsMIF1 as a positive control. The absorbance of the samples was immediately measured at OD_{475} every 45 seconds for 5 minutes at exactly 30^{o} C using a microplate reader.

3.8 Limulus Amebocyte Lysate (LAL) assay

To quantify the presence of any residual endotoxin in the purified recombinant proteins, the LAL assay (Pierce™ LAL Chromogenic Endotoxin Quantitation Kit, Thermo Fisher Scientific, 88282) was performed on the purified protein using a microplate procedure. All steps were carried out at a temperature of 37^{o} C. Briefly, the microplate and samples were equilibrated for 10 minutes. 50µL of each standard and unknown sample was added to the wells and incubated for 5 minutes. 50µL of the LAL reagent was added to the plate, shaken and incubated for exactly 10 minutes after which 100µL of the substrate solution was added. The plate was shaken and incubated for another 6 minutes. To stop the reaction, 50µL of 25% acetic acid was added, plate shaken to mix and read at OD_{410}.

3.9 Assessment of the effects of MIF1 on TLR4 activation

Cells (HEK-Blue™ hTLR4 at, HEK293 pNifty2-SEAP and HEK293 WT) were seeded in HEK-Blue™ Detection media or phenol red free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin at 5 x 10^4 cells/mL in a 96-well plate and then stimulated with LPS (from Escherichia coli O111:B4 L4391, Sigma Aldrich), TNF α (Biolegend), TsMIF1, MmMIF1 or pharmacological inhibitors such as Polymyxin B sulfate (PMB) (Acros Organics) prepared in 1x DPBS. Within each assay there were a minimum of three experimental replicates and each assay was repeated a minimum of three times (n=3). The final volume in each well was 200µL. Two methods for measuring secreted alkaline phosphatase in the culture supernatants were validated and used interchangeably through the study: the- p-Nitrophenyl phosphate (pNPP) assay and commercially available HEK-Blue™ Detection media.

For the LPS assay, cells were immediately stimulated after seeding with 20 µL/well of different Lipopolysaccharide (LPS) (Sigma Aldrich) concentrations (0.1 ng/mL- 1000 ng/mL) with or without 1 µg/mL Polymyxin B sulfate (PMB) (Acros Organics). For bioassays with MIF1, different wells containing HEK-Blue™ hTLR4 were immediately treated after seeding with 0.5 ng/mL, 1 ng/mL or 10 ng/mL of LPS, co-administered with different MIF1 (either TsMIF1 or MmMIF1) concentrations (0.01 µg/mL, 0.1 µg/mL). Treatment with 100 ng/mL of TNF α (Biolegend) was used as a positive control for triggering NFκB and AP-1 activation. HEK293 pNifty2-SEAP cells treated with 100ng/mL TNF α were used as an activation control for NFκB reporter plasmid. SEAP activity was measured 20 hours after treatment with one of the two methods. With the pNPP method, a 1:3 ratio of Alkaline Phosphatase (ALP) buffer (10mM Tris-HCl, 150mM NaCl, pH 8.0) and pNPP solution (2.5 mg/ml pNPP in 100 mM diethanolamine,

150 mM NaCl, 2 mM MgCl2, pH 9.5) was prepared and 100 µL was added to 50 µL of each sample. The plates were incubated for 20 minutes at 37^{o}C and 5% CO2 and the absorbance was read at OD_{405} on a multi-fold microplate reader (BioTek, Synergy™ HTX). When HEK-Blue™ Detection media was used, the plate was shaken after 20 hours and SEAP activity was measured at $OD_{620-655}$ using the same reader.

3.10 Generation of single cell clones

To produce a control reporter strain, HEK293 WT cells were transfected with a reporter plasmid found in the HEK-TLR4-Blue cell line, pNiFty2-SEAP using jetPRIME® transfection reagents (Polyplus transfection). Briefly, WT HEK293 cells were seeded in a 6-well plate at a density of 2.5 x 10^5 cells per well in 2 mL of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin (standard media). The following day, a transfection mix consisting of 200 µL of jetPRIME® buffer and 4 µL of jetPRIME® for every 2 µg of DNA was prepared and added dropwise to the seeded cells. The plate was shaken gently to mix and placed in the incubator. Cells were maintained in standard media for two days before subjecting them to selection. From the third day onwards, cells were maintained under zeocin selection in DMEM media with 10% FBS, 1% Penicillin/Streptomycin. Initially the polyclonal line transfected with 1 µg of reporter plasmid were used as NFκB activation control to determine if MIF recombinants modulate activation of NFkB in a TLR4 independent manner. Single cell clones were later generated from the selected polyclonal population of HEK293 WT transfected with pNiFty2-SEAP. Cells were seeded at a density of 0.5 cell/well or 1 cell/well in standard media. After approximately two weeks, wells containing single cell clones were isolated, subjected to selection with zeocin and tested for reporter activity using the NFκB inducer TNFα.

3.11 Statistical Analysis

The data obtained from experimental procedures were analysed using GraphPad Prism version 3.0 or version 4.02. Datasets were examined for normality of distribution using Column Statistics. Significance of statistical analysis was evaluated using Students T-Test or One-way ANOVA for parametric data while Kruskal Wallis test, Mann-Whitney or Wilcoxon Signed-rank was used for non-parametric data. The Bonferroni Post Hoc test was used to compare multiple columns while the Dunnet's Post Hoc Test was used to compare each column with the control. Data was considered statistically significant at P value of ≤0.05.

4.0 Results

4.1 MmMIF1 cloned into pET29b and obtained in BL21 Codon plus cells

MmMIF1 (*Mus musculus* MIF1) cDNA was successfully subcloned into pGEM-T and then cloned into the expression vector pET29b. The expression vector MmMIF1-pET29b was then transformed into *E.coli* BL21 Codon plus (Fig. 4.1). Sections I, II and III of fig. 4.1.A show the PCR amplicons verifying that the MmMIF1 was successfully cloned into pET29b by using three different set of MmMIF1 specific and vector specific primers: I) MmMIF1 insert specific primers (expected size: 354 bp); II) pET29b plasmid specific primers (expected size: 663 bp); III) pET29b forward primer and MmMIF1 reverse primer (expected size: 500 bp). As seen from Fig. 4.1.A, the bands correspond approximately to the expected size which confirms the ligation of MmMIF1 into pET29b. Sequencing results (Fig. 4.1.C) further confirmed the presence of the MmMIF1 insert with the adjacent His-tag in pET29b.

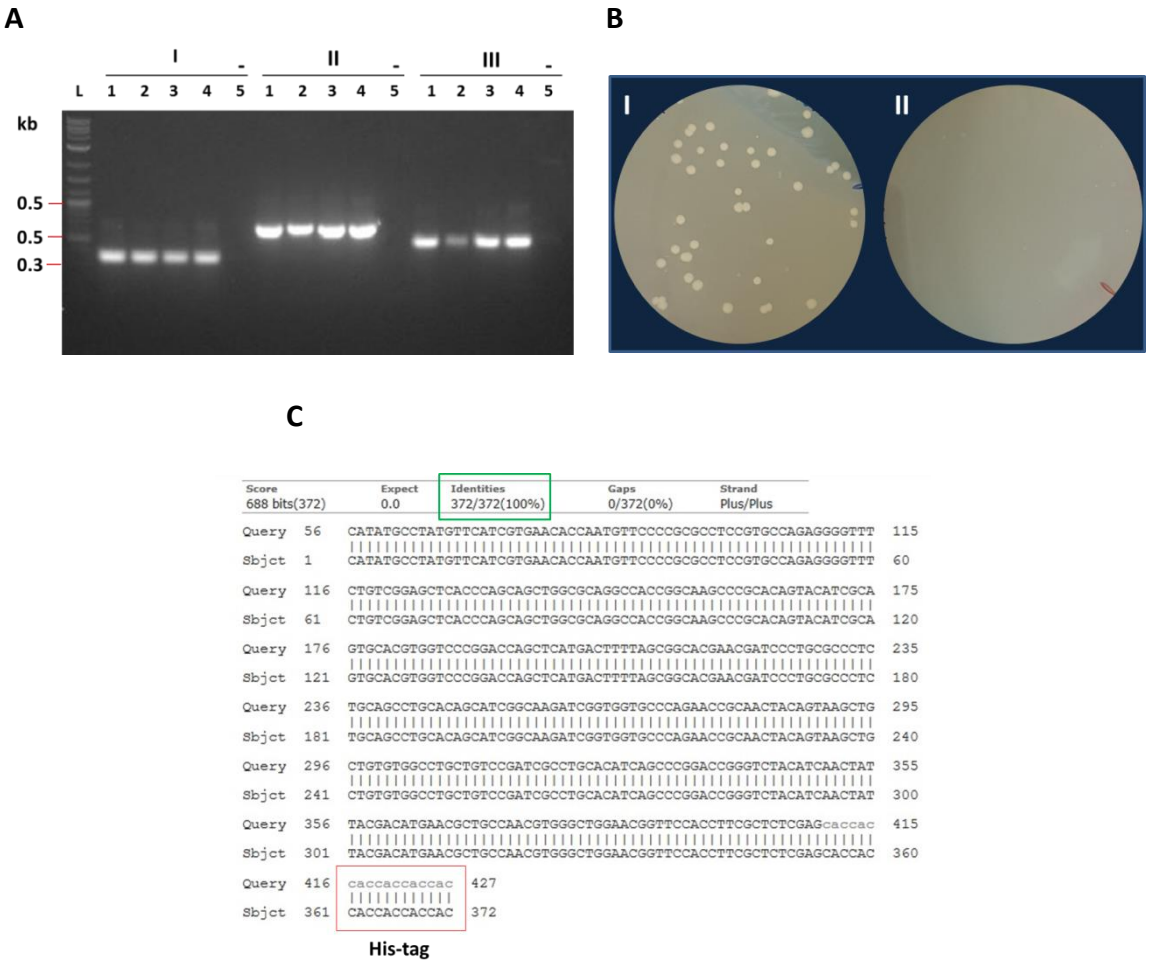

Figure 4.1: **MmMIF1 was successfully cloned into pET29b.**

A: L- 2-log DNA ladder. 1.5% gel showing PCR products with I1- I4: MmMIF1 specific primers, ~354bp; II1-II4: pET29b specific primers, ~663 bp; III1- III5: pET29b forward and MmMIF1 reverse primers, ~500 bp; I5, II5, III5: no-DNA control. **B:** BL21 Codon plus *E.coli* transformed with the pET29b-MmmIF1 construct (I) and no-plasmid control (II). **C:** Expected (subject) and obtained (query) sequences aligned using BLAST and found to be 100% identical (% highlighted in green) for the MmMIF1 sequence and the His tag (highlighted in red) in pET29b.

4.2 Expression, isolation and purification of MmMIF1

4.2.1 Soluble, enzymatically active MmMIF1 is expressed by the E. coli strain BL21 Codon plus

Induction of MmMIF1 expression in BL21 Codon plus with either 1000µM or 50µM of IPTG produced soluble protein (Fig. 4.2). However recombinant protein expression using Overnight Express media did not seem to induce the same level of expression as seen in Fig. 4.2.C and 4.2.D. Coomassie stained 15% polyacrylamide gel in fig. 4.2.A shows the total protein induced by both IPTG and overnight express media. Samples were OD normalised and equal volumes of sample were loaded onto the gel, thus levels of expression were directly comparable. As seen from Fig. 4.2.A, lanes 4 and 8 show a distinct band at approximately 12.5 kDa. This band is absent in the pre-induction controls (lanes 3 and 7) which confirm MmMIF1 expression upon induction. BL21 Codon plus containing pET29b without the MIF insert was used as a negative control for all experiments as seen in Fig. 4.2 to confirm the 12.5 kDa band that corresponds to MmMIF1. Coomassie analysis of the soluble and insoluble fraction of total protein can be seen in fig. 4.2.B and 4.2.C. Upon IPTG induction, MmMIF1 was found to express in both, the soluble and insoluble fraction similar to that of the positive control *Trichinella spiralis* MIF1 (TsMIF1) (Fig. 4.2.B). However, when induced with Overnight Express media, MmMIF1 expression was observed only in the insoluble fraction (Fig. 4.2.C, lane 8). In order to isolate the recombinant protein of interest from the insoluble fraction, additional procedures such as denaturing and refolding would need to be performed. To facilitate a simpler method for isolation of MIF1 protein, it was essential for expression of MIF1 in the soluble fraction. Western blot analysis (Fig. 4.2.D) using anti-His-tag antibody confirmed successful MmMIF1 expression in the

soluble fraction. These results indicate that induction with IPTG was the best method for producing MmMIF1 and subsequent large-batch cultures were induced using this method.

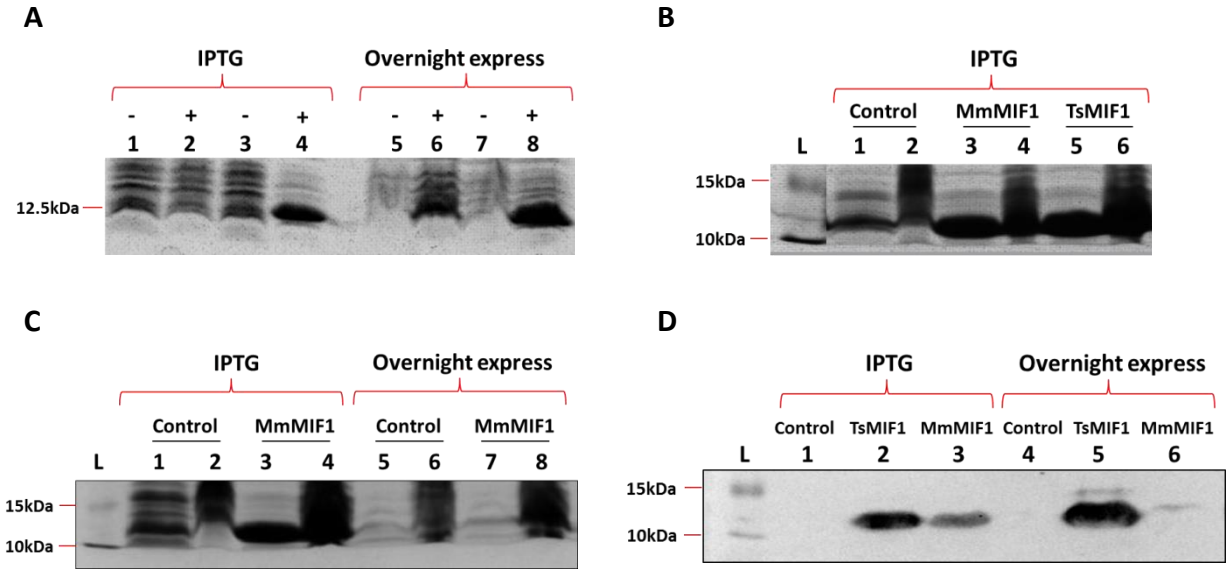

Figure 4.2: **BL21 Codon plus cells express murine MIF1 in the soluble fraction.** A-C: Coomassie- stained polyacrylamide gels, D: Western blot. **A:** Expression of MmMIF1 in total protein with 1mM IPTG or Overnight Express media pre (-) and post (+) induction. 1,2,5,6: pET29b in BL21 Codon plus without MIF insert; 3,4,7,8: MmMIF1pET29b. **B**: MmMIF1 expression in the soluble and insoluble fraction. 1: soluble pET29b without MIF insert, 2: insoluble pET29b without MIF insert; 3: soluble MmMIF1, 4: insoluble MmMIF1; 5: soluble TsMIF1 (positive control), 6: insoluble TsMIF1 (positive control). **C:** Comparison of MmMIF1 expression with IPTG and overnight express media in the soluble and insoluble fraction. 1, 5: soluble pET29b without MIF insert and 2, 6: insoluble pET29b without MIF insert; 3,7: soluble MmMIF1 and 4,8: insoluble MmMIF1. **D:** Western blot confirming MmMIF1 expression in the soluble fraction with anti-his-tag. Control is pET29b without MIF insert.

4.2.2 MmMIF1 can be purified from the soluble extracts of E. coli BL21 Codon plus

MmMIF1 was isolated from the soluble fraction of the bacterial protein lysates using Fast protein liquid chromatography (FPLC) (ÄKTA Prime Plus) affinity chromatography and purified by filtration and anion exchange chromatography. Eluted fractions containing MmMIF1 can be seen in Fig.4.3. Lane 1 in Fig. 4.3.A shows total protein after induction while lanes 2-7 show isolated MmMIF1 obtained from sequential purification runs on the ÄKTAprime plus. Each run shows a band at 12.5 kDa, which confirms successful isolation of MmMIF1 from total protein. Additional higher molecular weight bands can also be seen in lanes 2, 3, 4, 5 and although it is possible that those bands are contaminants, their molecular weights indicate that they are aggregates of MmMIF1 protein which were not visible after the samples were denatured thoroughly and for longer periods. Such a conclusion is drawn due to the observation that elutes of lower concentration in lanes 6 and 7 display clean bands at about 12.5 kDa. This is also seen after the Amicon-Ultra 15 filtration, where coomassie analysis of the filtered protein (fig. 4.3.B) shows that higher molecular weight bands are no longer present and after the last step of purification, where coomassie analysis (fig. 4.3.C) shows clean bands at 12.5 kDa (lanes 2 and 3) without any other additional bands indicating that pure MmMIF1 is obtained as observed on a 15% polyacrylamide gel.

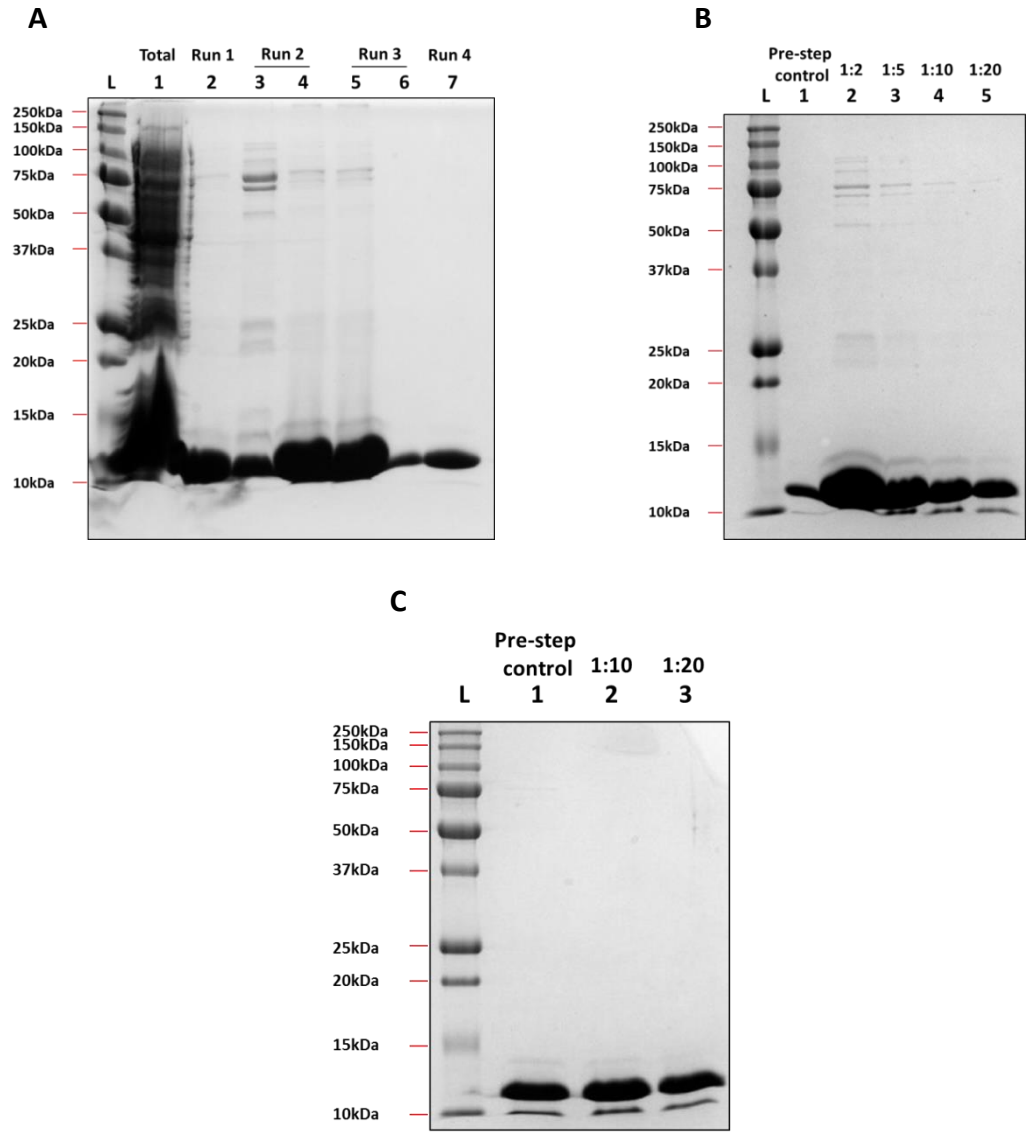

Figure 4.3: **MmMIF1 was successfully purified from *E. coli* BL21 Codon plus.** Coomassie-stained polyacrylamide gels showing- **A:** Isolated MmMIF1 from total soluble protein after Ni^{2+} affinity chromatography on the ÄKTAprime plus. 1: Total soluble protein (control); 2-7: Elutes containing MmMIF1 from different runs. **B:** MmMIF1 after removal of salts and imidazole and after buffer exchange. 1: Mixed elutes after Ni2+ affinity chromatography (diluted 1:10). 2-5: Different dilutions of MmMIF1. **C:** MmMIF1 after reduced levels of residual endotoxin in the protein sample that were removed by anion exchange chromatography. 1: Pre- anion exchange control (diluted 1:20); 2, 3: Dilutions of purified MmMIF1.

At each stage, MmMIF1 was quantified using the Bicinchoninic acid assay (BCA) with a final concentration of 17 mg/mL after anion exchange chromatography. MmMIF1 was verified for presence of any residual endotoxin and was found to be 1.2 EU/mL. These levels were acceptable for the subsequent bioassays since dilution of MmMIF1 to concentrations needed in the bioassay (0.01 µg/mL and 0.1 µg/mL) resulted in a final LPS concentration of ≤ 0.00007 ng/mL. The concentration of TsMIF1 was also measured and was found to be 30.7 mg/mL. Experiments for endotoxin detection in TsMIF1 using the LAL assay did not confirm the exact amount of endotoxin levels since measurements obtained even from a 1:300 diluted sample did not fit into the standard curve. However, from the absorbance values and endotoxin units (EU) obtained, it can be estimated that the endotoxin levels within the sample per mg was approximately 4.1 ng/mL. The tautomerase activity of both TsMIF1 and MmMIF1 was tested after the final stage of purification (Fig. 4.4). It was found that TsMIF1 sustained higher tautomerase activity than MmMIF1.

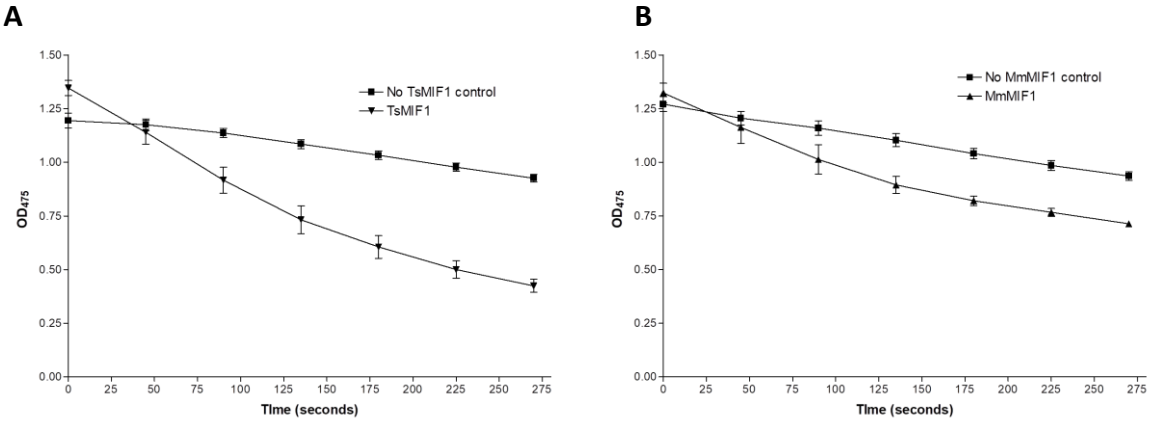

Figure 4.4: **TsMIF1 and MmMIF1 have tautomerase enzyme activity.** Depletion of L-dopa methyl ester in the presence or absence of TsMIF1 **(A)** or MmMIF1 **(B)** measured as an absorbance at OD_{475}.

4.3 Validation of the sensitivity and specificity of the HEK-Blue™ hTLR4 reporter cells

The sensitivity and specificity of HEK-Blue™ hTLR4 cells was determined by detection of NFκB/ AP-1 activation and SEAP secretion into the media. HEK cell responses to different concentrations of LPS (a TLR4 ligand) were measured to make a standard curve showing their range of LPS sensitivity. Parallel experiments were also performed which included inhibitor polymyxin B sulfate (PMB) to confirm the specificity of the cell line for both activation and inhibition of TLR4 signalling. To allow normalisation of results between biological replicates the observed OD values were converted into a response ratio in Fig. 4.5.D and Fig. 4.5.F where $response\ ratio = \frac{optical\ density\ of\ treated\ cells}{optical\ density\ of\ untreated\ cells}$. As seen in figure 4.5.C, 4.5.D and 4.5.E, 4.5.F, the observed trend remains the same. All subsequent data obtained using this bioassay is represented as a response ratio.

As seen from Fig. 4.5 and Fig. 4.6, NFκB/ AP-1 activation levels were found to increase with increasing concentration of LPS and decreased substantially upon co-treatment with inhibitor PMB. Results in fig 4.5 shows a steep increase in the activation of TLR4 in HEK-Blue™ hTLR4 cells when treated with 0.5 ng/mL LPS. Activation levels were found to peak at 1 ng/mL of LPS and do not increase significantly at higher concentrations. When cells were co-treated with 1 µg/mL PMB, LPS mediated activation of the SEAP reporter was abolished at concentrations ranging from 0.1 ng/mL to 10 ng/mL of LPS (Fig 4.6.A) with SEAP levels equivalent to the DPBS control. However, at higher concentrations of LPS (100ng/mL, 500ng/mL, 1000ng/mL) activation was inhibited but not abolished (Fig. 4.6.A). Based on these initial results the subsequent assessment of

MIF's effects on this reporter assay were performed at the LPS concentrations ranging from 0.5 -10 ng/mL.

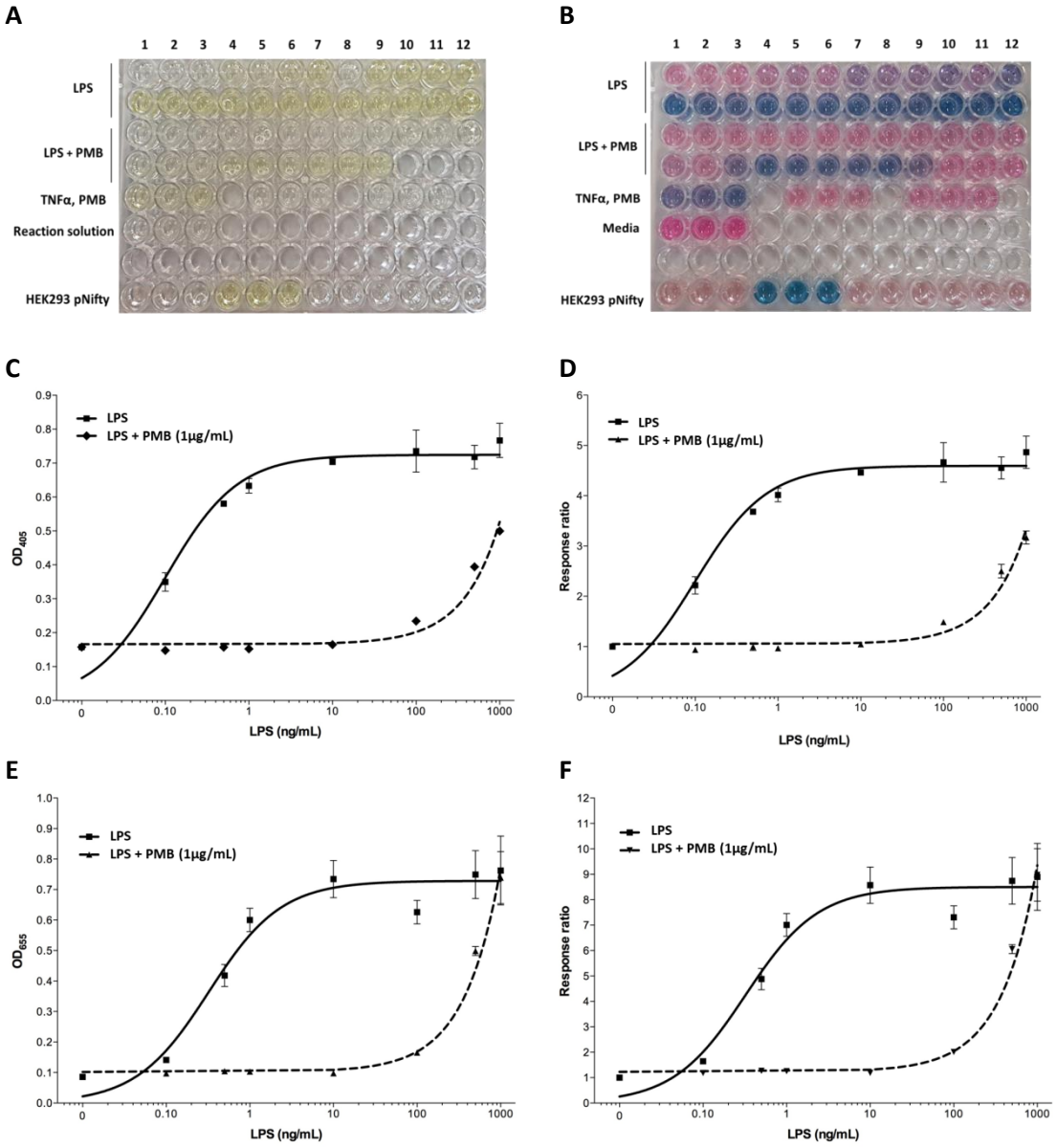

Figure 4.5: **HEK-Blue™ hTLR4 cells respond to LPS in a dose dependant manner. A, B:** 96-well plates illustrating the two calorimetric methods: p-Nitrophenyl phosphate (pNPP) (A) and HEK-Blue™ Detection (B). HEK293 pNifty: 1-3: DPBS control, 4-6: 100 ng/mL TNFα, 7-9: LPS 100 ng/mL, 10-12: LPS 500 ng/mL. **C, E:** HEK-Blue™ hTLR4 cells simulated with increasing concentrations of LPS (0.1 ng/mL - 1000 ng/mL) with or without inhibitor PMB. SEAP activity in the media was measured using pNPP solution at OD_{405} (C) or HEK-Blue™ Detection media at OD_{655} (E) respectively. **D, F:** The response ratios of the LPS treated HEK-

Blue™ cells as determined by the SEAP activity measured by pNPP solution (D) or HEK-Blue™ Detection (F). The values are represented as a response ratio where $response\ ratio = \frac{optical\ density\ of\ treated\ cells}{optical\ density\ of\ untreated\ cells}$. Values are mean for ± SEM, n=3.

To show that activation of the HEK-Blue™ hTLR4 cells is due to the expression of a functional TLR4 receptor which is otherwise lacking in wild type (WT) HEK 293, HEK 293 WT cells were transfected with the pNiFty2-SEAP reporter plasmid. Both HEK-Blue™ hTLR4 and HEK293 pNifty2-SEAP cell populations were treated with LPS and responses were measured (Fig. 4.6). As seen in Fig. 4.6, HEK-Blue™ hTLR4 cells respond to LPS (Fig. 4.6.A) while HEK293 pNifty2-SEAP were unresponsive to LPS (Fig. 4.6.B). To confirm the reporter construct was active in both cell lines they were also treated with a positive control 100 ng/mL TNF α (Fig. 4.6.A and 4.6.B), which triggers NFκB and AP-1 production via the IKK complex, via a TLR independent pathway. Both cell populations were shown to actively respond to TNFα stimulation with a response ratio of 2.5 and 5.4 for HEK-Blue™ hTLR4 cells (Fig. 4.6.A) and HEK293 pNifty2-SEAP (4.6.B) respectively. This indicates that the response to LPS is dependent on the expression of the TLR4 receptor and does not occur through other endogenous receptor. It also confirms that the reporter plasmid functions efficiently.

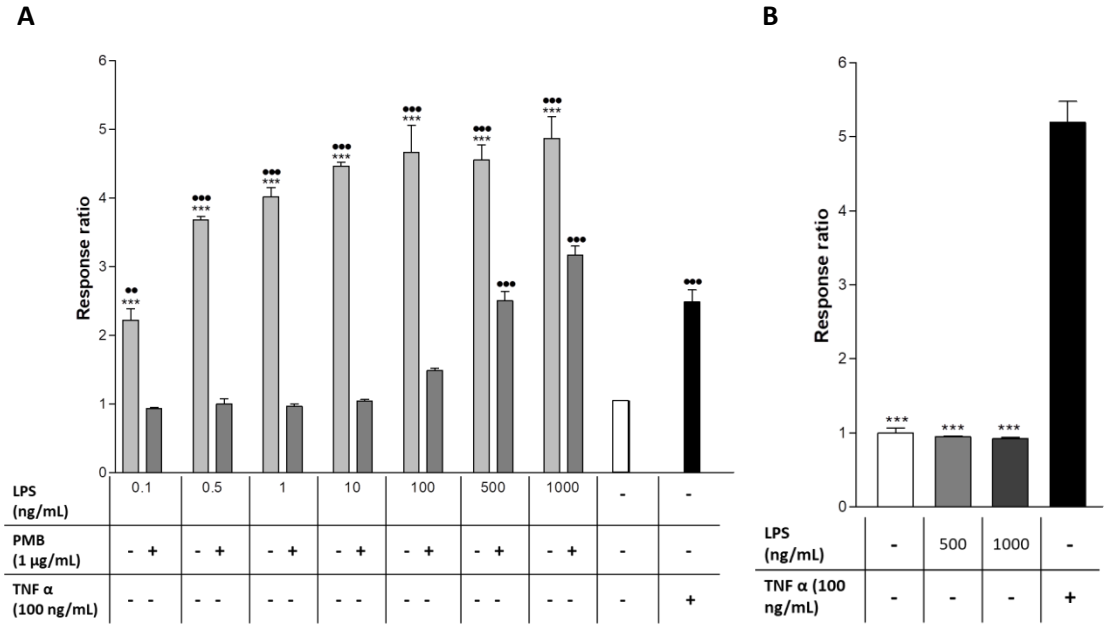

Figure 4.6: **HEK-Blue™ hTLR4 cells respond to LPS only in the presence of a TLR4 receptor. A:** HEK-Blue™ hTLR4 cells treated with different LPS concentrations (0.1 ng/mL - 1000 ng/mL) with (+) or without (-) 1 µg/mL of the inhibitor PMB. SEAP reporter activity was measured using pNPP solution at OD_{405}. Values are mean for ± SEM, n=3. P≤ 0.05 (*), P≤ 0.01 (**), P≤ 0.001 (***) for LPS vs LPS+PMB; P≤ 0.05 (●), P≤ 0.01 (●●), P≤ 0.001 (●●●) for untreated vs treated. **B**: HEK293 pNifty2-SEAP stimulated with 500 ng/mL and 1000 ng/mL of LPS or 100 ng/mL TNF α. P≤ 0.001 (***) for TNF α vs LPS and TNF α vs untreated. Statistical analysis determined with One Way ANOVA and Bonferroni Post Hoc test for multiple comparisons.

4.4 Mus musculus MIF1 has no significant role in the activation of the TLR4 pathway

After confirming the sensitivity and specificity of the HEK lines, cells were co- treated with *Mus musculus* MIF1 (MmMIF1) to determine if it influenced activation of the TLR4 receptor. Cells were treated with a range of LPS concentrations (0.5 ng/mL, 1 ng/mL or 10 ng/mL) and 0.01 μg/mL or 0.1 μg/mL of MmMIF1. Representative data of biological replicates is shown in Fig. 4.7. Additional experimental replicates are shown in Fig A.1 in the appendix. While there was a trend in some samples showing a reduction in TLR4 activation after MmMIF1 treatment these differences were not statistically significant. MmMIF1 alone did not result in NFκB and AP-1 activation and co-treatment of cells with MmMIF1 and TNFα did not show any significant differences in the activity of the SEAP reporter (Fig. 4.7). This data indicates that MmMIF1 neither directly modulates LPS action via the TLR4 pathway nor TNFα action via the IKK complex.

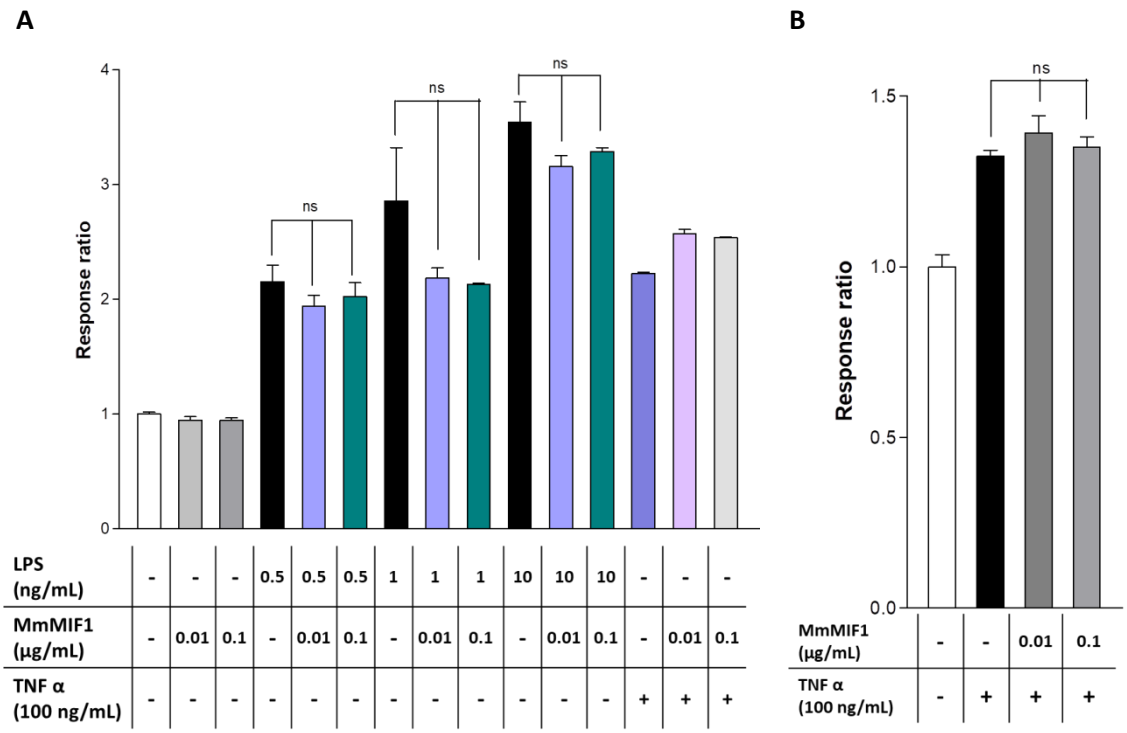

Figure 4.7: **Co-administering MmMIF1 with LPS did not cause significant difference in activation of TLR4 pathway. A:** HEK-Blue™ hTLR4 cells were co-treated with 0.01 µg/mL or 0.1 µg/mL MmMIF1 and either 0.5 ng/mL or 1 ng/mL or 10 ng/mL LPS. The activation of the SEAP reporter is compared to cells treated with LPS alone. HEK-Blue™ hTLR4 cells treated with TNFα with or without MmMIF1 used as a positive control. Values are mean for ± SEM, n=3. P-value is non-significant (ns) for LPS vs LPS + MmMIF1. **B:** HEK293 pNifty2-SEAP subjected to 100 ng/mL TNFα with or without 0.01 µg/mL or 0.1 µg/mL of MmMIF1. Values are mean for ± SEM, n=3, P-value ns = non-significant, P > 0.05. Statistical analysis for both 4.7.A and 4.7.B determined with One Way ANOVA and Bonferroni Post Hoc test for multiple comparisons.

4.5 Trichinella spiralis MIF1 inhibits the activation of the TLR4 receptor by LPS.

To compare the activity of a pathogen derived MIF homolog to that of an endogenous mammalian MIF, cells were co-treated with a range of LPS concentrations (0.5 ng/mL, 1 ng/mL, 10 ng/mL) and two concentrations of TsMIF1 (0.04 μg/mL, 0.4 μg/mL). Representative data of biological replicates is shown in Fig. 4.8. Additional experimental replicates are shown in Fig A.2 in the appendix. Unlike MmMIF1 it was observed that treatment with both 0.04 μg/mL, 0.4 μg/mL of TsMIF1 lowered activation of the SEAP reporter relative to the cells treated with LPS alone. This inhibition was observed across all three LPS concentrations. However, inhibition levels were not at the same level for all three LPS concentrations and TsMIF1 appears to have the highest inhibitory activity when administered at lower concentrations to cells treated with 0.5-10 ng/mL LPS. This observation may be partially explained by the TsMIF1 only controls where the higher concentration of TsMIF1 also show activation of the SEAP reporter indicating there may be residual LPS contamination with in the recombinant protein preparations. When cells were co-treated with TsMIF1 and 100 ng/mL TNFα, there was no significant difference in activity of the SEAP in comparison to responses with TNFα alone in either the HEK-Blue™ hTLR4 cells (Fig. 4.8.A) or HEK293 WT (Fig. 4.8.B). Thus, it is tempting to speculate that the suppressive action of TsMIF1 is through pathway components specific to TLR4 receptor signalling rather than the common components shared with the TNF transduction pathway such as the IKK complex.

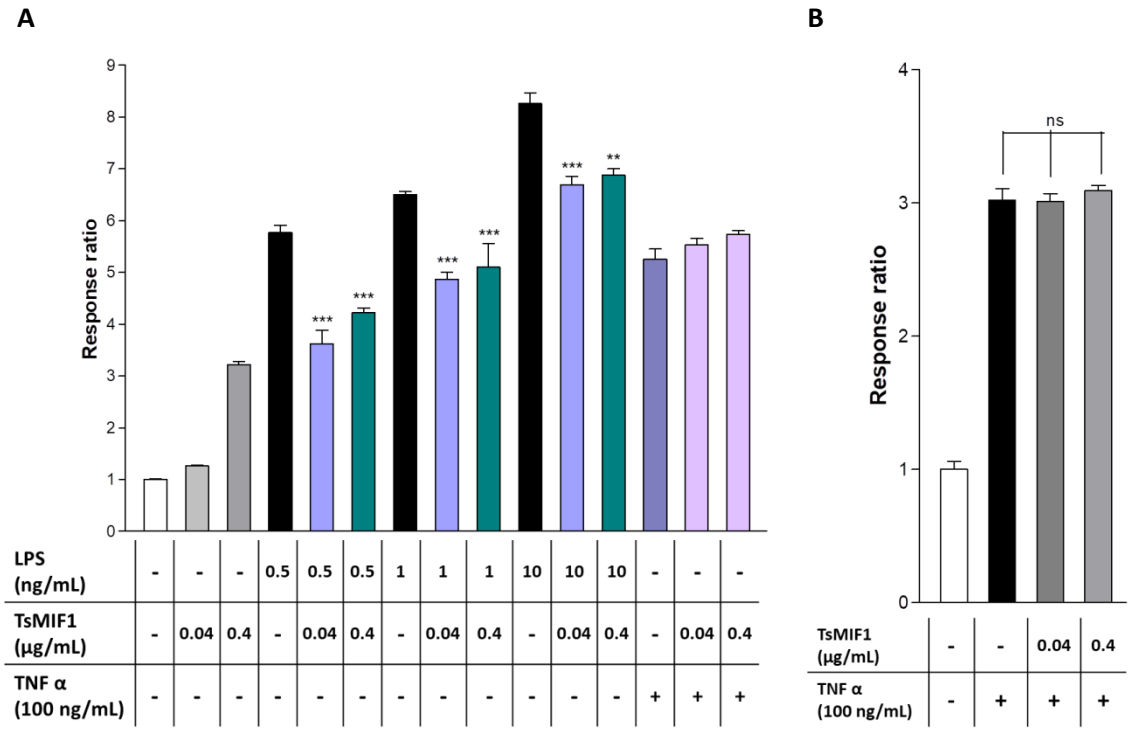

Figure 4.8: **TsMIF1 inhibits the action of LPS in HEK-Blue™ hTLR4 cells. A:** 0.04 µg/mL or 0.4 µg/mL TsMIF1 co-administered with either 0.5 ng/mL or 1 ng/mL or 10 ng/mL LPS in HEK-Blue™ hTLR4 cells and compared to cells treated with LPS alone. HEK-Blue™ hTLR4 cells treated with TNFα with or without TsMIF1 used as a positive control. Values are mean for ± SEM, n=3. P≤ 0.05 (*), P≤ 0.01 (**), P≤ 0.001 (***) for LPS vs LPS+ TsMIF1. **B:** HEK293 pNifty2-SEAP subjected to treatment with 100 ng/mL TNFα alone with or without 0.04 µg/mL, 0.4 µg/mL TsMIF1. Values are mean for ± SEM, n=3, p- value non- significant (ns) for TNFα vs TNFα + TsMIF1. Statistical analysis for both 4.9.A and 4.9.B determined with One Way ANOVA and Bonferroni Post Hoc test for multiple comparisons.

5.0 Discussion

In order to investigate the potential immunomodulatory properties of MIF1 in the activation of TLR4, a primary aim of this project was to clone, express and purify enzymatically active recombinant protein *Mus musculus* MIF1 using the pET29b bacterial expression vector. The effects of this recombinant were then tested in a TLR4 activation assay using HEK-Blue™ hTLR4 cells. In addition to testing the activities of MmMIF1 in the TLR4 bioassay, the effects of a parasite derived MIF1 homologue, *Trichinella spiralis* MIF1 was also assessed.

5.1 Cloning, Expression and Purification of enzymatically active MmMIF1 from bacteria

Murine MIF1 was successfully cloned into the bacterial expression vector pET29b (Fig. 4.1.A) and incorporated into BL21 Codon plus *E. coli* (Fig. 4.1.B). The DNA gel electrophoresis of gene and plasmid specific diagnostic PCR products and sequencing (Fig. 4.1.C) confirmed the presence of the MmMIF1 cDNA and it's orientation within the expression plasmid. Using IPTG as the protein induction stimulus, soluble MmMIF1 was produced (Fig. 4.2). It was also confirmed by Western blot using a His-antibody (Fig. 4.3.D) that the MIF1 protein had an intact His-tag. Overnight express media was also used for protein induction, however, it did not induce detectable protein in the soluble fraction. This may be due to inadequate oxygenation/aeration as the supply of oxygen is limited after dense growth of the culture (Studier, 2005). Temperature might be another factor and optimisation by reducing the temperature of incubation to lower temperature such as 25^{o}C - 30^{o}C might improve the yield of soluble protein (Novagen). A number of studies suggest that the availability of oxygen and amino acids can dramatically influence the outcome of recombinant protein biosynthesis (Carnicer

et al., 2012, Blommel *et al.,* 2007, Rosano and Ceccarelli, 2014). Although IPTG is a synthetic structural analogue of allolactose and is sometimes toxic when used in excessive amounts, the relatively low concentration used in this study (50 µM) may have been adequate for the induction of reasonable amounts of protein but was not so high that it results in the entire recombinant product being sequestered in inclusion bodies.

Thus, the soluble fractions were successfully used to obtain purified MmMIF1 (Fig. 4.3) with a final concentration of 17 mg/mL. The purified protein was also free from residual endotoxin as determined by LAL assay with relevance to the physiological concentrations at which the MmMIF1 was diluted to in the TLR4 bioassay. This was further confirmed in the bioassays where MmMIF1 on its own did not trigger NFκB and AP-1 activation in HEK-Blue™ hTLR4 cells above basal levels (Fig. 4.7). Since it was established from dose-dependent experiments (Fig. 4.5 and Fig. 4.6.A) that HEK-Blue™ hTLR4 cells are responsive to LPS, the presence of any residual endotoxin in MmMIF1 should have been observable in the results. This indicates that MmMIF1 is endotoxin free and also that by itself it does not initiate TLR4 activation. MmMIF1 also showed tautomerase activity between 0-135 seconds of the reaction (Fig. 4.4.B). However, the depletion in the substrate was much higher for TsMIF1 than MmMIF1. This is due to the difference in the activity across species (Fig. 4.4) which has been shown in a study by Tan *et al.,* 2001. They demonstrated that TsMIF1 had 6- fold higher specificity and tautomerase activity towards L- dopachrome methyl ester than mammalian MIFs. It may also be due to the effect of the anion exchange chromatography column because it was observed that tautomerase activity of MmMIF1 before this procedure was higher (data not shown). It is possible that the cellulose based column matrix had

unexpected effects on the enzymatic activities of MIF and alternative matrix materials like polystyrene/divinyl benzene based columns such as Mini Q from GE Healthcare might help ensure that the protein remains stable during the polishing step of the purification.

Similarly, TsMIF1 was also quantified with a final concentration of 30.7 mg/mL. The amount of residual endotoxin was not clearly established for TsMIF1 which may lead to the possibility that the endotoxin levels were high enough to interfere within the bioassay at higher TsMIF1 working concentration. This is observed in Fig. 4.8.A where HEK-Blue™ hTLR4 cells treated with 0.4 µg/mL of TsMIF1 alone resulted in higher activation of the SEAP reporter when compared to cells treated with either 0.04 µg/mL of TsMIF1 or DPBS. If TsMIF1 was inherently capable of activating TLR4 we would predict it would also act at lower concentrations such as 0.04 µg/mL concentration so the most likely explanation is that residual endotoxin within the recombinant protein caused base line activation observed in the assay and may have obscured inhibitory activities seen at the lower concentrations. To confirm this possibility the recombinant protein requires further purification via anion exchange chromatography and the TLR4 bioassays repeated.

5.2 Sensitivity and Specificity of the HEK-Blue™ hTLR4 Bioassay

Initial experiments using the HEK-Blue™ hTLR4 cells and WT HEK293 pNifty2-SEAP control cells isolated during this study showed that cell lines and the SEAP reporter used in this study specifically respond LPS treatment (Fig. 4.5 and Fig. 4.6). Cells lacking the TLR4 receptor showed minimal SEAP activity and control experiments treating cells with TNFα which has an intact receptor and signalling complex in WT HEK cells confirmed that SEAP responsiveness to signalling via the IKK complex. Although WT

HEK293 cells cannot express surface TLR4, MD-2 and CD14 which are essential for successful TLR4 activation with LPS, it contains all downstream proteins common to the TNFR1 pathway which can be activated in TLR4 independent manner. The sensitivity of the HEK-Blue™ hTLR4 cells was also assessed using HEK-Blue™ Detection media and p-Nitrophenyl phosphate (pNPP) assays (Fig. 4.5) which confirmed that both the methods have similar trends and are interchangeable in terms of both sensitivity and specificity. Within these assays the sensitivity range of the assay was also established with concentrations of LPS at which SEAP activity were within a linear concentration range spanning 0.1-10ng/mL. These optimised assay conditions for the reporter cells were then used to assess the effects of MmMIF1 and TsMIF1 treatment on TLR4 signalling with LPS simulation concentrations ranging from 0.5-10ng/mL.

5.3 TsMIF1 inhibits activation of the HEK-Blue™ hTLR4 cells by LPS unlike MmMIF1

TsMIF1 was found to significantly inhibit activation that was stimulated and initiated by LPS (Fig. 4.8). It can be inferred that this effect of TsMIF1 was through the TLR4 receptor specific signalling processes because there were no differences in the activation levels when it was co-administered with TNFα in both HEK-Blue™ hTLR4 cells and HEK293 pNifty2-SEAP. At a low concentration (0.04 µg/mL) TsMIF1 treatment on its own did not cause significant activation of the SEAP reporter. As discussed earlier, this is potentially due to residual endotoxin contamination within the recombinant protein. Therefore, we speculate that TsMIF1 may modulate the action of LPS through the TLR4 receptor at a point upstream of the IKK complex. As it was shown in Fig. 1.7, TGFβ activated Kinase 1 (TAK1) is the common point at which the two pathways intersect. Since TsMIF1 altered SEAP activation levels only when co-administered with

LPS and not with TNFα, the scope of signalling intermediates that TsMIF1 might affect would range from TLR4 receptor itself at the surface to TRAF6 which is the protein just upstream of TAK1 (Fig. 1.7). Identifying the intermediates may be achieved by analysing changes in the mRNA expression levels of these proteins within the pathway with or without MIF treatment using quantitative real-time PCR (qPCR). Detecting phosphorylated intermediates such as TAK1 by immunoblotting may provide insights into activated proteins in the pathway with or without MIF. MmMIF1 did not show any significant changes in the activation levels triggered either through the TLR4 pathway or the TNFR1 cascade.

One additional complication of this study is that one of the putative MIF receptor complexes - CD74/CD44 is expressed at low levels in HEK cells which may mean they are less responsive via this pathway than relative to other cell types such as APCs. Previous studies have shown that mammalian MIFs trigger phosphorylation of ERK1 and ERK2 via CD74/CD44 (Leng *et al.,* 2003 Shi *et al.,* 2006). However, other known MIF receptors such as Jab1 are expressed in HEK cells and the results found after treatment with TsMIF1 may indicate they play an important role in regulating TLR4 signalling events. Increasing expression levels of the CD74/CD44 receptor complex in the HEK reporter cells either via transfection or transduction will allow the relative contribution of the different MIF receptors to the phenotype observed in this study to be specifically evaluated.

It is also important to understand whether enzymatic activities of the recombinant MIFs (tautomerase and oxidoreductase) are linked to effects observed in this study. Use of specific enzyme inhibitors such as: 4-iodo-6-phenylpyrimidine (4-IPP) that covalently modifies Proline 2 at the N-terminal and shown to inhibit internalisation of

MIF/CD74 (Varinelli *et al.,* 2015); or p425, an allosteric inhibitor of MIF's tautomerase activity that has been shown to inhibit MIF/CD74 interaction and modulate the pro-inflammatory activities of MIF (Bai *et al.,* 2012). Another approach would be to generate mutant recombinant proteins lacking these enzyme active sites. These approaches will further our understanding on whether the tautomerase site of MIF is involved in the effects observed with NFκB and AP-1 activation. If the SEAP activity shows any changes upon this inhibition, measuring differential activation of proteins in the MAPK or the MIF/CD74 pathway will provide further insight about the exact mechanisms underlying MIF's modulation of this pathway.

6.0 Concluding remarks

To conclude, enzymatically active MmMIF1 and TsMIF1 were successfully cloned, expressed and purified for bioassays. Initial results indicate that TsMIF1 suppressed the LPS induced activation of the SEAP reporter while MmMIF1 did not seem to show any significant effects in this bioassay. Further characterisation and comparison of TsMIF1's activities relative to mammalian MIFs is particularly important as it might help further our understanding of how this parasite evades host immune responses and could potentially be a starting point for the development of novel therapeutic agents for GI inflammation since it inhibits the activation of at least one inflammatory responses pathway that is important in a number of human pathological conditions.

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Appendix

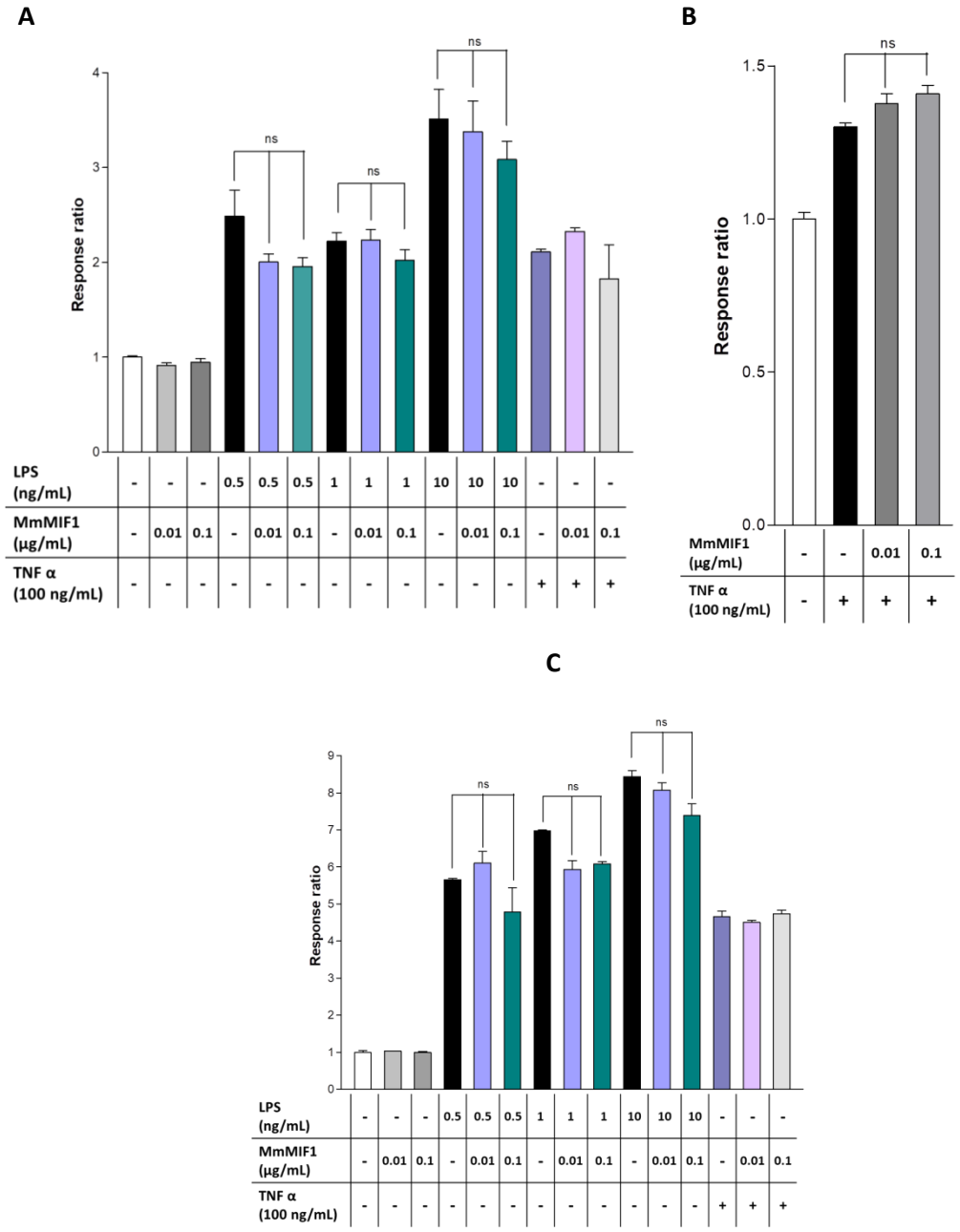

Figure A.1: **Co-administering MmMIF1 with LPS did not cause significant difference in activation of TLR4 pathway. A, C:** HEK-Blue™ hTLR4 cells were co-treated with 0.01 µg/mL or 0.1 µg/mL MmMIF1 and either 0.5 ng/mL or 1 ng/mL or 10 ng/mL LPS. The activation of the SEAP reporter is compared to cells treated with LPS alone. HEK-Blue™ hTLR4 cells treated with TNFα with or without MmMIF1 used as a positive control. Values are mean for ± SEM, n=3. P-value is non-significant (ns) for LPS vs LPS + MmMIF1. **B:** HEK293 pNifty2-SEAP subjected to 100 ng/mL TNFα with or without 0.01 µg/mL or 0.1 µg/mL of MmMIF1. Values are mean for ± SEM, n=3, P-value ns, non-significant, $P > 0.05$. Statistical analysis for both A.1.A and A.1.B determined with One Way ANOVA and Bonferroni Post Hoc test for multiple comparisons.

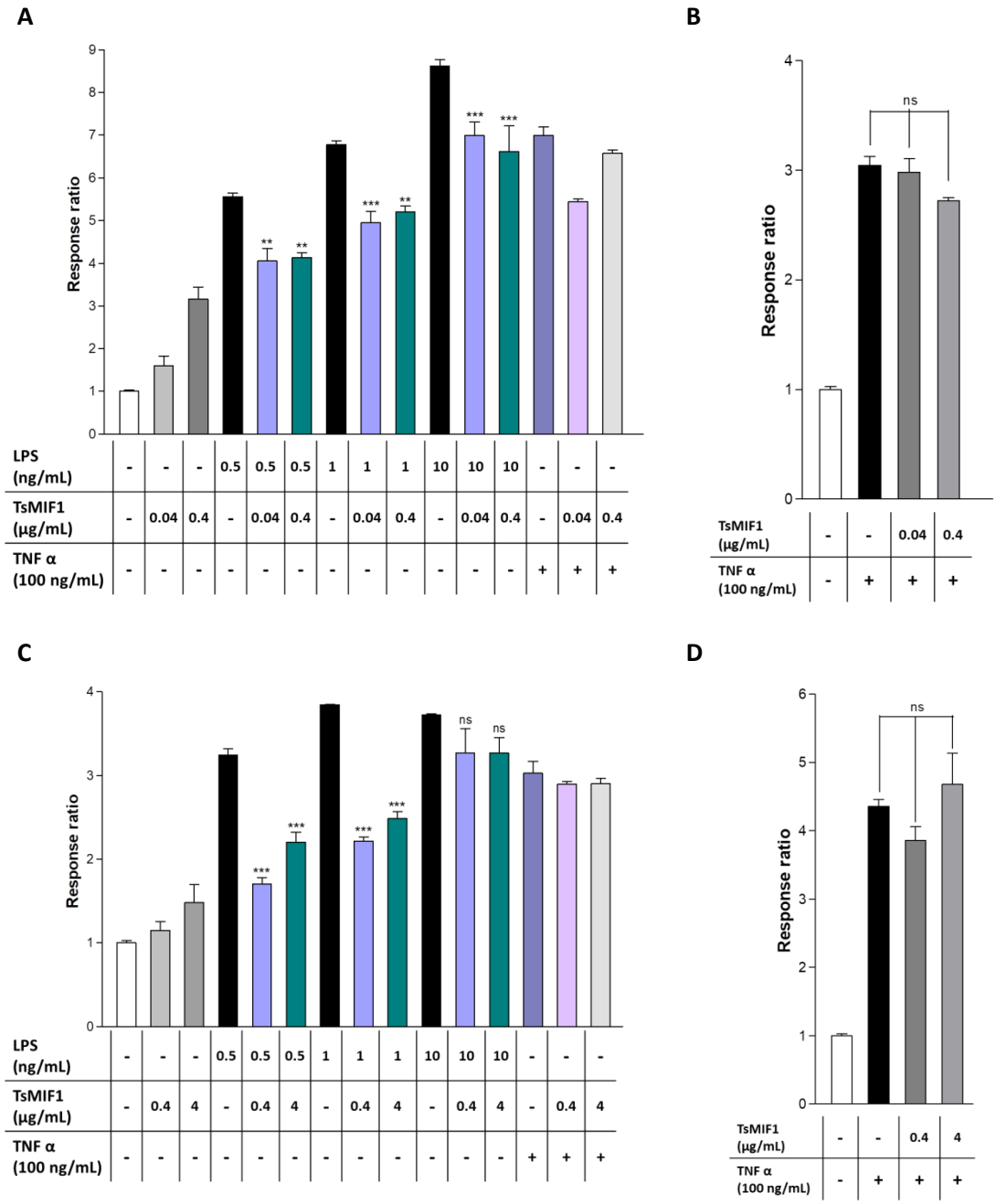

Figure A.2: **TsMIF1 inhibits the action of LPS in HEK-Blue™ hTLR4 cells. A, C:** 0.04 µg/mL or 0.4 µg/mL TsMIF1 (A) or 0.4 µg/mL or 4 µg/mL (C) TsMIF1 co-administered with either 0.5 ng/mL or 1 ng/mL or 10 ng/mL LPS in HEK-Blue™ hTLR4 cells and compared to cells treated with LPS alone. HEK-Blue™ hTLR4 cells treated with TNFα with or without TsMIF1 used as a positive control. Values are mean for ± SEM, n=3. P≤ 0.05 (*), P≤ 0.01 (**), P≤ 0.001 (***),P ≥ 0.05 (ns) for LPS vs LPS+ TsMIF1. **B, D:** HEK293 pNifty2-SEAP subjected to treatment with 100 ng/mL TNFα alone with or without 0.04 µg/mL, 0.4 µg/mL (A); 0.4 µg/mL, 4 µg/mL (C) TsMIF1. Values are mean for ± SEM, n=3, P- value non- significant (ns) for TNFα vs TNFα + TsMIF1. Statistical analysis determined with One Way ANOVA and Bonferroni Post Hoc test for multiple comparisons.