Modulation of the Gastrointestinal Immune Environment by Macrophage Migration Inhibitory Factors (MIF) and Helminth-Derived Cytokine Homologues of MIF

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Declaration

I hereby certify that the work presented within this thesis is the result of my own investigation, except where reference has been made to published literature and where acknowledgment is made for unpublished data. During the course of this research programme I have not been registered or enrolled to another award from any academic or professional institutions.

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Abstract

Macrophage Migration Inhibitory Factor (MIF) is a pleotropic cytokine first discovered over 50 years ago. MIF and many MIF-like proteins contain an evolutionarily conserved proline residue that confers an enigmatic tautomerase activity. Mammalian MIF proteins also contain an additional oxidoreductase domain whose activity is abrogated by substitution of two critical cysteine residues at position 57 and 60. MIF is secreted constitutively by intestinal epithelial cells and is highly upregulated when barrier function is compromised such as in the case of inflammatory bowel diseases. MIF homologues are also secreted by many parasitic organisms, one of which is the intestinal helminth, *Trichinella spiralis. T. spiralis* secretes vast quantities of MIF upon entering the gastrointestinal tract though to date the biological relevance of *T. spiralis* derived MIF in modulating host responses is undetermined.

In this study the generation MIF proteins and mutants devoid of enzymatic sites enabled the analysis of MIF's role within the intestinal immune environment including the transcriptomic assessment of *ex vivo* intestinal explants and primary macrophages. Recombinant WT and tautomerase deficient proteins generated as part of this body of work modulated TLR-4 mediated NF-kB activation in the presence of LPS in a HEK and HT29 cell model indicating that MIF can modulate epithelial driven immune responses via a master regulator. Consistent with this, *ex vivo* studies utilising murine intestinal explants revealed that murine and *Trichinella* derived MIF homologues modulate cytokines such as IL-6, TNF-a and IL-22 to drive distinct immune responses. In addition, the modulation of IL-22 and TNF-a was highly dependent on the presence of the tautomerase site for Mm-MIF-1 and Ts-MIF-1, respectively. Likewise, analysis of cytokine profiles

from MIF treated macrophages in the presence of TLR4 ligand, LPS, confirmed MIF's role in modulating immune responses.

Further characterization of BMDM macrophages using RNA seq technologies demonstrated that MIF homologues, and in particular, the tautomerase site, modulate the macrophage transcriptome priming cells for two discrete responses upon pattern recognition receptor (PRR) activation. Mm-MIF-1 treated BMDM macrophages downregulated several genes associated with the TNF-a processing and secretion, *ADAM28, Trp63* and *Rab27b*. Conversely, the parasite-derived Ts-MIF-1 upregulated genes responsible for cell cycle regulation, differentiation and cellular architecture such as *IGFBP2, BMP3, BMP7* and several *Krt* genes.

Overall, the data presented in this thesis provides clear evidence of discrete roles for murine and parasite-derived MIF in modulating innate immune responses and demonstrates that, while the activity of the tautomerase site is context dependent, loss of the enzymatic activity leads to dysregulation MIF responses.

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List of abbreviations

APC	Antigen presenting cell
BCA	bicinchoninic acid
BMDM	Bone Marrow derived Macrophage
BMP	Bone Morphogenetic Protein
CCL	Chemokine ligand
CD	Cluster of differentiation
cDNA	complementary DNA
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
CY-7	Cyanine -7
DAMP	damage associated molecular pattern
DC	Dendritic cell
D-DT	D-Dopachrome Tautomerase
DEG	Differentially expressed gene
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Foetal Bovine Serum
FDR	False Discovery Rate
GO	Gene Ontology
GT	Gastrointestinal tract

HEK	Human embryonic kidney
His	Histidine
HRP	Horseradish peroxidase
IEC	Intestinal epithelial cell
IFN-y	Interferon gamma
IL	Interleukin
ILC	Innate lymphoid cell
IPTG	Isopropyl _- d-1-thiogalactopyranoside
LB	Luria-Bertani medium
M cell	Microfold cell
MCP-1	Monocyte Chemoattractant Protein - 1
mg	microgram
mg	milligram
MHC	Major Histocompatibility complex
MIF	Macrophage Migration Inhibitory Factor
mRNA	messenger RNA
NF-kB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
NLR	Nod-like receptor
No RT	No reverse transcriptase
NTC	No template control
PAMP	pathogen associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
pNPP	p-Nitrophenyl Phosphate

PRR	Pattern recognition receptor
qPCR	quantitative PCR
RNA	ribonucleic acid
RNA seq	RNA sequencing
RPMI	Roswell Park Memorial Institute
RT	Room temparature
SBE	SMAD binding element
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SEAP	Secreted Alkaline Phosphatase
SXT	Somatic Extract
TAE	Tris-Acetic acid EDTA
Taq	Thermus aquaticus
TBS-T	Tris buffered saline-Tween 20
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethane-1,2-diamine
TGF-B	Transforming Growth Factor Beta
TGS	Tris, glycine, SDS buffer
Th	T helper
TLR	Toll-like receptor
TNF-a	Tumour Necrosis Factor alpha
UV	Ultra violet
X-gal	5-Bromo-4-Chloro-3-Indolyl _-D-Galactopyranoside
YT	Yeast Tryptone

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Chapter 1: Introduction to the Immunology of the Gastrointestinal Tract and Macrophage Migration Inhibitory Factor (MIF)

1.1 Background and Purposes of this study.

The gastrointestinal tract (GT) is perpetually subjected to attack from potentially hostile microbes, many of which secrete immunomodulatory molecules that mimic host responses in order to subvert local responses.

Macrophage Migration Inhibitory factor (MIF) or MIF-like proteins are produced by a vast number of organisms including mammals, bacteria and parasitic helminths, many of which have been widely investigated and their functions are understood (Bernhagen *et al*., 1994; Falcone *et al*., 2001a; Leng *et al*., 2003; M *et al*., 2012; Prieto-Lafuente *et al*., 2009a; Tan *et al*., 2001; Vermeire *et al*., 2008a). Various microorganisms and helminths that colonise the GT secrete homologues of MIF, although to date, the role(s) these proteins play in infection processes remain to be determined. In mammals, MIFs are produced in large amounts within the GT in response to an infection. Similarly, within the tumour microenvironment MIFs are also highly expressed proteins though controversially, in these contexts, they have been demonstrated to exert seemingly contradictory actions (Balogh *et al*., 2018; Dessein *et al*., 2010; Figueiredo *et al*., 2018).

A common trait shared between many MIF homologues is an evolutionarily conserved N-terminal proline site which is required for its tautomerase activity. Substitution of the tautomerase-conferring proline site to an alternative residue leads to a reduction or complete loss of enzymatic activity (Brown *et al*., 2009;

Fingerle-Rowson *et al*., 2009; Robert Kleemann *et al.*, 2000b; Senter *et al*., 2002a). Regardless of the fact that the proline site is conserved in most MIF-like proteins, studies investigating the biological relevance of MIF's enigmatic tautomerase activity have provided very little clarity on how it specifically links to specific biological functions or provided an example of a biological substrate.

The work encompassed in this PhD project focusses on understanding the potential roles mammalian and parasitic nematode derived MIFs have in modulating innate immune responses particularly in the intestine. It also tests the relationship between the biological activity of these MIFs and their tautomerase activity.

1.2 The intestinal immune environment.

1.2.1 Intestinal architecture.

The gastrointestinal tract (GT) is a complex organ system covered by absorptive mucosal epithelia and an underlying layer of muscle, connective tissue, blood, lymphatic vessels and immune cells. Continuously exposed to a wide variety of insults, including pathogen and self-derived antigens, the ability of the GT to maintain barrier and absorptive functions is a critical feature. Immune homeostasis is governed by several factors including the commensal microbiota, dietary components and endogenous regulatory mechanisms, all of which help to maintain barrier integrity. A key feature of the immune system within the GT is its ability to differentiate between commensal and pathogenic microorganisms (Eberl and Lochner, 2009; Kaper and Sperandio, 2005).

The intestinal immune architecture has a distinct structure and function which is largely dependent on a specific anatomical location within the GT (figure 1.1). In the upper GT there is minimal lymphoid tissue, hence most GT immune

responses occur within the small and large intestine. The small and large intestine are covered in a single layer of columnar epithelial cells interspersed with junctional proteins which form the protective surface of this tissue, constantly responding to external stimuli in order to maintain mucosal barrier integrity (Peterson and Artis, 2014). Within the small intestine, epithelial cells are covered by villi, sometimes referred to as a brush border, which serve to increase the surface area for absorption of nutrients and secrete enzymes into the local area, whilst contrastingly, epithelial surfaces in the large intestine are smooth and lacking villi largely since the majority of nutrients are absorbed prior to this point (Agace and McCoy, 2017; Santaolalla *et al*., 2011). The epithelium undergoes constant replenishment from immature stem cells which arise from structures known as crypts of Lieberkühn. Here, multipotent stem cells mature into absorptive enterocytes or other intestinal cells such as goblet cells which secrete mucus forming the protective glycocalyx. Immune responses in the intestinal tract occur primarily within the epithelial layer and the lamina propria and, despite being adjacent to one another, they confer distinct immune functions. Studies have shown that the type of immune responses elicited at these sites vary significantly and may be compartmentalized due to the specific resident microflora they contain. This ensures that immune responses at these sites are contained, thereby preventing unnecessary escalation of systemic immunity whilst limiting localised infections (Belkaid and Naik, 2013; Mowat and Agace, 2014).

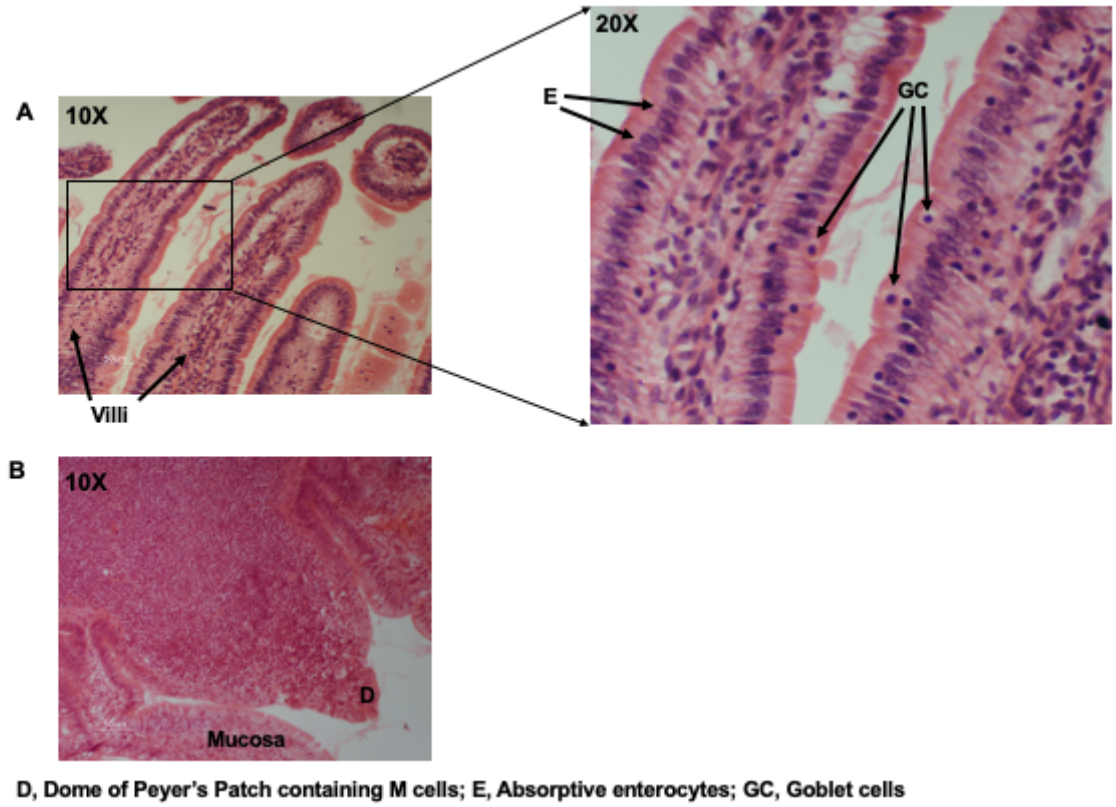


Figure 1. 1 Haematoxylin and Eosin staining of the small intestine. A) Duodenum architecture at 10X and 20X magnification. Black box represents magnified area. At 10X the Villi are clearly observed whilst at 20X the epithelial derived absorptive enterocytes and mucus-secreting goblet cells can be seen. **B)** Ileum structure at 10X. The mucosa forms the protective barrier of the intestinal surface separating the luminal contents while absorbing essential nutrients. Peyer's patches containing the highly specialised antigen sampling M-cells are found in the dome of Peyer's patches.

Within the GT, specialised gut-associated-lymphoid-tissue (GALT), consisting largely of innate lymphoid cells, mesenteric lymph nodes and Peyer's patches which are confined to the small intestine, play a critical role in both the development of mucosal adaptive immune responses as well as the development of tolerance to normal commensal microorganisms and dietary antigens.

Microfold cells (M cells), which reside on the luminal side of the Peyer's patches (PPs) constitutively traffic antigen through the epithelial layer within the GT forming a critical part in eliciting immune responses. Many of the immune responses within the GT are mediated by innate immune cells, of which, a large proportion are intestinal macrophages that can activate local CD4+ or CD8+ T-cells, many of which display markers associated with memory effector cells. Intestinal macrophages, though being constantly replenished by extravasating blood monocytes, have unique characteristics such as the production of high levels of IL-10 whilst still retaining the capacity to become classically inflammatory when appropriately activated (Morhardt *et al*., 2019).

The significance of immune homeostasis within the GT has been extensively studied and it is well-documented that intestinal immune dysregulation has the capacity to cause systemic pathologies including autoimmunity, diabetes and tumour development. For instance, chronic state of inflammation and exposure to inflammatory mediators, such as reactive oxygen species within this tissue has been directly linked to the progression of GT cancers (Wang *et al.,*2016). Additionally, cytokines which may alter the kinetics of immune response resolution, such as Macrophage Migration Inhibitory Factor (MIF), prolonging or altering inflammatory states, have been shown to influence, generally promoting tumour development and growth (Simpson *et al*., 2012, Yaddanapudi *et al*., 2013).

1.2.2 Epithelial cells in the GT.

Historically, intestinal epithelial cells were considered passive in terms of mediating immune responses in the GT, their principal role being to serve as a physical barrier between the luminal milieu and the internal environment. In more recent years, the importance of IEC's in maintaining mucosal homeostasis has

been extensively researched and, it is now understood that they form a critical role as sentinels, mediating local immune responses due to their unique positioning along the GT. IEC's can secrete a wide array of cytokines and chemokines, including, but are not limited to: IL-1β, IL-6, IL-8, IL-18, TGF-β, MIF, TNF-α, CCL-2 and CXCL-8, at varying levels in response to innocuous and noxious antigens (Bauché and Marie, 2017a; Harrison *et al*., 2015; Jung *et al*., 1995; Kucharzik *et al*., 2005; Maaser *et al.*, 2002a; Takada *et al*., 2010a; Vujicic *et al.*, 2018a) . Besides this, IEC's also secrete innate effector molecules such as antimicrobial peptides like REGIIIγ and several defensins that disrupt bacterial membrane or cell wall components including peptidoglycan.

IEC's respond to specific molecular patterns endogenous to harmful antigens or tissue damage by communicating with local innate immune cells. Accordingly, stimulation of intestinal epithelial cell line, Caco2, with purified flagellin derived from enterohaemorrhagic *Escherichia coli,* 0157:H7 led to rapid secretion of IL-8 and subsequent recruitment of leukocytes (Fraser-Pitt *et al*., 2011). IL-8 and the murine homologue, MCP-1 recruit monocytes and macrophages to the site of inflammation (Bauermeister *et al*., 1998). IEC's also play a significant role in modulating immune response to intestinal parasites. Nair *et al* (2008) demonstrated that IEC's infected with the parasitic nematode, *Trichuris muris,* secreted resistin-like molecule beta (RELM-β), activating local macrophages to upregulate MHC class II and secrete pro-inflammatory cytokine IL-12 further driving local antigen-specific Th1 responses contributing to infection longevity. Another example of IEC modulation of local immune cells was discovered during infection of murine IEC's with *Vibrio cholerae* which resulted in secretion of thymic stromal lymphopoietin and CCL20, attracting local dendritic cells to the site which led to the induction of a Th2 phenotype characterised by chemokines CCL17 and

CCL22 (Bhowmick *et al.*, 2012). Th2 responses to *V.cholerae* induce IgG4 and IgE production, in addition to, the recruitment of mast cells to resolve infection and provide future protective immunity (Bhuiyan *et al.*, 2009).

1.2.3 Innate immune cells in the GT.

The GT contains vast and varied types of immune cells in order to respond rapidly to harmful antigens whilst maintaining tolerogenic properties at steady state. Classical antigen-presenting cells, such as macrophages and dendritic cells, make up a large percentage of cells along the GT. Despite this, in the colon, macrophages constitute the major cell population and increase drastically in number from the beginning of the small intestine peaking in the caecum and colon (Denning *et al*., 2011; Nagashima *et al*., 1996). Spatially, colonic macrophages are located in very close proximity to the epithelial layer demonstrating their importance in mediating epithelial driven immune responses (Carlsen *et al*., 2006; Mahida *et al*., 1989). Colonic macrophages typically express high levels of markers associated with activation including MHC class II, CD163 and CD40 (Andrade *et al*., 2005; Mahida *et al*., 1989) in order to be able to respond rapidly once epithelial integrity is compromised. Paradoxically, they also produce substantial quantities of IL-10 promoting anti-inflammatory processes, the survival of $FOXP3^{+}_{Treg}$ cells, and maintenance intestinal tolerance (Murai *et al*., 2009). While intestinal macrophages are phenotypically and functionally specialised, being influenced to polarise to specific subtypes by the metabolic by-products of resident commensal microbes, they are constantly replenished by bone marrow derived monocytes (Desalegn and Pabst, 2019).

Though macrophages form the largest innate immune cell subset in the colon, several other innate immune cell types coexist with them including subset 1 $CD103^{+}$ $CD11b^{-}$ conventional dendritic cells (cDC1) which, are a distinct subtype

from those found in the small intestine. Depletion of $CD103^+$ $CD11b^-$ cDC1's in Clec9A–diphtheria toxin receptor (DTR) mice resulted in severe dextran sodium sulphate – induced colitis at doses as low as 2% (DSS) (Muzaki *et al*., 2016) while depletion of subset 2 $CD103^+$ $Cd11b^+$ cDC2s had no effect on intestinal inflammation demonstrating the importance of cDC1 in maintaining colonic epithelial integrity.

More recently, a third group of cells, innate lymphoid cells (ILCs) have been noted as critical mediators of mucosal immunity with their nomenclature divided into several subsets existing including ILC1, ILC2 and ILC3. ILCs are considered to be the innate arm of their respective T helper cell subsets, for example, ILC1 subsets promote the differentiation of Th1 cells including the secretion of IFN-γ in response to archetypical Th1 associated cytokines, IL-12 and IL-18 whilst being regulated by the Th1 associated transcription factor, T-bet (Fuchs *et al.*, 2013). ILC2 cells, much like their Th2 counterpart, are involved in the expulsion and clearance of helminth infections and allergy-associated inflammation, and respond to Th2 associated cytokines such as IL-2, IL-25 and IL-33 by producing IL-13 (Mjösberg *et al*., 2011). In addition to the aforementioned ILC subsets, ILC3 cells form an interesting subset that play a significant part in maintaining barrier function within the intestine. ILC3 cells are governed by the Th17 associated transcription factor, RORγt and secrete IL-17 and IL-22 in response to stimuli such as IL-1β and IL-23 from IECs and other innate immune cells (Melo-Gonzalez and Hepworth, 2017). To date, several studies have shown that ILC1 cells are abundant throughout the human intestine but ILC2 and ILC3 decrease in absolute number as the small intestine transitions into the colon (Krämer *et al*., 2017; Mowat and Agace, 2014).

1.2.4 Innate immune receptors in the GT.

One of the principal mechanisms by which immune responses are regulated in the GT is with the differential expression of innate pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and NOD-like receptors (NLRs), in IECs and innate immune cells. The PRR repertoire is extensive and has evolved to distinguish the many existing pathogens based on their unique pathogen or damage-associated-molecular-patterns (PAMPs/DAMPs) such as lipopolysaccharide (LPS), Flagellin and the high motility group box 1 protein (HMGB1) (Johnston and Corr, 2016). It has long been known that the 'janitorial' innate immune cells within the GT, such as macrophages and dendritic cells, express high levels of TLR-2 and TLR-4 at the cell surface. Contrastingly, IECs express low levels of TLR-4 and the TLR-4 co-receptor MD-2, to prevent constitutive activation by intraluminal antigens such as the gram negative bacterial endotoxins (LPS), and further studies mapping the location of TLR4 in IECs have demonstrated that it is predominantly expressed at the basolateral surface in healthy colonic epithelium (Hornef *et al*., 2003; Santaolalla *et al*., 2013). That said, once the epithelial barrier is compromised antigens can rapidly diffuse into the basolateral compartment, be recognised and cleared due to countless surveying and resident innate immune cells in local proximity such as the aforementioned macrophages. One of the most widely expressed TLR's in IECs is TLR4 which recognises the bacterial endotoxin, LPS, and causes a classical inflammatory response. Canonical activation of TLRs leads to a signal cascade mediated by TIRAP, TRIF and MYD88 and, typically results in the phosphorylation of master regulator NF-κB leading to upregulation of proinflammatory cytokine/chemokine expression and production. In murine models and human mutations, a number of the PRRs such as NOD2 have been

shown to not only predispose individuals to dysbiosis of the commensals within the GT leading to more frequent opportunistic infections, but, also lead to loss of barrier functions which can lead to chronic inflammation, autoimmune disorders and potentially cancer (Fukata and Arditi, 2013; Noguchi *et al*., 2009). Interestingly, NOD2 has recently been shown to act as a sensor for TLR4 signalling, driving responses based on the strength and duration of the TLR4 mediated signal. This unique mechanism of action provides some evidence for synergism between intracellular and extracellular PRR's (Kim *et al*., 2015).

1.3 The cytokine Macrophage Migration Inhibitory Factor (MIF).

1.3.1 The origins of MIF

Since its initial discovery as the first cytokine over forty years ago (Remold *et al*., 1971), MIF an evolutionarily conserved pleotropic cytokine and hormone, has been implicated in numerous biological processes from counteracting the immunosuppressive effect of naturally occurring glucocorticoids, to the regulation of glucose and lipid metabolism (Finucane *et al*., 2014). However, MIF has recently been shown to be a significant player in the progression of many disease pathologies, as shown in table 1.1, which are a currently a serious cause for concern worldwide, such as autoimmune/autoinflammatory disease, cardiovascular disease and cancer (Murakami *et al*., 2002, Morand *et al*., 2006, Stosic-Grujicic *et al*., 2008, He *et al*., 2009, Sreih *et al*., 2011). MIF's immunomodulatory activities have been extensively explored and it has been identified as a key regulator of both the innate and adaptive arms of the immune system. MIF has chemokine-like activities influencing both the recruitment of cells via migration (Fan *et al*., 2011; Hermanowski-Vosatka *et al*., 1999) and cytokine-like activities regulating the effects of other inflammatory mediators. However, more recently it has been shown that MIF can also regulate more fundamental

biological process such as cell cycle progress via p53, cell division and metabolic processes in both immune and non-immune cells (Brock *et al*., 2014). MIFs precise role(s) in directing immune responses is controversial and to date and the molecular events underpinning MIFs mechanisms of actions remains to be fully elucidated.

Table 1.1 A selection of disorders with pathophysiologies linked to MIF.

Disease	**Mechanism of action**	**Reference**
Rheumatoid Arthritis	Upregulates RANKL expression leading to osteoclastogenesis.	Kim *et al*., 2011
Colorectal Cancer	Increase in MMP9 and VEGF promoting angiogenesis.	He *at al*., 2009
Diabetes Mellitus	Increases lymphocyte proliferation and adhesion driving inflammation.	Stosic-Grujicic *et al*., 2008
Systemic Lupus Erythematosus	Increase macrophage and B cell survival by inhibiting apoptosis.	Sreih *et al*., 2011
Crohn's Disease	Activates dendritic cells and increases IL1β and IL8	Murakami *et al*., 2002

1.3.2 MIF protein structure and enzymatic activities.

MIFs are small proteins ranging in size from 12-15 kDa. Human MIF-1 (accession number CAG30406.1) is composed of 115 amino acids and Human DDT/MIF-2 (accession number CAG30317.1) is 118 aa. MIF monomers are made up of two anti-parallel α-helices and a four-stranded β-sheet. MIFs assemble into a

homotrimer where two additional β-strands from each monomer interact with the β-sheet from adjacent monomers forming the monomeric interface. The overall quaternary structure results in the three co-joined β-sheets forming a barrel-shaped solvent channel at the centre of the trimer as shown in figure 1.2 (H W Sun *et al*., 1996). Unlike most cytokines, mammalian MIFs have two enzymatic activities conserved within an N-terminal proline residue and in MIF-1-like MIFs the CXXC motif. The protein structure of MIF has a similar topology but no sequence similarity to two bacterial enzymes: 4-oxalocrontonate tautomerase (4-OT) and 2-carboxymethyl-5-hydroxymucante isomerase (CHI) (Subramanya *et al*., 1996). One unusual feature of MIFs, 4-OT and CHI, is that catalytic N-terminal Proline residues Pro2 have a pKa of 7.0 when, comparably, a typical N-terminal Proline would have a pKa of 3 pH units lower (Stivers *et al*., 1996). Studies have demonstrated that MIFs have the ability to keto-enol tautomerise two substrates *in vitro*, the naturally-occurring *p*-hydroxyphenylpyruvate and the artificial substrate L-dopachrome methyl ester (Rosengren *et al*., 1996, Bendrat *et al*., 1997, Rosengren *et al*., 1997, Lubetsky *et al*., 1999) and this activity is dependent on the formation of a trimeric complex. However, as neither of these tautomerase substrates exist in humans, the significance of such enzymatic activity and its role in MIFs varied biological activities are poorly understood. Additionally, a second enzymatic site, containing a CXXC motif, found in MIF-1-like family members, has been shown to be capable of functioning as protein oxidoreductase. *In vitro,* this has been demonstrated in the reduction of the mature insulin β-chain. The CXXC motif within human MIF has been implicated in a number of biological responses, from inducing intracellular signalling via Jab1 to enhancing cytoprotection in the context of myocardial reperfusion injury

(Luedike *et al*., 2012), and is believed to play critical roles in MIFs ability to act as a signalling molecule.

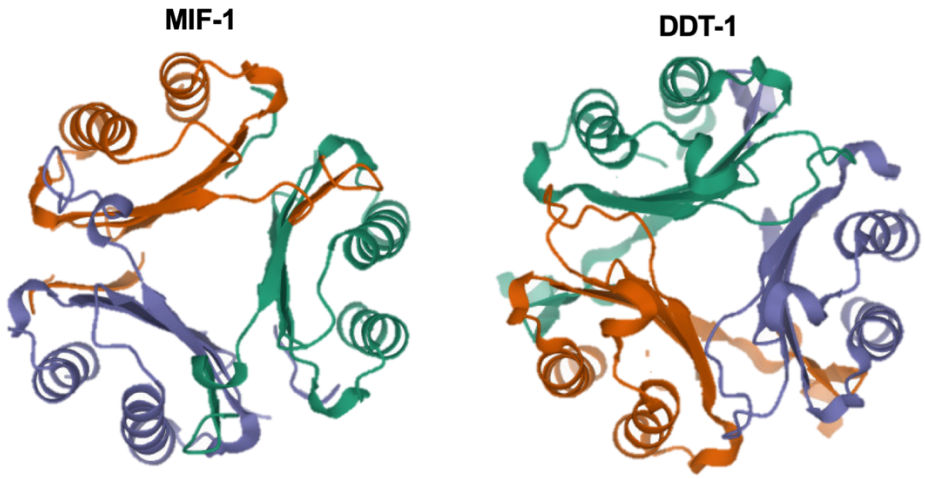


Figure 1. 2 MIF's three dimensional structure. Human MIF-1 (PDB: 1MIF) and DDT-1 (PDB: 1DPT) form a barrel-like structure formed from three subunits joined by the inherent b-sheets. The barrel-like structure contains the Pro2 catalytic core. Structures obtained from Protein Data Bank (PDB).

1.3.3 MIF receptors.

1.3.3.1 CD74.

MIF's signalling mechanisms have been widely studied and several cell surface and intracellular receptors have been proposed as transducer or receptors of MIF (figure 1.1). The most well-established receptor to date is the MHC class II invariant chain, Ii or CD74, which exists as both a transmembrane and soluble forms. CD74 was isolated as a MIF receptor in 2003 after a study by Leng *et al* (2003) demonstrated that antibody blockade of CD74 significantly decreased the amount of phosphorylated ERK 1/2 in response to 50ng/ml MIF treatment in fibroblastic cell line, CCL210. MIF has been shown to bind to CD74 with high

affinity (K_d = 1.4nM) (Merk *et al*., 2012) triggering a signal cascade that leads to the recruitment of a further cell surface receptor, CD44, which lead to the activation of ERK 1/2. Conflicting data from further studies indicate that, while CD44 is essential for canonical MIF signalling, the absence of CD44 does not completely abrogate MIF signal transduction. Interestingly, CD74 is expressed on the cell surface of non MHC class II cells such as epithelial cells suggesting a role, distinct from its function as chaperone for MHC-II and component of the antigen presenting machinery (Henne *et al*., 1995). In B-cells, MIF signalling via CD74, induces the activation of NF-κB and downstream transcription of survival factors such as Bcl-X (Starlets *et al*., 2006). Similarly, CD74 activation by MIF in murine colonic epithelial cells led to rapid upregulation of the anti-apoptotic Bcl-2 and cyclin E increasing cell survival (Maharshak et al., 2010a). Importantly, a tautomerase deficient MIF generated by knock-in of a mutant MIF gene where Pro2 was changed to Glycine, exhibited reduced binding to CD74 with an equilibrium dissociation constant of 39nM and 9nM, respectively (Fingerle-Rowson *et al*., 2009). These and other studies indicate MIF's tautomerase activity or this site of the protein may be partially responsible for CD74 binding. Beyond these initial studies how MIF's tautomerase site modulates receptor binding still remains relatively unexplored.

1.3.3.2 CXCR-2 and CXCR-4.

Aside from MIF's actions as a cytokine, it also confers chemokine-like properties binding to CXCR-2 and CXCR-4 which was demonstrated by MIF competing for binding to CXCR-2 and CXCR-4 against their cognate ligands such as CXCL-1. Additionally, MIF-binding to CXCR-2 promoted the formation of a receptor complex with CD74 leading to rapid chemotaxis of immune cells such as monocytes and T-cells (Bernhagen *et al*., 2007). Likewise, inhibition of CXCR-4

or CD74 in murine splenic B cells completely inhibits MIF-mediated chemotaxis suggesting that CD74 and CXCR-4 may work cooperatively to mediate MIF signalling (Klasen *et al*., 2014). MIF shares several structural motifs with other chemokine-like ligands, namely a *pseudo-ELR* motif (Asp-44–X-Arg-11) which in one study, upon mutation of Arg-11 and Asp-44, led to a significant reduction in the MIF/CXCR-2 receptor complex (Weber *et al*., 2008) and subsequent leukocyte recruitment. More recently, MIF been demonstrated to mediate, via CXCR-4, resistance to chemotherapeutic agents such as methotrexate and 5-fluorouracil in HT-29 cell and promotes an invasive phenotype. Additionally, enzymatic inhibition of MIF's tautomerase activity by ISO-1 significantly reduced the characteristics associated with metastasis indicating a potential functional role for MIF's tautomerase activities in CXCR-4 binding (Dessein *et al.*, 2010).

1.3.3.3 JAB/1.

In addition to the surface receptor mediated signalling described above, MIF also exerts other effects intracellularly via c-Jun activation domain-binding protein-1 (Jab1). Jab1 forms a critical part of the COP9 signalosome involved in promoting cell proliferation by disarming tumour suppressors such as p53 and $p27^{Kip1}$ (Shackleford and Claret, 2010). MIF has been shown to bind and inhibit Jab1 and AP1 leading to negative regulation of many genes such as *cJun*. Additionally, MIF stabilises levels of $p27^{kip1}$ by inhibiting cullun-dependant proteolysis and degradation. In this case, MIF did not affect the activation levels of NF-κB as was previously demonstrated indicating that in the context of a macrophage cell line, RAW 264 MIF inhibits AP-1 transcription independent of NF-κB (R. Kleemann *et al.*, 2000a). MIF's cysteine residues within its CXXC motif have been suggested as possible binding sites for the formation of MIF/Jab1 complex. Nguyen *et al* (2003), identified a 16-meric peptide from amino acids 50

– 65 covering the CXXC motif that could bind Jab1 and increase ERK 1/2 phosphorylation to the same extent as a full-length MIF protein. However, cysteine mutants (C57S/C60S), which are devoid of any oxidoreductase activity, still bound Jab1 with identical capacity indicating that MIF binding to Jab1 may be driven or potentiated by other domains of the protein. However, despite MIF cysteine mutants still binding to Jab1, the typical downstream activation of ERK 1/2 was absent indicating that loss of Cys57 and Cys60 may drive modulation of ERK 1/2 via an alternative MIF receptor such as CD74. Interestingly, MIF's interactions with Jab1 have also been implicated in its well-known role as a p53 antagonist and this is likely due to Jab1-mediated regulation of protein turnover of p53.

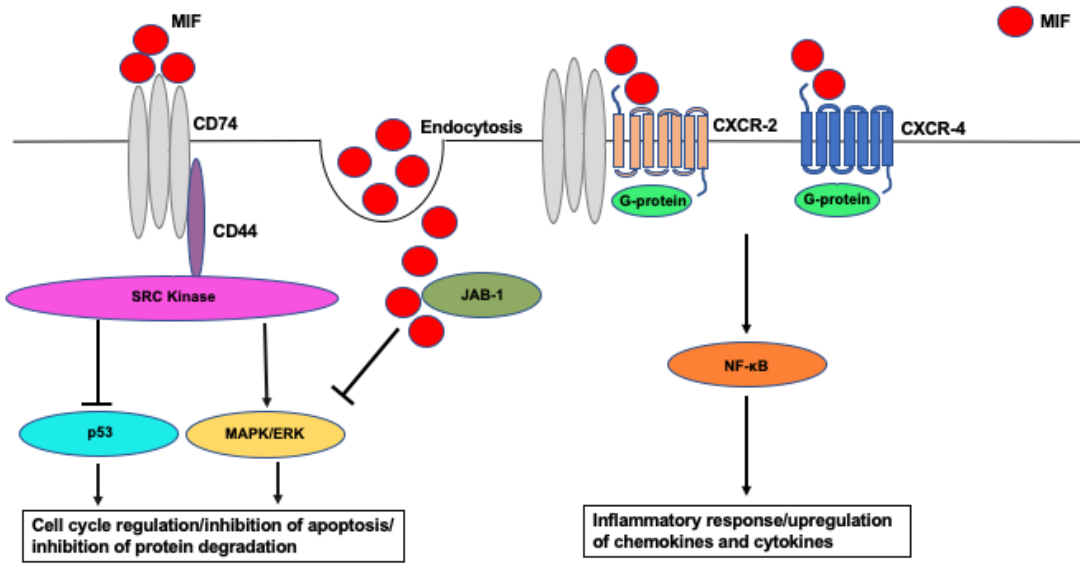


Figure 1. 3 Summary of MIF signalling pathways. MIF signals via several potential receptors. CD74/CD44 – Binding of MIF to CD74 leads to recruitment of CD44, phosphorylation of SRC kinase and various downstream responses including inhibition of p53 and activation of MAPK/ERK pathway. JAB1 – MIF can be endocytosed and signal intracellularly by binding to JAB-1, including inhibiting MAPK/ERK mediated gene expression. CXCR-2 and CXCR-4 – Binding of MIF elicits a G-protein coupled response and activates or supresses NF-κB signalling modulating gene expression of cytokines and chemokines. Both CXCR-2 and CXCR-4 can form a complex with CD74.

1.3.4 MIF modulation of intestinal immunity and GT functions.

The role of MIF in systemic inflammatory responses has been widely researched. However, little is known about its role in mucosal immunity and the GT. Previous studies have focussed on MIFs ability to direct immune cell differentiation and migration within the GT. Colonic epithelial cell derived MIF inhibits the migration of monocytic U937 cells towards the chemotactic agent SDF-1α (Maaser *et al*., 2002a). Additionally, recombinant human MIF converts colonic cancer cell line Caco2 to antigen sampling M cells within the Peyer's patches. Likewise, co-

culture of MIF-producing cell line, Raji B cells, with Caco2 cells induces the M cells phenotype and this is ablated in the presence of a MIF-antibody (Man *et al*., 2008a). Despite efforts to understand MIF's role within the GT, the effect on the epithelial cells that line the entire GT and serve as the initial point of contact for most invading pathogens, remains relatively unexplored. Studies utilising murine MIF KO models have potentially identified a role for MIF in positively regulating intestinal permeability. Absence of MIF in epithelial cells led to a significant increase in the mRNA of tight junction proteins, Zona Occludins 1 and Claudin 2 and the aberrant expression of IL-18 which is known to cause disruption of epithelial junctions (Vujicic *et al.*, 2018b). Evidence from a limited number of studies indicates MIF could play a key role in mediating protective immunity as demonstrated widely in the context of parasitic infections. MIF deficient mice are susceptible to infection with *Heligmosomoides polygyrus* despite immunization and, whilst Th2 responses were typically unaffected, the innate arm of the immune response was severely comprised including reduced eosinophilia and delayed polarisation of macrophages to an M2 phenotype preventing expulsion (Filbey *et al*., 2019a). Similarly, host MIF can stimulate responses to eliminate parasites such as *Leishmania* (Gupta *et al.*, 2013) and *Toxoplasma gondii* (Cavalcanti *et al.*, 2011a) by inducing the production of pro-inflammatory cytokines like IFN-y, IL-12 and nitric oxide (Bozza *et al*., 2012). Furthermore, it has been demonstrated that endogenous MIF is produced by the gastric and intestinal epithelium (Maaser *et al.,* 2002). However in cancers MIF mRNA and tautomerase activity is significantly increased within epithelial cells from human sporadic colorectal adenocarcinomas (Wilson *et al*., 2005). Additionally, MIF dysregulation within the GT also appears to be linked to the development several infection-induced pathologies. One example of this is gastric colonization and

ulcer formation induced by the bacteria *Helicobacter pylori.* In this system production of too much MIF is linked to the inflammation of the mucosa, ulceration, and the development of gastric cancers (Beswick *et al*., 2006). However, the details of both the sources of MIF in the GT and its immunomodulatory functions remain largely undefined.

Tumorigenesis is a multi-step process involving a complex array of steps where tumorigenic cells acquire the ability to overcome normal growth control process, colonize new tissue spaces and evade immune surveillance mechanisms that would typically destroy them (Dunn *et al*., 2002, Schreiber *et al*., 2011, O'Sullivan *et al*., 2012, Mittal *et al*., 2014). Genetic and molecular studies indicate MIF participates in each of these key steps (Choi *et al*., 2012). This includes promoting cell growth via inhibition of the master tumour suppressor gene p53 as well as facilitating the colonization of new tissue spaces by allowing tumour cell migration or induction of angiogenic responses (Coleman *et al*., 2008; Choudhary *et al*., 2013). Critically, MIF is now believed to play a key role in the development of favourable immunological microenvironments for the growing tumour, suppressing local immune responses leading to the failure of immunosurveillance (termed cancer immunoediting) (Dunn *et al*., 2002, Dunn *et al*., 2004, O'Sullivan *et al*., 2012, Schreiber *et al*., 2011). Among the cancers that develop in the GT, colorectal cancers have been shown to secrete large amounts of MIF into the local environment (He *et al*., 2009). This is thought to assist tumour progression by inhibiting immune effector cell migration and promoting the transition of epithelial-to-mesenchymal cells with immunosuppressive activities (Boissiere-Michot *et al*., 2014). Based on these observations MIF is now being explored as target for cancer and understanding the mechanisms by which MIF contributes

to each of these aspects of tumour development will be critical for the effective development of novel therapeutic strategies.

1.3.5 Parasite MIFs modulate mucosal immunity mimicking the tumour microenvironment.

Remarkably, many intestinal parasites have been shown to produce their own cytokine homologues of MIF including *Entamoeba histolytica* (Vermeire *et al*., 2008), *Strongyloides ratti (Younis et al*., 2012), and *Heligosomoides polygyrus* (Filbey *et al.*, 2019b). Additionally, *Trichinella spiralis,* a nematode which completes an entire life cycle within a single host, has been shown to produce two MIF cytokine homologues upon contact with the acidic gastric environment (Tan *et al*., 2001; Guiliano., unpublished). Infection with this nematode typically leads to a potent Th2-type immune response which in the case of *T. spiralis* but not *S. ratti* or *H. polygyrus.* Numerous studies have demonstrated *T. spiralis'* propensity to modulate immune responses in a stage-specific manner in order to promote their own survival. *T. spiralis* has been shown to increase the production of Th1 cytokines, such as IL12 and INF-γ, in early infective stages in order to regulate the number of new-born larvae whilst limiting worm expulsion via Th2 mediated mechanisms (Helmby and Grencis., 2003). Paradoxically, intravenous administration of *T.spiralis* new-born larvae leads to the rapid induction of IL-10-producing innate immune cells which, in turn, promotes larval survival whilst limiting host collateral damage (Huang *et al*., 2014). The molecular mechanisms utilised by this and other intestinal helminths to modulate host immunity in a way that promotes their survival bears a striking resemblance to that seen in some tumours (Elliott and Weinstock, 2012, Weinstock and Elliott, 2013).

Analysis of parasite derived MIFs show that while they are quite divergent in terms of their sequence homology (~40-50% amino acid similarity) they still have

a similar quaternary structure and maintain some of the critical enzymatic and other biological activities. However, these studies have also highlighted that they are capable of eliciting distinct responses from immune cells relative to endogenous human MIF. This indicates they may have additional or novel immunomodulatory properties. Comparative analysis of the human and parasite derived molecules may provide novel insights into the sites and mechanisms of action of these molecules.

1.4 Research aims and novel contributions.

The primary aim of this study is to develop an understanding of MIF's role within the intestinal immune environment with particular emphasis on colonic epithelial cells and macrophages one of the innate immune cells that form the initial immune response to pathogens and facilitate adaptive immune responses within the GT. Forming a significant part of this study is the production and comparative analysis of recombinant wild-type and tautomerase-deficient mammalian (human and murine) and *Trichinella spiralis*-derived MIF homologues to gain insight into the relatively unexplored biological relevance of the highly conserved enzymatic activities. This would allow for the identification of specific MIF domains as a therapeutic target.

In addition, part of this PhD work focused on developing a set of tools that would enable us to assess MIF's function as a modulator of key immune protein complexes, such as NF-κB, a critical regulator of both epithelial and macrophage driven responses. This includes the generation of several stable isogenic reporter cell lines derived from colonic cancer cell lines which upregulate expression of the fluorescent proteins GFP or mCherry in response to NF-κB activation. In addition, several other reporter cell lines, including HEK 293 cells

expressing GFP after activation of a Smad-binding element, or signal HEK 293 TLR-4 activation by secreted alkaline phosphatase were also used in conjunction with these MIF recombinants to explore its potential modulatory properties on these key innate signalling pathways.

Similarly, a second focus point for this work included the examination of the effects of recombinant mammalian and parasite MIF treatment on cytokine production by murine bone marrow derived macrophages and whole tissue colonic explants under normal conditions or conditions mimicking loss of barrier function or infection such as PAMP stimulation. To assess the relative contribution of the tautomerase activity to the biological activity of these enzymes, mutant recombinants were included as comparators.

Finally, to uncover additional insight into how MIF alters the transcriptome and molecular signalling pathways in innate immune cells RNA-sequencing analysis was used to measure changes in gene expression in murine bone marrow derived macrophages after treatment with mammalian and parasite derived MIF homologues. The analysis of these datasets has allowed additional novel links between MIF, its immunomodulatory activities and several key signal transduction pathways to be uncovered.

Chapter 2: Materials and Methods

2.1. Bacterial strains and plasmids

2.1.1. Growth of Bacterial strains

Bacterial strains were maintained on either LB agar plates or, for long-term storage, in 20% glycerol at -70°. Two bacterial strains were utilised within this study: TOP10 *E.coli* cells for propagation and stability of plasmids, and BL21 expression derivatives for induction and protein expression. All growth media is listed in table 2.1. Bacterial strains and plasmids used in this study are listed in table 2.2.

Table 2. 1 Media recipes used in this study.

Media	Component	w/v
LB broth	Bacto-Tryptone	10g/L
	Yeast Extract	5g/L
	NaCl	5g/L
	Distilled H_2O	1 litre
LB agar	As above + agar	15g/L
2 x YT broth	Bacto-Tryptone	16g/L
	Yeast extract	10g/L
	NaCl	5g/L
	Distilled H_2O	1 litre
2 x YT agar	As above + agar	15g/L
Tryptone Soy broth	Oxoid (CM0129)	30g/L
Tryptone Soy agar	As above + agar	15g/L

Table 2. 2 Genetic characteristics of strains and plasmids used in this study.

Strain or plasmid	Genotype		Source
Strains			
TOP10	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K λ⁻ *rps*L(Str^R) *end*A1 *nup*G		Guiliano lab
BL21(DE3)	F⁻ *omp*T *hsd*S$_B$ (r_B^-, m_B^-) *gal dcm* (DE3)		Guiliano lab
BL21-CodonPlus	F⁻ *omp*T *hsd*S(r ⁻ m ⁻) *dcm*$^+$Tet^r *gal end*A Hte [argU proL Cam^r]		Guiliano lab
Plasmids		**Inducer**	
pGEM-T EASY	Derivative of pGEM®-5Zf(+) vector, Amp^r		Promega (Cat no. A1360)
pET29b	Bacterial expression vector, contains C-terminal ^{6}Histidine-tag, Kan^r	IPTG	Merckmillipore (Cat no. 69872)
pIRES	Mammalian expression vector, Amp^r, Neo^r	-	Takarabio (Cat no. 631605)
pGro7	Contains chaperones groES-groEL, Cam^r	L-Arabinose	Takarabio (Cat no. 3340)
pKJE7	Contains chaperones dnaK-dnaJ-grpE, Cam^r	L-Arabinose	Takarabio (Cat no. 3340)
pG-Tf2	Contains chaperones groES-groEL-tig, Cam^r	Tetracycline	Takarabio (Cat no. 3340)
pTf16	Contains chaperones Tig, Cam^r	L-Arabinose	Takarabio (Cat no. 3340)
pG-KJE8	Contains chaperones dnaK-dnaJ-rpE groES-groEL, Cam^r	L-Arabinose, Tetracycline	Takarabio (Cat no. 3340)

Amp^r, ampicillin resistant; Cam^r, chloramphenicol resistant; Kan^r, kanamycin resistant.

2.1.2. Preparation of chemically competent TOP10 and BL21 E.coli cells.

TOP10 and BL21 *E.coli* cells were made chemically competent following an adaptation of the well-established Inoue protocol (Inoue *et al.*, 1990). A single bacterial colony was picked from an agar plate containing the bacterial competent cells, inoculated into 5mL of 2 x YT broth containing the appropriate antibiotics and incubated overnight at 37°C. Overnight cultures of *E.coli* were resuspended in 2xYT broth and grown to OD_{600} 0.5-0.7. Cells were collected by centrifugation at 4700 rpm at 4°C for 10 minutes. Ensuring that cells always remained on ice, pellets were resuspended in 80mL of RF1 buffer (100mM RbCl, 50mM MnCl2, 30mM KOAc, and 10mM CaCl2, 15% w/v glycerol, pH 5.8) and incubated on ice for 1 hour. Bacteria were centrifuged as described previously, resuspended in 40mL RF2 buffer (10mM MOPS, 10mM RbCl, 75mM CaCl2, and 15% w/v glycerol) and incubated on ice for an additional 15 minutes. 500 µL and 100µL cells were then aliquoted into cryotubes (pre-chilled at - 70°C) and immersed into a Dewar flask containing liquid nitrogen before being stored at - 70°C until required.

2.1.3. Transformation of *E.coli* TOP10 and BL21 cells

Aliquots of 500µL or 100µL of frozen *E.coli* TOP10 and BL21 competent cells were allowed to thaw on ice and 0.1-1µg plasmid DNA, or 10µL ligation reaction mixture, were added and gently mixed. After incubation for 15 minutes on ice, cells were heat-shocked in a water bath at 42°C for 45 seconds and then promptly transferred to ice for 5 minutes. 500µL of 2xYT broth were added to the cells and incubated at 37°C for 1 hour with constant agitation before being aliquoted on LB agar plates containing the appropriate antibiotic for selection of positive transformants.

2.2. Molecular Techniques.

2.2.1. RNA Isolation and purification.

2.2.1.1. Cells

RNA from U937, HT-29, HEK 293 WT, C57BL/6 splenocytes and C57BL/6 BMDM was isolated using a commercial RNA isolation kit (ISOLATE II RNA mini kit – Bioline). Briefly, C57BL/6 spleens were homogenised by being pressed between two frosted glass slides, pipetted up and down until a single cell suspension was achieved, and then centrifuged at 1500rpm.

Cell lines were washed in PBS to remove residual media and then centrifuged at 1500rpm. All pellets were resuspended in lysis buffer and β-ME and loaded into an isolate filter column and spun at 11,000 x g. Flow through was precipitated with 70% EtOH, put through an isolate mini column, centrifuged at 11, 000 x g. Flow-through was discarded and the remaining column membrane desalted in preparation for DNase I treatment. After DNA digestion, the column membrane was washed three times and then RNA eluted using 40µl RNase-free water. RNA was quantified using a NanoDropTM 1000 spectrophotometer and quality checked by determination of absorbance ratio at 260 nm:280 nm (2.0-2.3) and 260 nm:230 nm (>2.0).

2.2.1.2. Tissue

RNA from intestinal biopsies was isolated using a TRIzol Chloroform protocol with an additional column purification step (Zymo Research Direct-zol RNA MiniPrep kit). Briefly, three biopsies were placed into 500µl TRIzol (Thermo Fisher), homogenised using 1mm glass beads in a FastPrep-24 homogeniser (MP Biomedicals) for six cycles of 10 seconds at 6.5 m/sec each. 100µl Chloroform was added to each sample, vortexed briefly and left to incubate for 5 minutes at

room temperature before centrifuging a 11,000 x g for 15 minutes. The upper aqueous phase (approximately 200µl) containing total RNA was carefully removed to avoid protein contamination, an equal volume of 100% EtOH added and carefully mixed before loading into a Direct-zol column. Samples were centrifuged at 11,000 x g, flow through discarded and the membrane washed through with RNA wash buffer in preparation for DNase I digestion. After DNAse digestion, RNA isolation columns were washed through three times and RNA eluted using 50µl RNase free water.

2.2.1.3. DNase I treatment.

5µl (6 U/µl) of previously resuspended DNase I was mixed with 75µl DNA digestion buffer, gently mixed and 80µl added directly to the centre of the RNA isolation column. Samples were incubated at room temperature for 15 minutes before inactivating the digestion reaction by the addition of RNA wash buffer.

2.2.1.4. RNA clean-up.

Samples exhibiting a low 260 nm:230 nm (<2.0) and therefore deemed to have a high residual salt contamination were subjected to a clean-up procedure to prevent any inhibitory actions on the RT reaction. Briefly, RNA was precipitated by adding 1/10th total 3M Sodium Acetate plus three volumes of 100% EtOH and incubating at room temperature for 15 minutes. Following this, samples were centrifuged at 13,000 rpm for 10 minutes and the pellet washed with 1mL of 75% EtOH. If the pellet was dislodged samples were re-centrifuged at 13,000 rpm for 5 minutes, supernatant removed and then the pellet left to air-dry for 10 minutes at room temperature. Pellets were resuspended in 25µL RNase free water.

2.2.2. Reverse Transcription.

Reverse transcription was performed using a Sensifast cDNA synthesis kit (Bioline). Briefly, 1µg RNA was added to a master-mix containing 4µL 5x TransAmp buffer (50:50 oligo dT/random hexamers), 1µL Reverse Transcriptase and DNase/RNase free water added to a total volume of 20µL. The synthesis reaction was performed in a PCR thermocycler (Bio-Rad) at 25 °C for 10 min, 42 °C for 15 min and 85 °C for 5 min to inactivate the reaction. cDNAs were placed in -70 °C for long-term storage.

2.2.3. End-point PCR.

PCR reactions were performed in a PCR thermocycler (Bio-Rad). MIF sequences were amplified with overhangs for NdeI (CATATG) and XhoI (CTCGAG) restriction sites. Generally, PCR cycles included the following standard cycling conditions: Cloning - initial denaturing at 98 °C for 30 seconds; 30 cycles of denaturing at 98 °C for 5 seconds, primer annealing at 52 °C – 60 °C for 20 seconds, extension at 72 °C for 30 seconds; followed by a final extension period of 72 °C for 10 minutes. Reactions contained 10 µl 5X Phusion High Fidelity buffer (NEB), 5µl 2mM dNTPs (Thermo Fisher), 2.5µl 10µM forward primer (Eurofins), 2.5µl 10µM reverse primer, 0.5µl Phusion polymerase, 50ng DNA and up to 50 µl nuclease-free water. PCR for qualitative assessment of target DNA/cDNA- initial denaturing at 95 °C for 30 seconds; 30 cycles of denaturing at 95 °C for 20 seconds, primer annealing at 52 °C – 60 °C for 30 seconds, extension at 68 °C for 1 minute; followed by a final extension period of 68 °C for 5 minutes. Reactions included 2.5µl 10X Standard *Taq* reaction buffer (NEB), 2.5µl 2mM dNTPs (Thermo Fisher), 0.5µl 10µM forward primer, 0.5µl 10µM reverse primer, 0.125µl *Taq* DNA polymerase (NEB), 50ng DNA and up to

25µl nuclease-free water. Primers used in this study (Eurofins, UK) are listed in the appendix table A.1.

2.2.4. Detection of PCR products.

PCR products were electrophoresed on a 1.5% - 2.5% agarose gel composed of 1X TAE buffer with 0.01% SYBRSafe (ThermoScientific) at 100V for 30 – 40 minutes. To identify the size of generated PCR products samples were mixed with 4X loading dye (NEB) and run alongside a 2-log marker (NEB) before being visualised using a ChemiDoc MP T100 system (Bio-Rad).

2.2.5. Cloning of MIF sequences.

After confirmation that the correct size amplicon (350 bp) was generated, PCR products were subject to on-column purification using a Qiagen QIAquick PCR purification kit according to the manufacturer's protocol. Purified fragments were quantified using a NanoDropTM 1000 spectrophotometer and quality checked by determination of absorbance ratio at 260 nm:280 nm (2.0-2.3) and 260 nm:230 nm (>2.0). Amplicons were initially ligated, using TA cloning, into pGEM-T EASY (Promega) (figure 2.1) insert:vector ratio of 6:1 as calculated using the online NEB ligation calculator tool. Vector and insert were added to a ligation mix containing 5µl 2X Rapid Ligation Buffer, 1µl pGEM-T EASY vector, 1µl T4 DNA ligase, PCR product (variable) and nuclease-free water to 10µl and incubated at 4°C overnight.

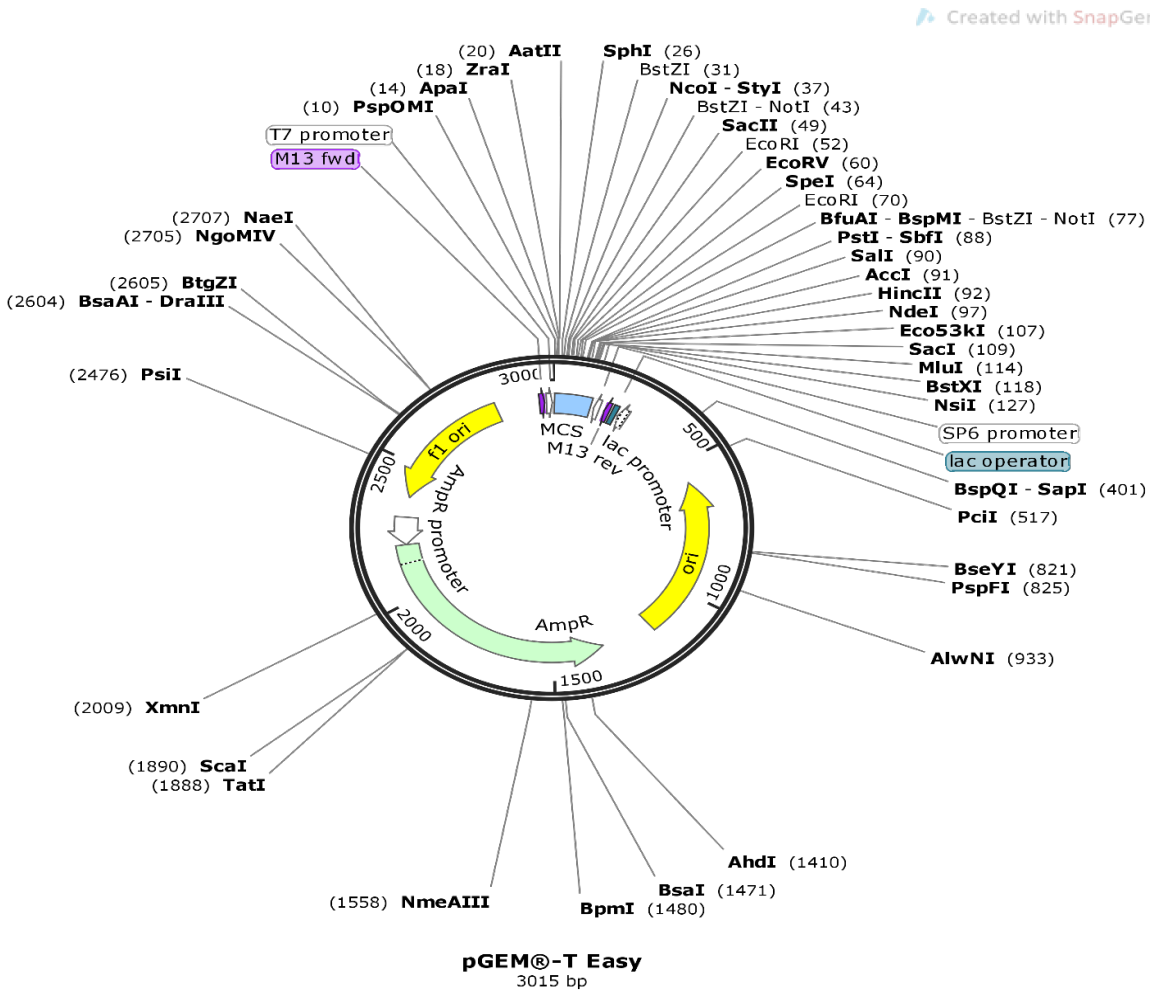


Figure 2. 1 pGEM-T Easy Vector Map

(https://www.snapgene.com/resources/plasmid-files)

Ligation reactions were subsequently propagated by transformation into chemically competent *E.coli* TOP10 cells. 50µl, 100µl or 150µl transformants were plated on LB agar containing Ampicillin, IPTG and x-gal to allow for selection using white/blue screening. Positive colonies were selected, re-plated and presence confirmed by colony screening using conventional PCR. Plasmids were purified using a Monarch Plasmid Miniprep Kit (NEB) prior to sequencing by Dundee Core Sequencing Facility.

To express proteins, MIF sequences were sub-cloned into pET29b expression vector which contains an endogenous ^{6}His-tag sequence (figure 2.2). Briefly, MIF sequences were digested from the pGEM-T EASY vector using restriction enzymes NdeI and XhoI and ligated to a previously digested pET29b using T4 ligase (NEB). Ligations to pET29b were performed at 16°C overnight and then heat inactivated at 65°C for 5 minutes. pET29b including MIF sequences were transformed into BL21-CodonPlus cells in preparation for protein expression. Full sequences including accession numbers can be found in table A.2.

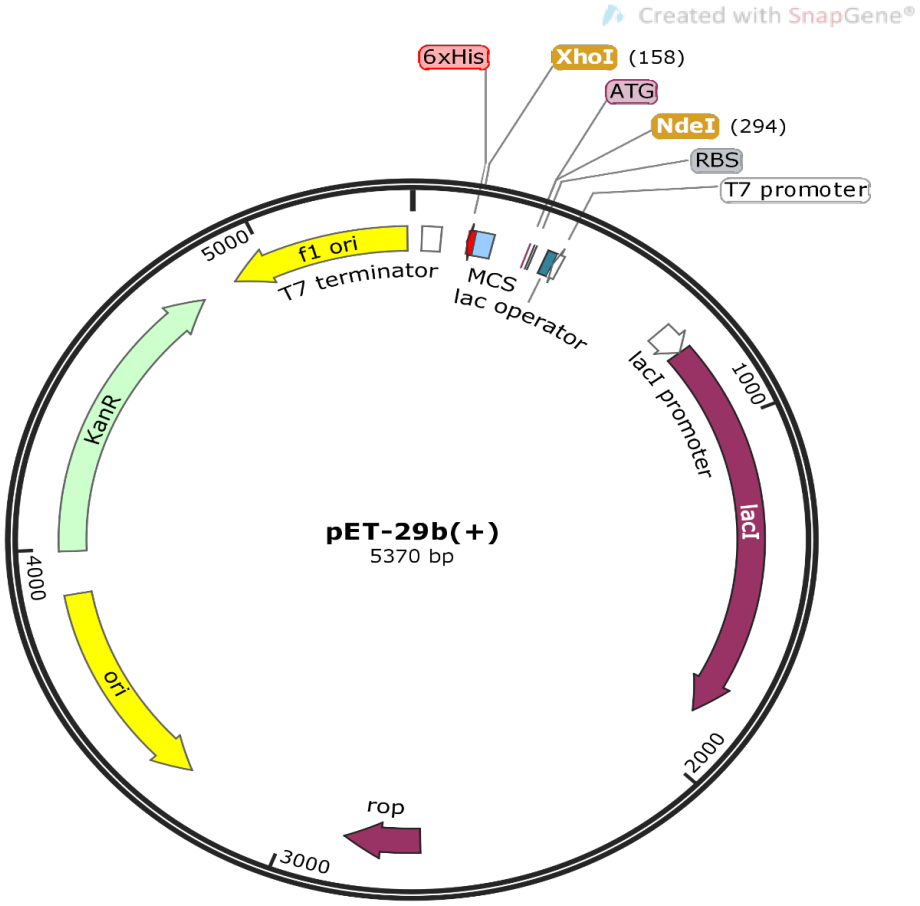


Figure 2. 2 pET29b Vector Map

(https://www.snapgene.com/resources/plasmid-files)

2.2.6. Cloning of pIRES_CD74 and pIRES_CD74/CD44.

Cloning of CD74 and CD44 containing restriction sites for NheI/XhoI and SalI/NotI, respectively, into pIRES was performed by Eurofins, UK. Briefly, sequences for CD74 (accession number: NM_004355.3) and CD44 (accession number: AY101193.1) were selected based on publications which confirmed the presence of these receptors on the cell surface (further discussed in chapter 4). pIRES is biscitronic vector containing two multiple cloning sites allowing for the simultaneous expression of two genes of interest (figure 2.3). The presence of CD74 and CD44 inserts were confirmed using qualitative endpoint PCR and visualising products on a 1.5% agarose gel.

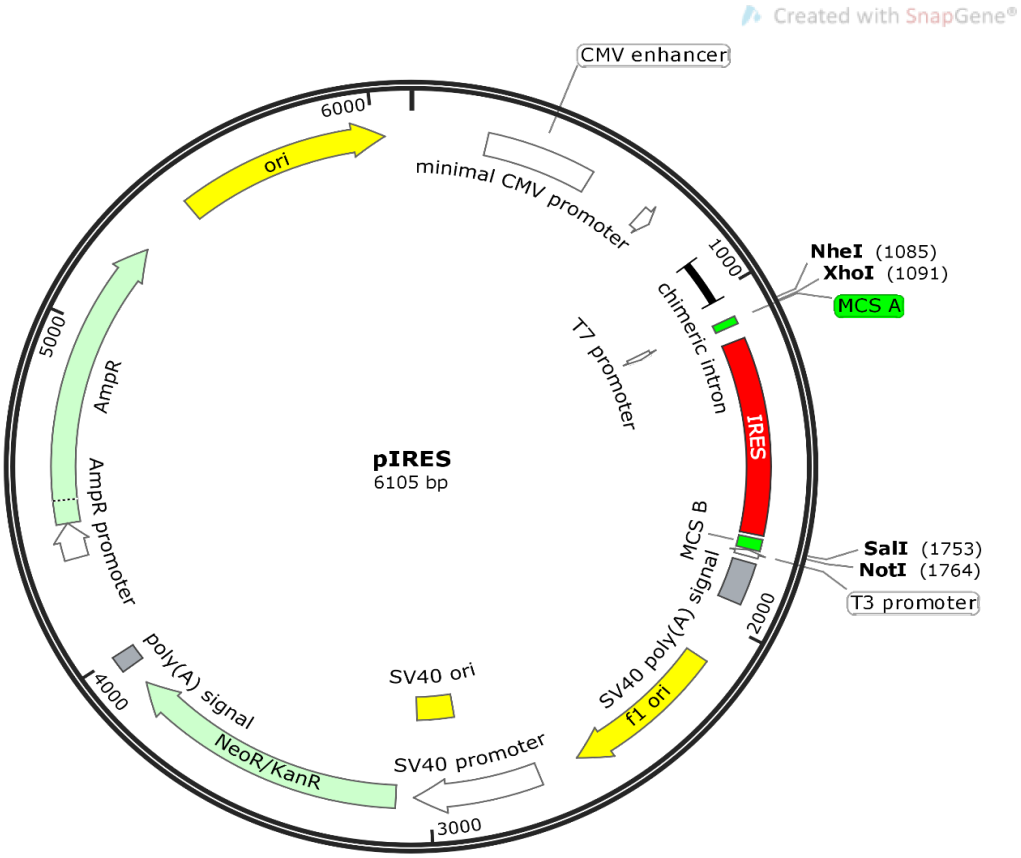


Figure 2. 3 pIRES Vector Map.

(https://www.snapgene.com/resources/plasmid-files)

2.2.7. Real-Time PCR – relative quantification.

cDNA from HEK 293 WT, HEK SBE-isogenic cells, C57BL/6 intestinal explants and BMDMs was utilised in assays to assess transcriptional changes using an Agilent Aria Mx Realtime PCR system. In initial assays, a titration for all primers, listed in the appendix table A.3. was utilised in order to firstly confirm the presence of a specific transcript and, secondly, to determine the correct primer concentration to use in the subsequent qPCR reaction. All products were run and detected on a 2.5% agarose gel. Following reaction condition optimization, qPCR analysis was performed using a SYBRgreen mastermix (Bioline) whereby a final amount of 10ng cDNA was used as template assuming that the RNA to cDNA conversion was 100%.

The efficiency of all reactions was obtained by generating a standard curve and deemed suitable when between 90-110%. Briefly, a series of cDNA dilutions at 0ng, 0.1ng, 1ng, 10ng and 100ng were added to the reaction containing primers and the mastermix. The efficiency of reactions was generated automatically by the Aria Mx software. Relative quantification was deduced using the following well-documented Pfafll equation: $Ratio = \frac{(Etarget\ \Delta Ct)\ (control-treated)}{(Ereference\ \Delta Ct)\ (control-treated)}$

2.2.8. RNA seq.

The quality of RNA samples to be sequenced was assessed using a bioanalyzer 2100 (Agilent) and deemed of sufficient quality as all samples had a RNA Integrity Number (RIN) of >9. Following this, mRNA capture, RNA fragmentation and cDNA synthesis was performed using a KAPA Stranded mRNA-Seq Kit (KAPA Biosystems). Samples were subsequently assigned a unique molecular identifier to prevent PCR amplification bias prior to sequencing. Libraries were subject to quality control procedures including gel electrophoresis (TapeStation, Agilent)

and quantification (Qubit, Thermo Fisher). RNA sequencing analysis was achieved using an Illumina NextSeq 500 single end run at 15M reads per sample.

2.2.9. RNA seq analysis.

Basic RNA seq analysis was performed by Dr Tony Brooks at the UCL genomics facility. Briefly, reads were aligned to the genome using the well-established RNA seq aligner, STAR (Dobin *et al.*, 2012). Subsequently, quality control and filtering/adapter trimming of all reads was performed using the Fastp preprocessor (Chen *et al.*, 2018). To test for differential expression between the groups Mm-MIF-1, Mm-MIF-1 P2G, Ts-MIF-1 and Ts-MIF-1 P2G, the reference group was set to control (untreated) and analysed using the R vignette, DESeq2 (Love *et al.*, 2014). Results were filtered to include those genes with a log2 fold change <1.5 or >1.5 and with an adjusted p value of < 0.05. Heatmaps were generated using the complex heatmaps vignette in R (Gu *et al.*, 2016) and all gene ontology analysis was performed using the widely established DAVID online software (Dennis *et al.*, 2003).

2.3. Protein expression

2.3.1. Expression of HsMIF-1, HsMIF1 P2G, MmMIF1 P2G, TsMIF1, TsMIF1 P2G.

In order to express functional MIF proteins, inserts were digested from pGEMt using restriction enzymes Nde1 and Xho1 and ligated overnight, at 16°, to a previously digested pET29b (Novagene) expression vector containing a sequence for a Histidine tag. pET29b containing MIF inserts were transformed into *E.coli* protein expression strain BL21-CodonPlus. Seed cultures were grown overnight in Tryptic Soy broth containing 50µg/mL Kanamycin and 34µg/mL Chloramphenicol, in a 37°C shaking incubator at 230 rpm. These were then sub-

cultured at a 1:100 dilution and allowed to grow to an OD^{600} 0.6 before being induced with 1mM IPTG for six hours at 37°C. Post-induction, cultures were spun down at 4000 rpm for 10 minutes and pellets were resuspended in PBS before being re-centrifuged at 4000 rpm for 10 mins. Following this, pellets were either resuspended in His-tag purification binding buffer (20mM Sodium Phosphate, 500mM Sodium Chloride, 20mM Imidazole, pH 7.4.) in preparation for sonication on ice, or incubated in lysis buffer (20mM Sodium Phosphate, 500mM Sodium Chloride, 20mM Imidazole and 0.5% NP-40) for 30 minutes on a rocker at 4°C. Subsequently, lysates were spun at 4000 rpm for 20 minutes.

2.3.2. Expression of Hs-MIF-1 C57S/C60S, Hs-DDT-1, Mm-DDT-1, Ts-MIF-2.

To obtain soluble extracts for Hs-MIF-1 C57S/C60S, Hs-DDT-1, Mm-DDT-1 and Ts-MIF-2, several adapted and optimised conditions were used. Briefly, for Hs-MIF-1 C57S/C60S, Hs-DDT-1, Mm-DDT-1 expression was induced once the OD^{600} reached 0.8 with 100µM IPTG. Additionally, cultures were incubated at 15°C at 150 rpm for 12 hours.

Ts-MIF-2, could not be found in the soluble protein fraction of BL21 CODON plus cells so several chaperonin cell lines (table 2.2), purchased from Takarabio, were tested (Chen *et al.*, 2018) and Ts-MIF-2 was found to express and be present soluble fraction of BL21 pGRO7. pGRO7 co-expresses the *E.coli* chaperonin GroEL/ES in the presence of L-Arabinose. Overnight cultures were diluted 1:50, 2mg/mL L-arabinose was added to ensure high level expression of GroEL/ES, cells were grown to OD^{600} prior to induction with 200µM IPTG. Following this, cultures were grown at 25°C, 180 rpm for 10 hours.

Table 2. 3 List of Takara Chaperonin plasmids used for protein expression.

Plasmid	Chaperone	Resistance	Chaperone Inducer
BL21-CodonPlus	-	Cm	-
pGRO7	groES-groEL	Cm	L-Arabinose
pGTf2	groES-groEL-tig	Cm	Tetracycline
pTf16	Tig	Cm	L-Arabinose
pGKJE7	dnaK-dnaJ-grpE	Cm	L-Arabinose
pGKJE8	dnaK-dnaJ-rpE groES-groEL	Cm	L-Arabinose, Tetracycline

2.3.3. SDS-PAGE Gel electrophoresis.

A 15% denaturing polyacrylamide gel was used to resolve all MIF proteins in TGS buffer (25 mM Tris, 192 mM glycine, 0.1% SDS). Resolving gel was composed of 375mM Tris-HCl, pH 8.8, 0.1% SDS, 0.05% ammonium persulfate and 0.1% TEMED whilst the stacking gel was 4% acrylamide, 125mM Tris-HCl, pH 6.8, 0.1% SDS, 0.05% ammonium persulfate and 0.1% TEMED. Protein lysates were OD corrected in a previous step to ensure an equal amount of protein across samples. Protein lysates were mixed with Laemmli buffer (Bio-Rad), including 20mM β-ME, in the following ratios: Total and insoluble fractions – pellet resuspended in 1X Laemmli; soluble – 1-part lysate to 3 parts 4X Laemmli buffer. Following this, samples were heated at 95°C for 5 minutes before loading into the gel. All protein gels were run at 100V for 45 minutes allowing complete separation of proteins and the Precision Plus pre-stained standard (Bio-Rad).

2.3.4. Coomassie Staining.

Proteins were initially detected using a Coomassie staining procedure prior to antibody-probing. Briefly, gels were incubated in a 0.25% Coomassie solution (0.25g Coomassie Brilliant Blue R250, 50% MeOH, 10% glacial acetic acid) rocking overnight at room temperature. Excess Coomassie was removed by

washing the stained gel using a de-staining solution (50% MeOH, 10% glacial acetic acid) and visualised using a ChemiDoc MP T100 imaging system (Bio-Rad).

2.3.5. Western Blot.

Resolved proteins were transferred to a nitrocellulose membrane (0.2 µm pore size, GE Lifesciences) using a Transblot Turbo Transfer System (Bio-Rad). Briefly, gels were sandwiched between two blotting pads and a nitrocellulose membrane and the stack pre-soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol) for 3 mins before being placed into the Transblot cassette and run at 25V for 8 minutes. Membranes were immediately placed into a blocking solution of 5% w/v non-fat powdered milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween 20), rocking for one hour at room temperature. Membranes were washed five times with TBS-T after blocking and then incubated overnight with an HRP conjugated monoclonal anti-His-tag antibody, used at 1:3000 dilution in 5% powdered non-fat milk in TBS-T. Membranes were washed as described previously and proteins detected and visualised using Clarity Western ECL substrate (Bio-Rad) in a ChemiDoc MP T100 imaging system (Bio-Rad).

2.4. Protein Purification.

2.4.1. Preparation of samples for purification.

Frozen pellets of bacterial cultures were resuspended in lysis buffer at 5mL per 1g wet weight and briefly vortexed to mix. Lysis buffer was selected over sonication methods due to how time consuming the sonication process was when numerous samples were involved. A direct comparison of MIF's enzyme activities was performed to ensure there was no evidence of protein denaturation.

Samples were incubated for 30 minutes on a rocker at 4°C, before being centrifuged at 4000 rpm for 20 minutes at 4°C to separate the soluble and insoluble fractions. Ts-MIF-2 could not be detected using this method so an alternative method was employed. Ts-MIF-2 was resuspended in 10mL His-tag binding buffer and sonicated on ice using 20 second pulses/20 second rest at 38% for a total period of 5 minutes. Following this, lysates were centrifuged at 4000 rpm for 20 minutes at 4°C ready for purification.

2.4.2. Ni-NTA purification.

Protein lysates were purified using a Protino Ni-NTA 1mL column (Macheray-Nagel). Prior to injection of the sample onto the column, using the ÄKTAprime plus (GE Life Sciences), His-tag binding buffer and His-tag elution buffer were washed through loops A and B at a 1mL/min flow rate, respectively, to ensure efficient binding of sample to the matrix. Sample injection was set at 0.5mL/min in order to increase the efficacy of sample binding then increased back to 1mL/min for washing and elution (precise breakpoints can be seen in table 2.4). Protein fractions were collected in 1mL fractions and analysed on a 15% SDS PAGE gel.

2.4.3. Buffer exchange and sample concentration.

His-tag purified fractions were collected and concentrated using a Vivaspin 20 Ultrafiltration Unit (Sartorius) with a molecular weight cut off of 3,000 Da. In addition to this, Vivaspin units were utilised to buffer exchange the samples allowing them to be subject to the final purification anion-exchange step. Briefly, 20mL lysates were loaded into the Vivaspin tubes and centrifuged at 4000 rpm for a total period of 90 minutes at 4°C with intermittent pipetting every 10 minutes to prevent blockage of the PES membrane. Following concentration of the sample from 20mL to 1mL, excess salts were removed by the addition of 15mL

Tris 50mM, pH 8.0 to allow for buffer exchange. This step was repeated three times ensuring all samples were compatible with anion exchange chromatography.

2.4.5. Anion exchange chromatography.

Anion exchange chromatography was performed using Pierce strong anion exchange columns in order to remove residual endotoxin from samples. Briefly, the anion exchange spin column was equilibrated using 50mM Tris buffer, pH 8, before concentrated MIF samples were applied to the column, spun at 7,000 rpm for 10 minutes and purified flow-through collected.

2.4.6. BCA assay.

Quantification of purified proteins was performed utilising a BCA assay (Thermo Scientific Pierce) which measures protein driven reduction of Cu^{+2} to Cu^{+1}. Briefly, a series of BCA protein standards from 0µg/mL – 2000 µg/mL were prepared in order to generate a standard curve. Purified protein samples were diluted 1:5, 1:10 and 1:20 and 10µL of each sample and standard added to a microtitre plate. Subsequently, 50-parts BCA reagent A were added to 1-part BCA reagent B and mixed to form the working BCA reagent; 190µL was added to all wells and incubated at 37°C for 30 minutes before being quantified spectrophotometrically at 562nm using a microplate reader (Synergy HTX, BioTek).

2.4.7. Limulus amebocyte lysate assay.

The quantification of residual bacterial endotoxin contamination in the purified recombinant proteins was performed using a LAL Chromogenic Endotoxin Quantitation Kit (Pierce). Briefly, a series of endotoxin standards between 0.1 – 1 EU/mL were prepared to act as reference point for sample endotoxin

concentrations. Samples were diluted 1:10 and 1:100 prior to being added to a microtitre plate at a volume of 50µL along with the standards and negative controls consisting of endotoxin-free water and 50mM Tris buffer (pH 8.0), 10% glycerol. 50µL of LAL reagent was added to all wells and plates incubated at 37°C for 10 minutes. Subsequently, 100µL of prewarmed chromogenic substrate was added and plates returned to incubate at 37°C for 6 minutes. Finally, 100µL stop solution (25% acetic acid) was added to halt the reaction before being analysed spectrophotometrically at 410nm using a microplate reader (Synergy HTX, BioTek).

2.5. Enzyme Assays

2.5.1. Tautomerase Assay.

The tautomerase activity of all MIF proteins was determined as previously described (Melissa Swope *et al*., 1998). Equal volumes of L-dopachrome methyl ester (10mM) and sodium periodate (20 mM) were mixed and incubated for 5 min to form the L-dopachrome methyl ester required for the assay. Tautomerase enzymatic activity was measured in 96-well microtitre plates containing a reaction mix of 25 mM potassium phosphate, 0.2% Tween 20, pH 6.0. 160ul of buffer was mixed with 20µl of L-dopachrome methyl ester. *T. spiralis,* murine or human MIF was added at a final concentration of 20µg/mL as this was found to have the most efficient rate of reaction. In some reactions MIF inhibitor (4-Iodo-6-phenylpyrimidine) was added at the start of the reaction at a final concentration of 25µg/mL. MIF-catalysed reduction of absorbance was monitored over time spectrophotometrically at 475nm.

2.5.2. Oxidoreductase Assay.

Oxidoreductase activity of Hs-MIF-1, Hs-MIF-1 C57S/C60S, Mm-MIF-1 and Ts-MIF-1 was determined using the well-established insulin reduction assay which leads to an increase in turbidity measured at 650nm. PDI-catalysed reduction of Thioredoxin was used as a positive control.

Oxidoreductase activity was measured in 96-well microtitre plates containing a reaction mix of 25 mm potassium phosphate, 0.2% Tween 20, pH 6.0. 160ul of buffer was mixed with 20ul of L-dopachrome methyl ester. Ts-MIF-1, Mm-DDT-1, Hs-DDT-1, Hs-MIF1-C57S/C60S was added at a final concentration of 20µg/mL as this was deemed to have the most efficient rate of reaction. MIF-catalysed reduction of absorbance was monitored spectrophotometrically over a time period of 60 minutes.

2.6. Cell Culture.

2.6.1. Reagents.

LPS (Salmonella enterica serotype typhimurium, L6143) was purchased from Sigma-Aldrich (now Merck). TNF-α, IFN-γ, TGF-β and rHMIF1 were all purchased from Biolegend UK.

2.6.2. Cells.

Cell culture media components used in this study are listed in table 2.3. Colorectal adenocarcinoma cells - Caco2, HT-29 and HT-29 NFκB-mCherry cells, embryonic kidneys cells - HEK 293 WT, HEK-Blue™-hTLR4, HEK 293 NF-κB-mCherry, NFκB-mCherry and HEK SBE-GFP cells were maintained in complete DMEM. Bone-marrow-derived macrophages were cultured in BMDM differentiation media. Isolated cells were seeded at 6 x 10^6 cells/petri dish in 10mL of PBMC media. On day 3 and 5, 10mL of fresh medium were added.

After 7 days cells were analysed for F4/80 expression using flow cytometry (BD Celesta). All cells were maintained in a 37° incubator under 5% CO_2. Cell lines were repeatedly checked for low-lying bacterial contamination by culturing in antibiotic-free media.

Table 2. 4 Cell culture media used in this study.

Media	Components
Complete DMEM	DMEM, high glucose, GlutaMAX(TM), pyruvate. 10% Heat inactivated FCS. 1% Penicillin/Streptomycin
Complete RPMI	RPMI 1640 10% Heat inactivated FCS 1% Penicillin/Streptomycin
HT-29 NFκβ-mCherry media	DMEM, high glucose, GlutaMAX(TM), pyruvate. 30% HT-29 conditioned media 10% FCS 1% Penicillin/Streptomycin
HEK-Blue™-hTLR4 media	DMEM, high glucose, GlutaMAX(TM), pyruvate. 10% Heat inactivated FCS 100µg/mL Zeocin 50µg/mL Blastocidin 1% Penicillin/Streptomycin
BMDM differentiation media	RPMI 1640 30% L-cell conditioned media 20% Heat inactivated FCS 1% Penicillin/Streptomycin

2.6.3. Generation of Bone Marrow Derived Macrophages.

Bone marrow cells from femurs of C57BL/6 mice were used to generate BMDMs using batch tested L929-cell conditioned medium as a source of macrophage colony stimulating factor. The cells were resuspended in 10 mL BMDM differentiation media at a density of 5 X 10^6 cells per non-tissue culture treated

petri dish. Cells were incubated at 37°C in a 5% CO_2 atmosphere. One day after seeding the cells, an additional 10 mL of fresh bone marrow differentiation media was added per plate and cells were incubated for an additional 3 days. At day 3 and 5, 10mL was removed and replaced with fresh bone marrow differentiation media. To obtain the BMDMs, the supernatants were carefully aspirated, and the attached cells were washed with 10 mL of sterile PBS and placed on ice for 2 minutes whilst scraping with a disposable plastic scraper. The cells were centrifuged at 1800 rpm for 5 minutes and resuspended in 2 mL of RPMI media, counted and assessed for F4/80 expression.

2.6.4. Generation of stable hTLR4-NF-κB-mCherry, HT-29-NF-κB-mCherry and HEK-SBE-eGFP reporter cell lines.

Polyclonal HEK 293 or HT-29 cells stably transduced with pHRSIN-SBE-eGFP or pHRSIN-NF-κB-mCherry (Breckpot *et al*., 2010a) reporter constructs were provided by D.B. Guiliano. In order to isolate isogenic single cell clones, cells were plated at 0.5 cells per well in a 96-well plate, using either complete DMEM or HT-29 NFκB-mCherry media, in order to ensure the growth of a single cell. Cells were periodically assessed for expression of GFP/mCherry using a BD FACS Celesta flow cytometer (BD Biosciences) and six positive single cell clones were subsequently expanded and stored in the liquid nitrogen storage facility for later use. The isogenic cell line with the greatest fold change and lowest background expression, after treatment with 2.5ng/mL TGF-β (SBE-eGFP) or 50ng/mL TNF-α (NFκB-mCherry) stimulation, were used in future reporter assays.

2.6.5. Transient Transfections of pIRES_CD74 and pIRES_CD74/CD44.

HEK 293 cells were seeded at 3 x 10^4/0.5mL in a 24 well plate using complete DMEM and allowed to adhere overnight prior to transfecting. Following this, a

titration of vector DNA (pIRES):carrier DNA (pBluescript_empty), to a total amount of 750ng DNA, was prepared to assess the optimum conditions for CD74 and CD44 expression. Briefly, varying amounts of pIRES plasmid DNAs were added to jetPRIME buffer (see table 2.4) and vortexed to mix. The jetPRIME reagent was subsequently vortexed and centrifuged at 11,000g for 1 min before adding 1.5µL to the DNA mix. Reactions were incubated for 10 minutes at room temperature before 50µL was added drop-wise to each well and gently rocked to ensure complete coverage. Plates were incubated for 5 hours before removing the transfection media and adding fresh complete DMEM and allowing cells to rest for an additional 24 hours prior to analysis for cell surface expression of CD74 and CD44 using flow cytometry (BD FACSCelesta). Antibodies used were PE-CD74 and PE-Cy7 CD44 (Biolegend).

Table 2.5 DNA (ng) used to transfect WT HEK 293 cells.

pBluescript (ng)	pIRES_CD74 or pIRES CD74_CD44 (ng)	jetPRIME buffer (µL)	jetPRIME reagent (µL)
0	750	75	1.5
250	500	75	1.5
500	250	75	1.5
750	0	75	1.5

2.6.6. HT29-NFκB-mCherry reporter assay.

HT29-NFκB-mCherry isogenic cells, IC5, were seeded at 5 x 10^5/mL and 0.5mL added to all wells of a 24-well plate. Following an 8-hour period of settlement cells were primed with 10ng/mL IFN-γ for 12 hours before removing the supernatant and replacing with fresh complete DMEM containing 100ng/mL LPS or 100ng/mL LPS and 100ng/mL Hs-MIF-1 and incubated for 20 hours before assessing mCherry expression using a BD FACSCelesta. 50ng/mL TNF-α was used as a positive control.

2.6.7. HEK-Blue™-hTLR4 reporter assay.

HEK-Blue™-hTLR4 (Invivogen) cells, between passage number 9 and 20, were seeded at 5 x 10^3 cells/100µL in a 96 well plate and allowed to adhere for 2 hours before the addition of 10ng/mL LPS and coadministration of 10ng/mL LPS and 100ng/mL Hs-MIF-1, 100ng/mL Hs-MIF-1 P2G, 100ng/mL Mm-MIF-1, 100ng/mL Mm-MIF-1 P2G and 100ng/mL Ts-MIF-1. Optimal LPS concentrations were previously determined using a dose response curve (S. Paraliker, 2017, unpublished) whilst MIF concentrations and experimental times were determined using previously published data by Kudrin *et al* (2006). Plates were incubated for 18 hours before being assessed for secreted alkaline phosphatase utilising a well-documented p-Nitrophenol phosphatase assay. 100 µL of freshly prepared pNPP (as before) reaction mix was added to 50 µL of each sample and the plates were incubated at 37°C for 20 minutes and 5% CO2. Reactions were assessed spectrophotometrically at OD^{405} using a microplate reader (BioTek, Synergy™HTX).

2.6.8. HEK-hTLR4-NFκB-mCherry reporter assay.

Isogenic HEK-hTLR4-NFκB-mCherry cells were cultured as described previously (2.7.2) and cultured with 10ng/mL LPS or 10ng/mL LPS and 100ng/mL Hs-MIF-1, 100ng/mL Hs-MIF-1 P2G, 100ng/mL Ts-MIF-1, 100ng/mL Ts-MIF-1 P2G and 100ng/mL Ts-MIF-2 before being assessed for mCherry expression using a BD FACSCelesta. Optimal LPS and MIF concentrations and experimental times were determined as previously mentioned (2.6.7).

2.6.9. TGF-β and BMP-4 timecourse assay.

Isogenic clone IC3 HEK-SBE-eGFP cells were seeded in triplicates at 3 x 10^3 cells per well in a 96 well plate and allowed to adhere overnight in a 37°C, 5% CO_2 incubator. Following this, 2.5ng/mL rTGF-β or 2.5ng/mL rBMP-4 (Biolegend)

was administered and cultured for 4, 8, 12 or 24 hours before assessing the expression levels of eGFP using a BD FACSCelesta.

2.6.10. TGF-β and MIF assay.

Isogenic clone IC3 HEK-SBE-eGFP cells were seeded and incubated as mentioned in section 2.7.5.1. Following this, 2.5ng/mL TGF-β or 2.5ng/mL TGF-β and MIF homologues were added to wells and incubated for 18 hours before assessing the levels of eGFP expression using a BD FACSCelesta.

2.6.11. LPS and MIF BMDM assay.

BMDMs were plated at 1 x 10^6 cells/mL in a 24 well plate before being allowed to rest overnight without L292-conditioned media prior to further experimental procedure. After overnight incubation in a 37°, 5% CO_2 incubator, 10ng/mL LPS or 10ng/mL LPS and 100ng/mL Mm-MIF-1, 100ng/mL Mm-DDT-1 100ng/mL Mm-MIF-1 P2G, 100ng/mL Ts-MIF-1, 100ng/mL Ts-MIF-1P2G or 100ng/mL Ts-MIF-2 were co-administered and incubated at 37° for 20 hours. After this time, supernatants were stored at -20 for future cytokine analysis whilst cells were trypsinised and resuspended in RNA later at -20° for mRNA analysis. Optimal LPS and MIF concentrations and experimental times were determined as previously mentioned (2.6.7).

2.6.12. *Ex vivo* intestinal explant assay.

Intestinal biopsies were taken from the ascending, transverse and descending colon of C58BL/6 female mice in order to obtain results representative of the entire colon. Punch biopsies were 3mm^2 (Miltex) and three biopsies were seeded, in complete RPMI, per well in a 24 well plate. Explants were stimulated with 100ng/mL LPS in the presence or absence of 100ng/mL Mm-MIF-1, 100ng/mL Mm-MIF-1 P2G, 100ng/mL Ts-MIF-1, 100ng/mL Ts-MIF-1 P2G or

100ng/mL Ts-MIF-2 and incubated at 37° for 24 hours. After treatment, supernatants were stored at -70 for future cytokine analysis whilst tissue was carefully removed using tweezers and placed in RNA later at -20°C for transcriptomic analysis. LPS and MIF concentrations were determined using previously published data (Suzuki *et al.*, 2003; Kudrin *et al.*, 2006).

2.7. Immunological assays.

2.7.1. ELISA

Supernatants from experimental intestinal explant cultures or BMDM's were assessed for secretion of cytokines utilising ELISA assays for TNF-α, IL-6 and IL-22 (Thermo Fisher). Briefly, plates were coated with 100µL capture antibody and allowed to incubate overnight at 4°C before washing anyway any unbound antibody using ELISA wash buffer (1 x PBS and 0.05% Tween 20). A series of standards were prepared according to manufacturer's instructions, and experimental samples diluted 1:10 or 1:20 before adding 100µL of each to the microtitre plate. Plates were incubated as previously described to allow for the optimum sensitivity. After 24 hours, plates were washed five times with washing buffer to remove excess standard and sample material before adding 100µL detection antibody to each well and incubating at room temperature for 1 hour. Plates were washed five times before 100µL of Avidin-HRP was added to each well and incubated for 30 minutes at room temperature before being washed as before. 100µL of substrate solution was added to each well and left to incubate for 15 minutes at room temperature before 50µL stop solution (1M phosphoric acid) was added to inhibit further reactions. Cytokine levels were quantified spectrophotometrically at 450nm using a microplate reader (Synergy HTX, BioTek).

2.7.2. Flow cytometry.

All samples were analysed using a BD FACS Celesta and analysed in FlowJo.

2.7.2.1. Antibody staining.

HEK 293 cells and BMDMs were washed with 1 x PBS before the addition of 50uLTrypsin to each well or scraping with a 1mL pipette tip (BMDM). Plates were incubated at 37°C for 5 minutes and then complete 150uL complete was DMEM added to stop further trypsinization. Plates were spun at 1800 rpm for 5 minutes at 4°C, supernatants removed, and cell pellets washed twice with 1 x PBS. Following this, cells were resuspended in 100uL FACs staining buffer containing Fc receptor block CD16/CD32 (eBioscience) and incubated on ice for 10 minutes. After this, cells were resuspended in 100uL freshly made FACS buffer (2% FCS, 1 x PBS, 1mM EDTA) to which antibodies were added at the specified concentration (table 2.5). Plates were incubated on ice for 60 minutes in the dark before being washed twice with FACS staining buffer. The cell pellets were resuspended in 200uL FACS staining buffer before being analysed for CD74-PE or CD44-PECy7 (HEK 293) or F4/80 – Brilliant Violet 421 (BMDM).

Table 2.6 Antibodies used in this study.

Antibody	Supplier	Volume used (per 1µL FACS buffer)
CD74-PE	Biolegend	5µL
CD44-PECy7	Biolegend	5µL
F4/80-Brilliant Violet 421	Biolegend	1µL
CD16/32	eBioscience	1µL

2.7.2.2. Detection of fluorescent proteins.

Following treatment with trypsin or scraping, HT-29 NF-κB-mCherry, hTLR4-NF-κB mCherry and HEK-SBE-eGFP cells were washed twice with 1 x PBS before

being resuspended in 500uL FACS buffer and strained through a 70μM cell strainer prior to running through the BD FACSCelesta.

2.7.3. Phagocytosis Assay.

BMDMs were seeded at 1 x 10^6/mL using 0.5mL per well in a 24-well plate and left to adhere for 2 hours in a 37°C, 5% CO_2 before the addition of 10ng/mL LPS or 10ng/mL LPS and 100ng/mL Mm-MIF-1, 100ng/mL Mm-MIF-1 P2G, 100ng/mL Ts-MIF-1, 100ng/mL Ts-MIF-1 P2G and 100ng/mL Ts-MIF-2. Stimulated BMDMs were incubated for 20 hours prior to incubation with Zymosan A Alexa Fluor 488 particles (Thermo Fisher). Briefly, Zymosan particles were resuspended in 1 x PBS and sonicated for 3 x 20 seconds to create a homogenous suspension. Particles were centrifuged at 11000 rpm for 5 minutes before being resuspended in mouse serum and incubated at 37°C for 1 hour to augment opsonisation before being washed 3 times with sterile 1 x PBS. Zymosan particles were resuspended in complete RPMI before being added to cells at a particle:macrophage ratio of 20:1 and plates spun at 1000 rpm to ensure particles were at the bottom of each well. Following an incubation period of 45 minutes at 37°C plates were briefly incubated at 4°C for 3 minutes to stop further phagocytosis. Cells were washed 3 times with 1 x PBS followed by the addition of 200μL Trypsin incubated at 37°C for 5 minutes to remove non-internalised Zymosan A particles. Finally, cells were removed by scraping with a 1mL pipette tip and resuspended in 500μL FACS buffer being strained through a 70μM strainer and analysed for Alexa Fluor 488 expression using a BD FACSCelesta.

2.9. Statistical Data Analysis

Prior to inferential statistical tests, all data were tested for normality using the D-Agostino Pearson Omnibus Normality and Kolmogornov-Smirnov tests. Student T-Test and ANOVA were utilised to infer whether differences between groups

were statistically significant. ANOVA tests were further analysed using the Tukey or Dunn post-hoc test in order to make judgements regarding differences within groups. Data was considered statistically significant at the following values: *=<0.05, **=<0.01, ***=<0.001 and ****=<0.0001.

Chapter 3: Cloning and Characterisation of Mammalian and Helminth-derived Homologues of MIF.

3.1. Introduction.

3.1.1. MIF's Protein Structure and Enzymatic Activity.

MIF is a 12.5-kDa, 114 amino acid polypeptide, widely expressed in vertebrates and is a fundamental regulator of innate immune responses. Aside from the canonical mammalian MIF-1 a number of other MIF paralogues have been isolated. In mammals a MIF homologue, D-dopachrome tautomerase, D-DT/MIF-2, was identified after the discovery that B cells from CD74 -/- mice display a pronounced susceptibility to apoptosis when compared to MIF deficient B cells (Gore *et al*., 2008). MIF and D-DT are ligands for the MIF receptor, CD74 (Meza-Romero *et al*., 2016); both upregulate MAPK/ERK (1/2) activity (Merk *et al*., 2011a); and lead to the expression and secretion of pro-angiogenic factors IL8 and VEGF (Coleman *et al*., 2008). Aside from mammals and other vertebrates, MIF homologues have been discovered in many eukaryotic pathogens including: *P. falciparum*, the protozoan parasite responsible for Malaria (Cordery *et al*., 2007); *B. malayi,* the causative agent of lymphatic filariasis (Pennock *et al*., 1998); and *E. histolytica,* which has been shown to promote secretion of IL-6 and TNF-α macrophage cell line, RAW 264.7 and is responsible for over 100, 000 deaths per annum (Moonah *et al*., 2014).

To assess the role of MIF and D-DT within the GT, including whether the evolutionarily conserved tautomerase plays a role in any observed responses, it was essential to clone and express MIF proteins using a suitable expression system which could be exploited in large scale protein production. Initial studies performed within this project indicated that the commercially available recombinant Hs-MIFs tested lacked the inherent tautomerase activity associated

with MIF proteins. In addition to this, the manufacturer's instructions relating to the use of these recombinants in macrophages migration assays utilised concentrations of MIF that were significantly higher (g vs ng) than those reported in the literature or would be biologically relevant. As part of this work sought to assess the role of MIF's tautomerase activity in influencing intestinal innate immune responses, it was critical to utilise enzymatically/biologically active MIF proteins.

For the purposes of this PhD project, it was necessary to produce recombinant human and murine MIF proteins as our studies aimed to assess MIF's role in intestinal epithelial cells and murine APCs. In addition, for comparative purposes, two additional MIF homologues were selected for use in the immunological bioassays. These MIFs were isolated from the gastrointestinal parasite *Trichinella spiralis*, a parasitic nematode capable of infecting a wide variety of species including humans. *T. spiralis* has a well characterised lifecycle during which the adult nematodes transiently colonize the GT. The roles of various immune cells and effector mechanisms in establishing protective responses are known in *T. spiralis* and the parasite is known to secrete a number of potentially immunomodulatory factors including two MIF homologues Ts-MIF-1 (Pennock *et al*., 1998; Tan *et al*., 2001) and Ts-MIF-2 (D. Guiliano, unpublished). T.spiralis larvae produce large amounts of MIF-1 upon contact with the acidic environment of the stomach as previously determined by D. Guiliano (unpublished) (figure 3.1) and despite the fact that MIF-1 expression is common amongst trichocephalids this study will focus on *T. spiralis*. Both Ts-MIF1 and Ts-MIF-2 share the conserved Proline residue (figure 3.2.) required for tautomerase activity with all vertebrates MIF sequences. However, neither have the CXXC domain which is conserved in vertebrate MIF-1 and some parasite MIF homologues. Studies of

Ts-MIF-1 have shown that it has a six-fold greater tautomerase activity than that of Hs-MIF-1 (Tan *et al*., 2001) rendering it a valuable comparator in MIF cellular studies.

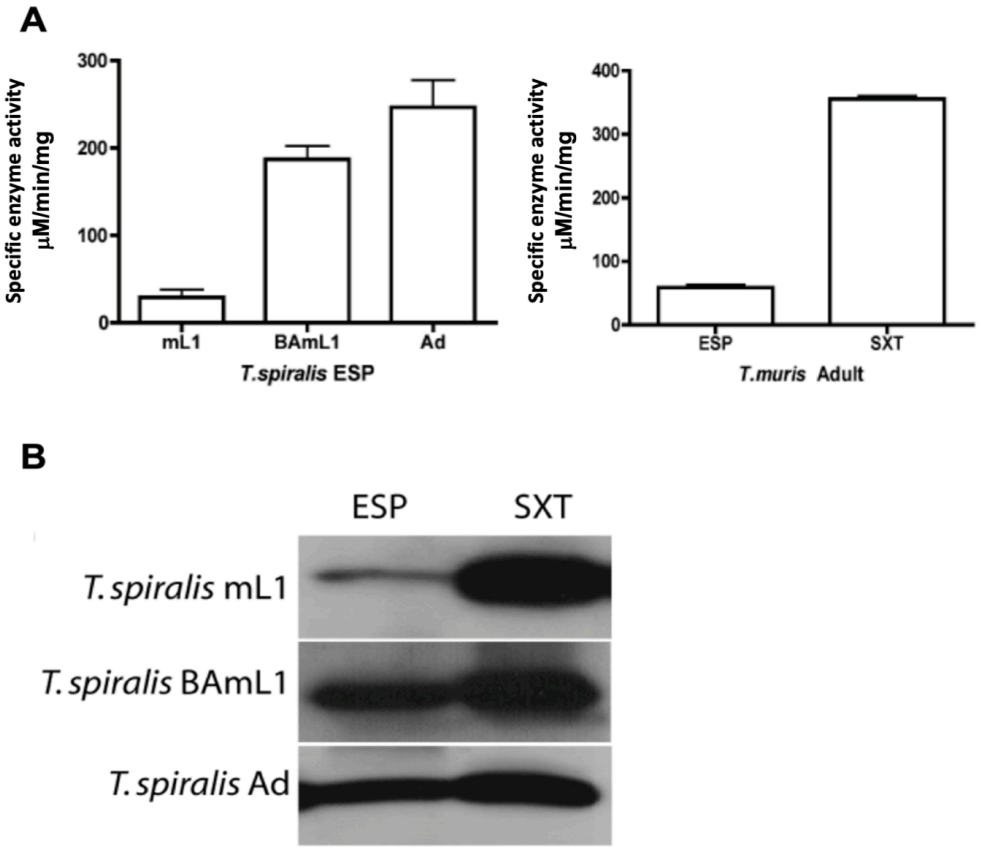


Figure 3. 1 MIF-1 is highly expressed by *T.spiralis* and confers the prototypical tautomerase activities. A) Excretory secretory products (ESP) from various stages of the *T.spiralis* and *T.muris* life cycle tautomerize the conversion of methyl ester of 2-carboxy-2,3-dihydroxyindole-5,6-quinone (*L*-dopachrome) to the methyl ester of 5,6-dihydroxyindole-2-carboxylate. mL1, infective muscle stage larvae; BAmL1, bile activated mL1; Ad, adult worms. **B)** Western blot depicting MIF-1 protein in T.spiralis life cycle stages (as before). Samples probed with an a-MIF-1 antibody. ESP, excretory secretory products; SXT, somatic extract. Experiments performed by Dr David Guiliano.

The fact that MIF's enzymatic activity may be instrumental in coordinating or subverting immune responses validates the importance of expressing and purifying an active correctly folded MIF protein, though so far, attempts to identify a physiological substrate for the tautomerase have been unsuccessful. Numerous studies have investigated the role of species-wide MIFs on immune responses, however, to date there are few that focus on the importance of the tautomerase activity due to conflicting opinions on whether it has biological relevance or is, in fact, a degenerate function. The limited research that has taken place has led to contradictory results where one study using MIF mutants which have Proline-2 (Pro2) substituted for an alternative amino acid, continue to retain the classical glucocorticoid-inhibition override activity while a subsequent study shows a loss of this activity (Hermanowski-Vosatka *et al*., 1999; Klaus Bendrat *et al*., 1997; Lubetsky *et al*., 2002). In addition, studies using other bioassays have shown that tautomerase-activity (or Pro2) is required for MIF mediated of increase MMP1 and MMP3 activity in synovial fibroblasts (Onodera *et al*., 2000) and superoxide generation in activated neutrophils (M Swope *et al*., 1998).

As previously mentioned, many vertebrate MIF proteins also contain a CALC (Cys57-Ala-Leu-Cys60) motif (figure 3.2) which forms an intramolecular disulphide bridge and functions as the catalytic centre of an oxidoreductase activity. Unlike Pro2, the CALC sequence has long been recognised as having a critical role in MIFs inflammatory actions. Utilising the well-established macrophage activation assay or *Leishmania* intracellular killing assay (Bernhagen *et al*., 1994; Thierry Calandra *et al*., 1995a) Kleemann et al (1998) revealed that Cys57 and Cys60 but not Cys81 MIF-1 mutants lacked the capacity to activate macrophages to the same extent as WT MIF-1. Additionally, Cys57

and Cys60 MIF mutants could not override glucocorticoid inhibition in a macrophage cell line, RAW 264.7 (Robert Kleemann *et al.*, 2000b). Interestingly, vertebrate D-DT/MIF-2 and Ts-MIF-1/Ts-MIF-2 do not contain a complete CALC site and therefore are not predicted to have any oxidoreductase activity.

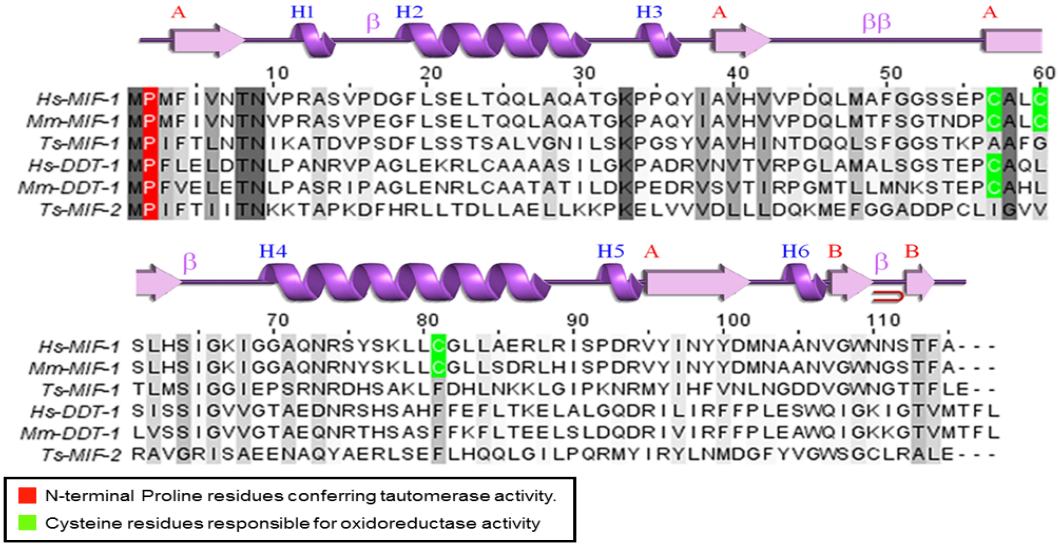


Figure 3. 2 Multiple sequence alignment shows conservation of enzymatic domains across species. Genbank protein sequences for Mm-MIF-1 (Accession number CAA80583.1), Ts-MIF-1 (Accession number CAB46354.1), Hs-DDT-1 (Accession number CAG30317.1), Mm-DDT-1 (Accession number NP_034157.1) and Ts-MIF-2 (D. Guiliano) were aligned (JalView) with the protein sequence for Hs-MIF-1 (Accession number CAA80598.1). Conserved residues are shown in the grey shaded areas. The tautomerase active site Proline is highlighted in red whilst the Cysteine resides associated with MIF's oxidoreductase activity is in green. The tautomerase-conferring proline residue at the *N*-terminal is retained across all species whilst the oxidoreductase catalytic centre, Cys-57 and Cys-60, can be found only in Hs-MIF-1 and Mm-MIF-1. The secondary structure of Hs-MIF-1 is depicted as a cartoon and was adapted from the CATH website (pdb summary of 1MIF).

MIF's three-dimensional structure is assembled by the joining of six α-helices and three β-sheets to form a solvent-exposed barrel-like structure (Figure 3.2); three active sites exist within the barrel with each monomer containing an *N*-terminal Proline residue acting as a catalytic nucleophile. The enzymatic activity of recombinant MIF is wholly reliant on the correct three-dimensional confirmation being formed *in vitro*. One important point to consider is that, although MIF has been detected as monomers, dimers and trimers, only the trimeric form is thought to confer enzyme activity. This reiterates the importance of obtaining MIF proteins with the correct tertiary structure and, analysis of MIF proteins within the tautomerase assay, allows for rapid determination of folding efficiency.

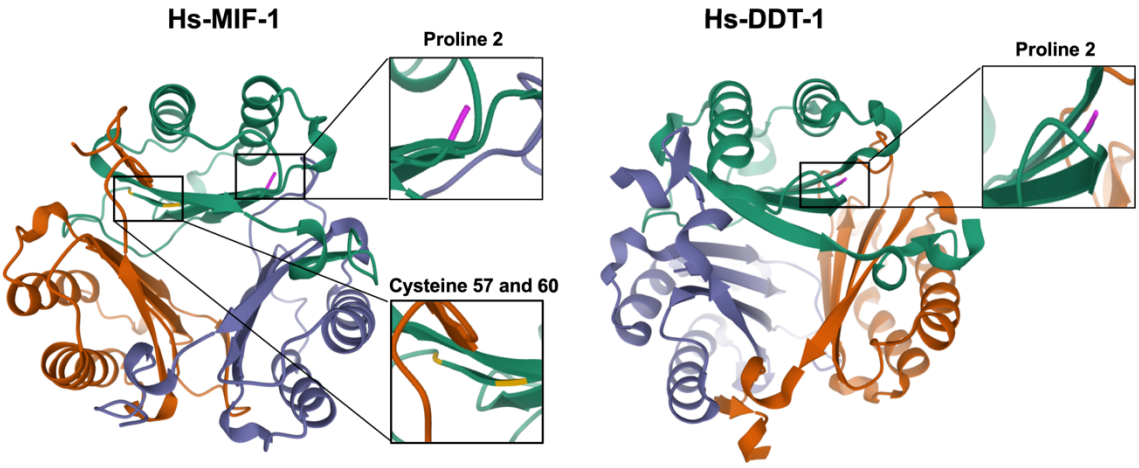


Figure 3. 3 The three-dimensional structure of Hs-MIF-1 and Hs-DDT-1/MIF-2 protein as determined x-ray crystallography. Left, human MIF -1 x-ray structure from PDB entry 1MIF. Right, human DDT-1/MIF-2 x-ray structure from PDB entry 1DPT. The tautomerase conferring site (Pro2) is highlighted in pink (magnified area) whilst the oxidoreductase conferring sites (Cys57 and Cys60) are highlighted in yellow (magnified area).

3.1.2. Production of recombinant MIFs using *Escherichia coli*.

Production of recombinant proteins within bacterial systems is often achieved using *Escherichia coli* as the expression host. Theoretically, over-expression of proteins in *E.coli* is straightforward due to its ease of culture and genetic manipulation. Typically, cloning the gene of interest into an expression vector, transforming the vector into an appropriate host and induction of protein expression results in a high level of the desired protein. However, many issues arise with the expression of eukaryotic proteins in prokaryotic organisms, such as *E.coli,* due to a lack of proper machinery required for translation and protein folding. Additionally, differences in codon bias between species the protein is isolated from and the production host can also present additional problems which negatively influence protein production. While it is not possible to fully recapitulate the eukaryotic translational environment in *E. coli* there are several widely used approaches including the use of plasmids under the control of a tightly regulated promoter, and transformation into competent cells that contain additional factors to increase translation efficiency and limit protein folding issues. The most commonly used bacterial cells for heterologous protein expression are a series of *E.coli* derivatives known as BL21.

3.1.2.1 Protein expression using BL21 derivative competent cells.

The origins of the BL21 competent cell line can be traced back to an E.coli B strain as early as 1942 (Daegelen *et al.*, 2009) and has several genetic characteristics consistent with other parental B strains, such as, the absence of the Lon protease responsible for degrading many unrelated proteins. Additionally, OmpT, an outer membrane protease which degrades extracellular proteins, is missing from the BL21 genome which is beneficial for recombinant

protein production thereby preventing the degradation of desired proteins (Grodberg and Dunn, 1988). In DE3 strains of BL21, the λDE3 prophage has been introduced to the genome and contains a T7 RNA polymerase which is controlled by the *lac*UV5 promoter. Neither BL21 nor BL21 (DE3) confer any antibiotic resistance which was importance for the purposes of this study to prevent antibiotic resistance incompatibility when using additional chaperonin plasmids which will be discussed further on in this chapter.

BL21-CodonPlus Competent cells are a codon bias-adjusted cell line originating from another BL21 derivative known as BL21-Gold. BL21-CodonPlus (DE3) have been utilised in this study to overcome to issues with codon bias in prokaryotic cells and contain the preferential tRNAs required by vertebrates to facilitate the production of heterologous recombinant proteins. Without this, low abundance tRNA's are rapidly depleted leading to terminated or truncated polypeptides significantly reducing the levels of protein expression. In this study, BL21-CodonPlus cells were used as an initial test of protein expression and only continued after significant amounts of soluble protein could be ensured.

An important point to consider when expressing proteins is the expression vector as incompatibility of vector and cell line can lead to, at best, aggregation of recombinant proteins. Problems can also arise when basal expression of the protein of interest is leaky, that is, not under the control of a tightly regulated promoter leading to constitutive expression. This is a common problem in traditional expression systems utilising the lac system; such consistent expression of a protein leads to issues such as inadequate growth of the host cell due to plasmid instability and toxicity of the target protein. Numerous expression vectors have been developed to counteract the problem with leaky protein expression. pET29b, a low-copy expression vector utilised in this study, aims to

overcome the issue with constitutive expression by exploiting a T7 lac promoter. Downstream of this lies a sequence for a lac operator and the lac repressor (lacI) which converge to suppress transcription of the host cell T7 RNA polymerase and the T7 lac promoter in pET29b to inhibit transcription by any T7 polymerase that may be produced ensuring a two-step checkpoint to prevent basal expression and potential toxicity issues due to expression of the recombinant protein prior to induction.

3.1.2.3 Co-expression of chaperone plasmids to increase folding efficiency.

Despite the development of numerous specialised competent cell lines for the production of heterologous recombinant proteins, problems can still arise in the absence of sufficient molecular chaperones to assist in the proper folding of the desired protein leading to the formation of inclusion bodies and insoluble protein. The co-expression of a chaperone such GroEL-GroES, DnaK-DnaJ-GrpE and the chaperone-like trigger factor, alongside a plasmid containing the protein of interest, can significantly increase soluble protein yield and is a beneficial tool in the case of error-prone proteins. As the chaperone plasmids confer chloramphenicol resistance, they can be used alongside the kanamycin resistant expression vector, pET29b, for co-expression purposes. Several studies have shown considerable success employing this method utilising a commercially available set of chaperone plasmids (refer to Materials and Methods). Nishihara *et al* (1998, 2000) employed the use of the aforementioned chaperones when attempting to co-express the relatively unstable protein, Japanese Cedar pollen, showing considerable success with increased protein production and stability within the soluble fraction. An important consideration when co-expressing two plasmids for the generation of recombinant proteins is the compatibility of plasmid and cell line in terms of antibiotic resistance. In this study, we utilised the BL21

(DE3) cells described above as they carry no antibiotic resistant genes ensuring that both expression plasmids can be successfully selected.

3.1.3. Production of MIF proteins using E. coli expression systems.

Historically, studies that have investigated MIF's structure and function have used bacterial expression systems such as the *E. coli* BL21-CodonPlus (DE3) as their host expression cells of choice. Typically, studies have suggested there are difficulties in obtaining protein within the soluble fractions of protein lysate; whether these studies attempted to optimise the growth and induction conditions to increase solubility is unspecified and numerous MIF studies relied on the unpredictable protocol of lysing inclusion bodies, denaturing the protein for purification and then performing a refolding procedure to renature the protein. However, the refolding methodology has several drawbacks: the process of dialysis to remove any denaturant is time-consuming; final protein yield is often minimal even in large scale cultures; and the entire process is largely experimental. In addition to this, studies that utilised earlier versions of pET vectors such as pET11b (Bernhagen *et al*., 1994; Fan *et al*., 2013; Kleemann *et al*., 1998a; Kudrin *et al*., 2006; H. W. Sun *et al*., 1996) and pKP1500 (Mozetic-Francky *et al*., 1997), which lack the endogenous ^{6}His-tag sequence found in pET29b, were reliant on purification techniques such as gel filtration and anion exchange chromatography which because of technical limitations do not allow for quick one-step purification of recombinant protein. In this study, we selected the pET29b vector containing a poly-histidine tag to clone sequences into as various studies expressing and purifying MIF homologues from *Brugia malayi* illustrate considerable success purifying using this system (Falcone *et al*., 2001b; Pennock *et al*., 1998; Prieto-Lafuente *et al*., 2009b).

3.2. Chapter aims and objectives.

To produce a set of functional, enzymatically active MIF proteins and tautomerase/oxidoreductase mutants for use in future cellular assays the following aims were proposed:

1. Clone human, murine and helminth MIF homologues into expression vector pET29b for large scale protein expression and purification.

2. Clone, express and purify MIF mutants lacking tautomerase activity (P2G) for Hs-MIF-1, Mm-MIF-1, Ts-MIF-1 and oxidoreductase deficient mutants (C57S/C60S) for Hs-MIF-1 using site-directed mutagenesis.

3. Assess all recombinant MIF proteins for native tautomerase and oxidoreductase enzyme activity to confirm they have been expressed and purified with a native structure and thus are suitable for downstream bioassays.

3.3. Cloning of MIF homologue expression constructs.

Hs-MIF-1, Hs-DDT-1, Mm-MIF-1 and Mm-DDT-1 sequences were selected based on published data and synthesized using first strand synthesis from RNA derived from human U937 monocyte cells and murine C57Bl/6 female mice and products were cloned into shuttle vector pGEM-T. Sequences were subsequently PCR amplified and subcloned into expression vector pET29b containing a C-terminal His-tag site, using restriction sites NdeI and Xho1 (Figure 3.3.A.). Ts-MIF-1 and Ts-MIF-2 in pET29b were kindly provided by Dr David Guiliano (UEL). All inserts were confirmed by sequencing as shown in figure 3.3.B.

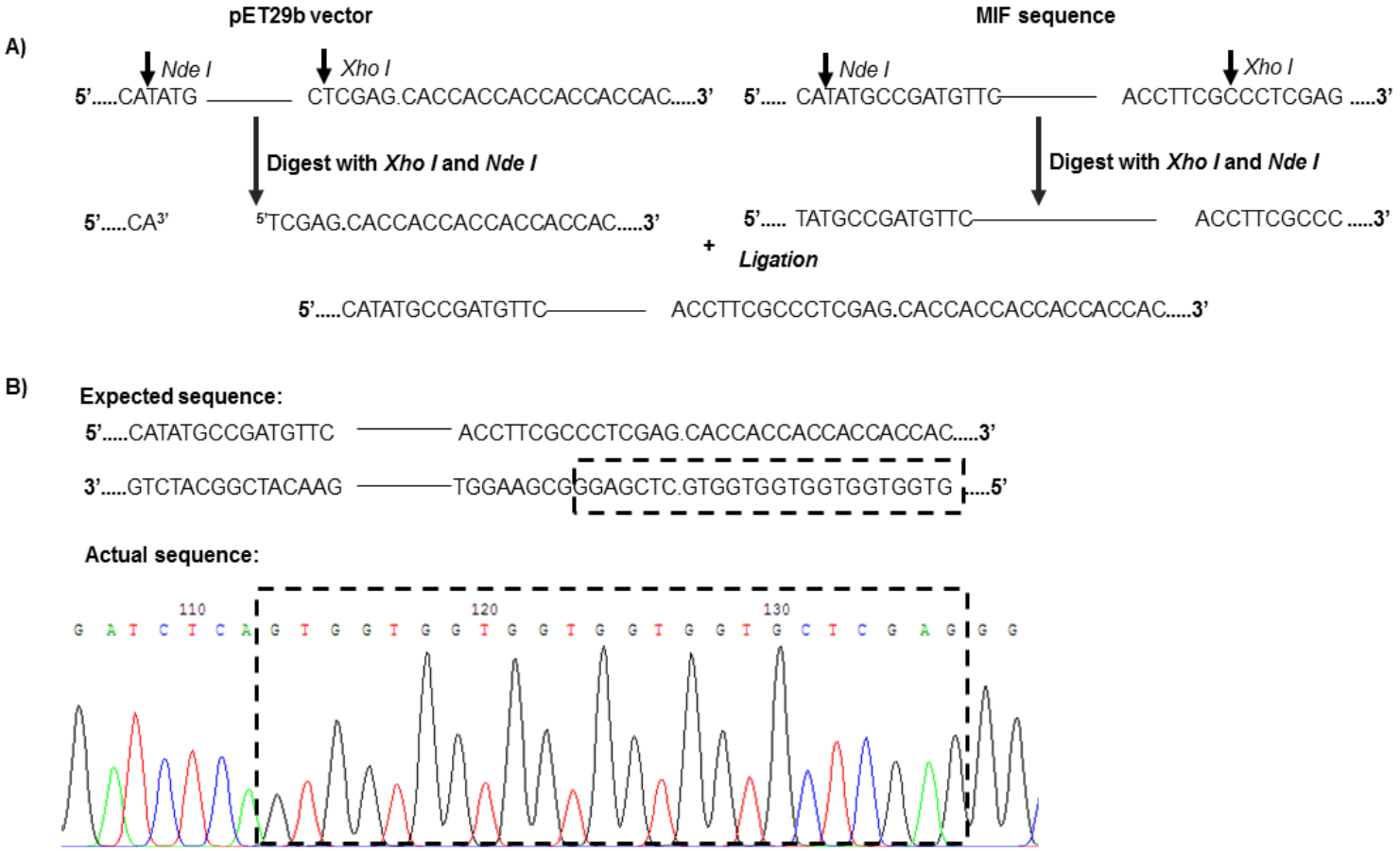


Figure 3. 4 Schematic representation of the cloning of the MIF homologues into pET29b. A) Cloning schema for MIF homologues. The pET29b vector and MIF cDNA sequences were digested with the restriction enzymes NdeI and XhoI. The MIF cDNAs were ligated to complimentary ends of pET29b. **B)** Shows a sample chromatograph confirming successful in-frame cloning of MIF cDNAs into pET29b with the dotted area indicate the location of the pET29b His-tag sequence.

Hs-MIF-1 P2G, Mm-MIF-1 P2G and Ts-MIF-1 P2G inserts were generated by synthesizing the forward PCR primers substituting the *N*-terminal Proline residues to Glycine and is schematically represented in figure 3.4.A. Glycine was selected based on several publications that demonstrated that substitution of Proline with other amino acids such as Serine, did not significantly limit the enzymatic activities and monocytic migration when compared to substitution with Glycine (Bendrat *et al*., 1997; Hermanowski-Vosatka *et al*., 1999; Lubetsky *et al*., 1999; M Swope *et al*., 1998). In addition to this, Glycine is the smallest amino acid and has no net charge, therefore, reducing the likelihood of the substitution affecting MIF's three-dimensional structure. Hs-MIF-1 C57S/C60S, lacking two critical cysteine residues that have been substituted for serine, was constructed using a crossover PCR reaction as depicted in figure 3.4.B. Cloning of all MIF sequences are shown by PCR in figure 3.5.

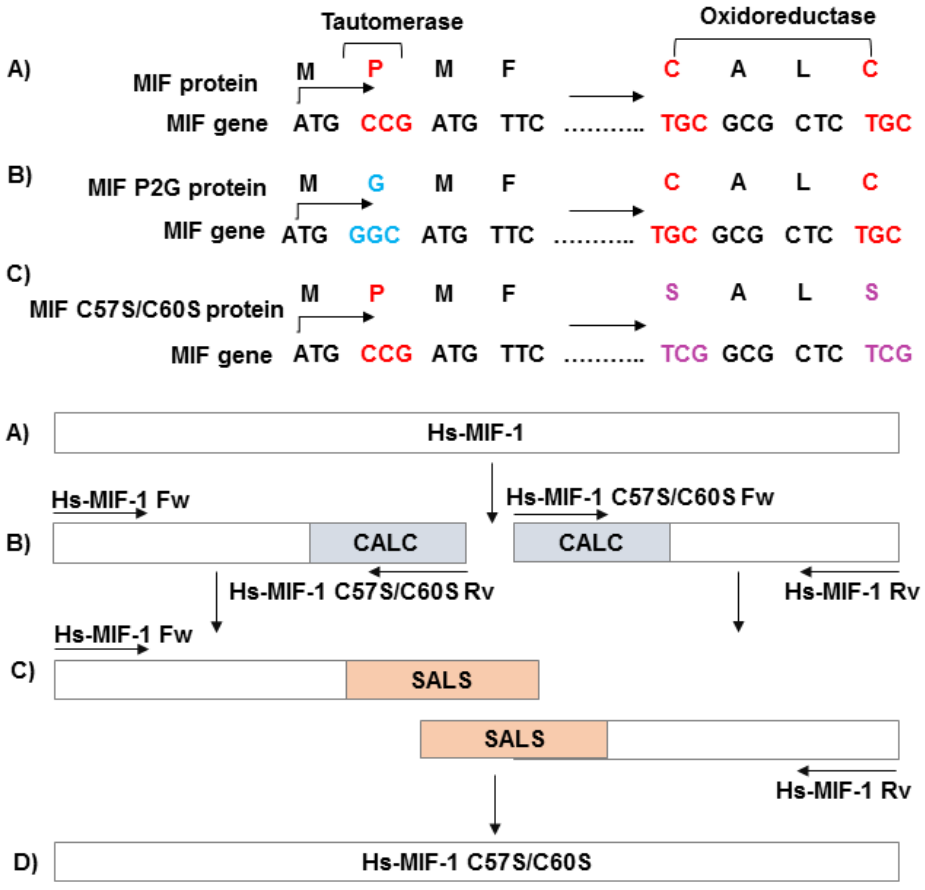


Figure 3. 5 Schematic representation of PCR mutagenesis and crossover PCR mutagenesis utilised to generate MIF-1 and MIF-2 homologue P2G mutants and the Hs-MIF-1 C57S/C60S mutant. A) tautomerase and oxidoreductase conferring amino acid ands and corresponding DNA sequence. MIF mutants tautomerase mutants substitute proline for glycine whist the oxidoreductase mutants substitute cysteine for serine residues. **B)** Overlapping fragments substituting cysteine residues for Serine residues were initially amplified from Hs-MIF-1 in pET29b. PCR purified fragments were then mixed, annealed to each other and a second round of PCR amplification performed utilising standard Hs-MIF-1 primers annealing to the 5' end of fragment 1 and 3' end of fragment 2 which amplifies the full sequence containing the SALS site. The final crossover cDNA fragment was confirmed by sequencing.

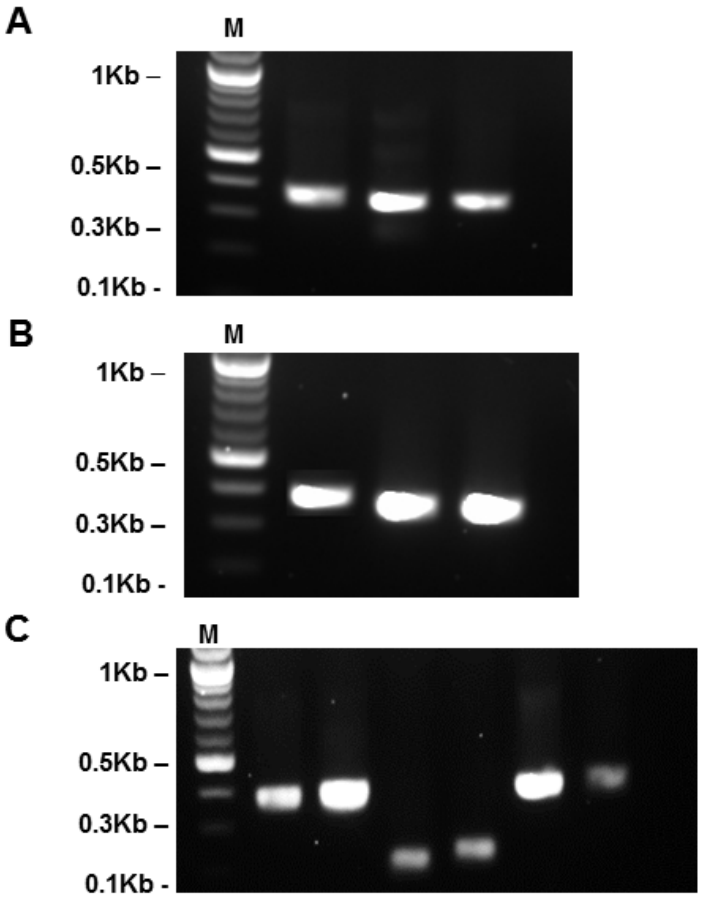


Figure 3. 6 Confirmation of successful cloning of MIF sequences into the pET29b expression vector. After isolation of pET29b transformants the presence of the MIF homologue cDNAs were confirmed using PCR and MIF homologue specific Fw and Rv primers. PCR products were run on a 1.2% agarose gel in TAE buffer. All of the resulting amplicons were the anticipated size for the cDNA fragments. A) Left to right - Ts-MIF-1, Ts-MIF-1_P2G, Ts-MIF-2. B) Left to right - Mm-MIF-1, Mm-MIF-1_P2G. C) Left to right - Hs-MIF-1, Hs-MIF-1_P2G, Hs-MIF-1_C57S/C60S fragment Fw, Hs-MIF-1_C57S/C60S fragment Rv, Full length Hs-MIF-1_C57S/C60S. M – NEB 2-log marker.

3.4. Purification of wild-type and mutant MIF homologues.

To obtain fully functional MIF proteins, pET29b vectors containing the MIF sequences were initially transformed into BL21-CodonPlus (DE3) expression cells and expressed as His-tag fusion proteins. Hs-MIF-1 and Hs-MIF-1 P2G were successfully induced and expressed within the soluble extract under standard conditions (1mM IPTG for 6 hours at 37°). However, we encountered numerous problems when attempting to obtain Hs-MIF-1 C57S/C60S and Hs-DDT-1 from the soluble extract as these were initially found solely within the insoluble fraction. This was consistent with previous studies revealing that substitution of Cys 60 for an alternative amino acid, impedes the formation of a disulphide bridge leading to misfolding of MIF and hence detection solely within the insoluble (Herrero *et al*., 2011; Kleemann *et al*., 1998b, 1998a, 1999a). Though the aforementioned studies successfully purified Cys60 mutants from the insoluble fraction using a denaturing and refolding protocol, this could not be achieved within the context of our study. However, numerous MIF homologues

such as Ts-MIF-1 and Ts-MIF-2 lack these cysteine residues however these homologues are still able to fold correctly. Therefore, assessed a number of different expression conditions for these proteins that have been reported to increase protein solubility. Normally proteins are induced when cultures are in the exponential phase of growth, typically at OD^{600} 0.6, however in proteins that are susceptible to misfolding or toxic to the cell it is often beneficial to induce at a later stage thereby slowing the rate at which proteins are expressed and folded. For this reason, several conditions were tested which revealed that induction of Hs-MIF-1 C57S/C60S and Hs-DDT-1 at a higher OD, OD^{600} 0.8 prevents misfolding. In addition to this, the induction conditions were adapted to growth at 15°, 150rpm, 100μM IPTG, for 12 hours. Figure 3.8.6 and 3.8.8 show the successful expression of Hs-MIF-1 C57S/C60S and Hs-DDT-1 within the soluble fraction.

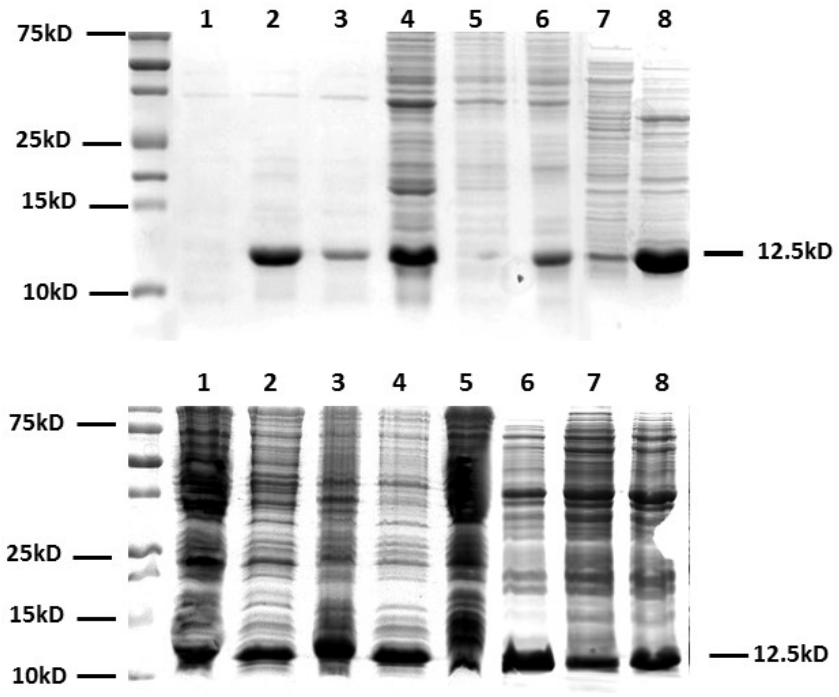


Figure 3. 7 Hs-MIF proteins are successfully expressed in BL21 CODON PLUS cells. Proteins were resolved on a 15% SDS PAGE gel and stained with Coomassie Brilliant Blue. A.1-8) Total protein: 1- non-induced Hs-MIF-1; 2- induced Hs-MIF-1; 3- non-induced Hs-MIF-1 P2G; 4- induced Hs-MIF-1 P2G; 5- non-induced Hs-MIF-1 C57S/C60S; 6- induced Hs-MIF-1 C57S/C60S; 7- non-induced Hs-DDT-1; 8- induced Hs-DDT-1. B.1-8) 1- insoluble Hs-MIF-1; 2- soluble Hs-MIF-1; 3- insoluble Hs-MIF-1 P2G; 4- soluble Hs-MIF-1 P2G; 5- insoluble Hs-MIF-1 C57S/C60S; 6- soluble Hs-MIF-1 C57S/C60S; 7- insoluble Hs-DDT-1; 8- soluble Hs-DDT-1.

Mm-MIF-1 was previously cloned into pET29b, expressed and purified under standard conditions (Paraliker, 2017). Figure 3.9.A shows the successful induction of Mm-MIF-1 P2G and Mm-DDT-1 in total protein lysates; Mm-MIF1-P2G was expressed predominantly within the soluble fraction, as shown in figure 3.8.B-2, whilst Mm-DDT-1 was initially found all in the insoluble fraction. Mm-DDT-1, like Hs-DDT-1, was grown in the adapted conditions discussed previously after which protein was easily detected within the soluble fraction (figure 3.9.C-2).

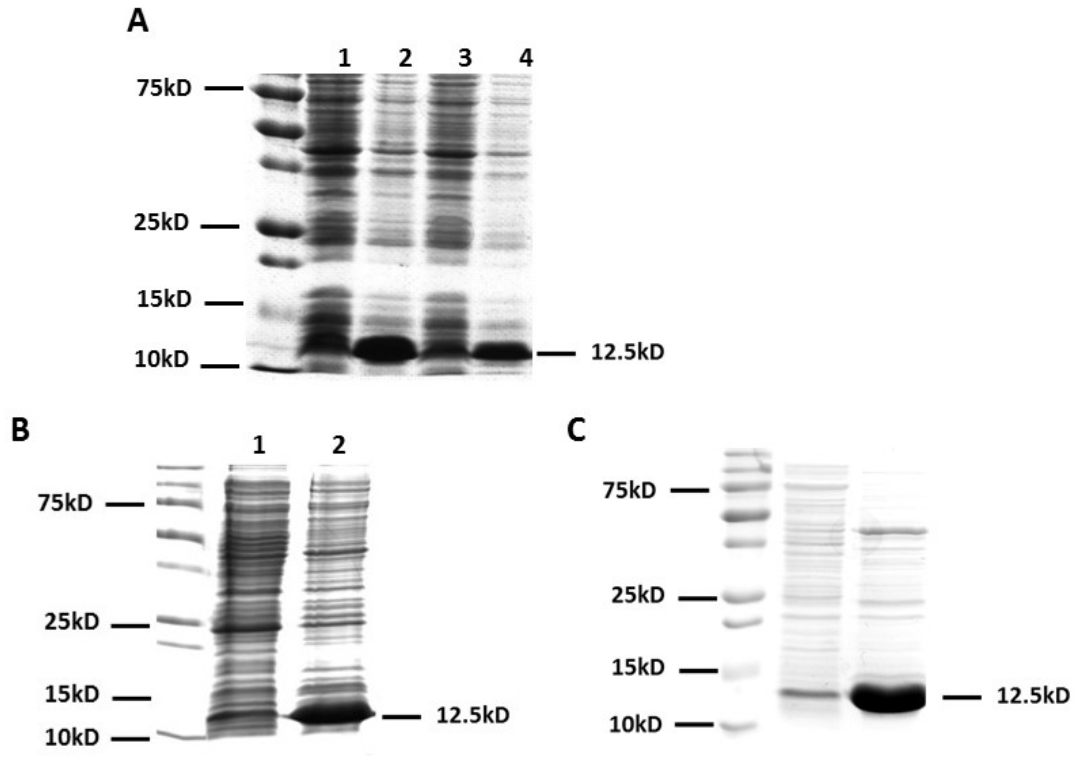


Figure 3. 8 Mm-MIF proteins are successfully expressed in BL21 CODON PLUS cells. Proteins were resolved on a 15% SDS PAGE gel and stained with Coomassie Brilliant Blue. A.1-6) Total protein: 1- non-induced Mm-MIF-1; 2- induced Mm-MIF-1; 3- non-induced Mm-MIF-1 P2G; 4- induced Mm-MIF-1 P2G; 5- non-induced Mm-DDT-1; 6- induced Mm-DDT-1. B.1-2) 1- insoluble Mm-MIF-1, 2- soluble Mm-MIF-1. C.1-2) 1- insoluble Mm-MIF-1 P2G, soluble Mm-MIF-1 P2G.

Initially, Ts-MIF-1, Ts-MIF-1 P2G and Ts-MIF-2 were induced using 1mM IPTG for a total period of six hours. Ts-MIF-1 and Ts-MIF-1 P2G were successfully induced when compared to their non-induced counterparts (figure 3.9.A), and both constructs were found to be largely within the soluble extracts of protein lysates, as depicted in figure 3.9.B indicating that Ts-MIF-1 and Ts-MIF-1 P2G were correctly folded *in vitro*. Despite Ts-MIF-2 successfully being induced in

BL21-CodonPlus (DE3) cells when assessing total protein content (figure 3.10.A), we did however, encounter several issues in generating any soluble protein (figure 3.10.B). In order to optimise the solubility of Ts-MIF-2 we screened a set of commercially available BL21 expression hosts which also co-express additional chaperonins to assist in protein folding. Ts-MIF-2 was successfully expressed within the soluble extract of pGRO7 cells after induction with 200μM IPTG and 2mg/ml L-arabinose at 25° for 10 hours and could not be found in the soluble lysates of any other chaperonin cell line (figure 3.10.C). pGRO7 cells co-express the heat shock proteins or chaperonin GroEL and co-chaperonin GroES in the presence of L-arabinose. GroEL forms a barrel-like structure which retains the protein whilst GroES acts as the 'lid' cooperatively protecting the folding protein from the cytoplasmic environment, thus preventing misfolding.

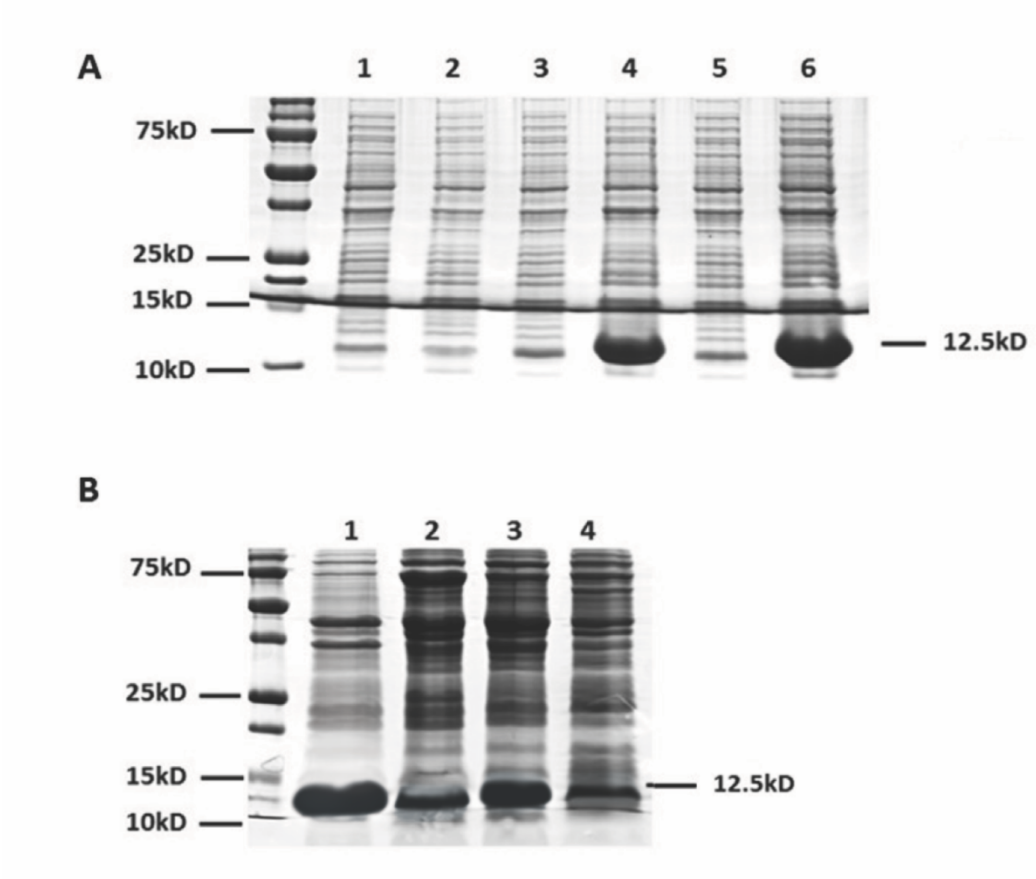


Figure 3. 9 Ts-MIF-1 and Ts-MIF-1 P2G successfully induced and expressed as soluble protein. Proteins were resolved on a 15% SDS PAGE gel and stained with Coomassie Brilliant Blue. A. 1-6) All total extract: 1-non-induced empty pET29b; 2- induced empty pET29b; 3-non-induced Ts-MIF-1; 4-induced Ts-MIF-1; 5-non-induced Ts-MIF-1 P2G; 6-induced Ts-MIF-1 P2G. B. 1-4) All induced: 1-soluble Ts-MIF-1; 2-insoluble Ts-MIF-1; 3-soluble Ts-MIF-1 P2G; 4-insoluble Ts-MIF-1 P2G.

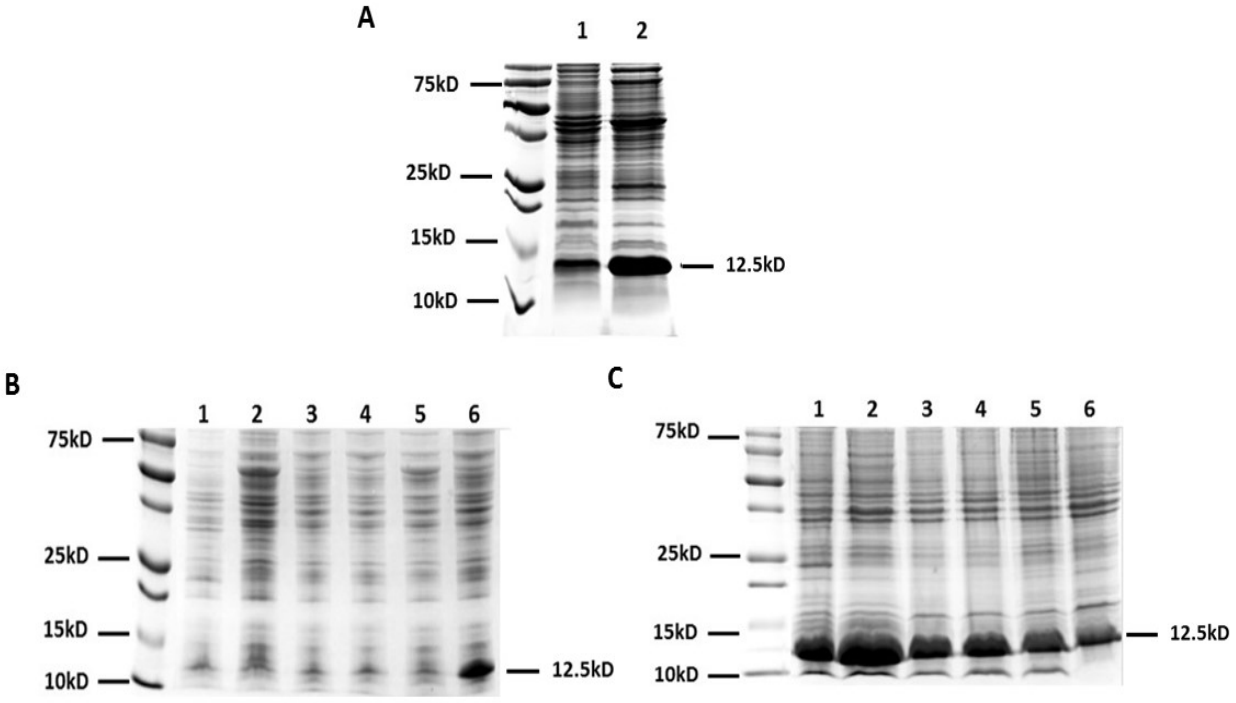


Figure 3.10 Ts-MIF-2 is successfully expressed in BL21 pGRO7 cells. Proteins were resolved on a 15% SDS PAGE gel and stained with Coomassie Brilliant Blue. A.1-2) Total protein extract: 1-Non induced Ts-MIF-2; 2-Induced Ts-MIF-2. B.1-6) Soluble: 1-CODON PLUS; 2-Tf2; 3-Tf16; 4-KjE7; 5-KjE8; 6-pGro7. C.1-6) Insoluble: 1-CODON PLUS; 2-Tf2; 3-Tf16; 4-KjE7; 5-KjE8; 6-pGro7.

Expressed proteins were purified utilising the C-terminal His-tag and Ni-affinity purification on an AKTA prime. All fractions collected and assessed for presence of MIF protein. Figure 3.11 shows a typical gel containing all fractions after purification; His-tagged MIF can be seen exclusively within the elution fractions. Elution fractions were subsequently concentrated, and buffer exchanged before the final anion-ion exchange purification in order to remove any residual LPS. Purified proteins were detected using a probe targeted to MIF's *C*-terminal ^{6}His-tag (figure 3.12). Endotoxin contamination was assessed utilising a limulus amebocyte assay and all samples contained <1ng/ml endotoxin per 1mg/ml MIF protein. Within our experiments all MIF proteins were diluted to a final concentration of 100ng/ml, so endotoxin levels throughout cellular assays were <0.0001ng/ml rendering them effectively endotoxin free.

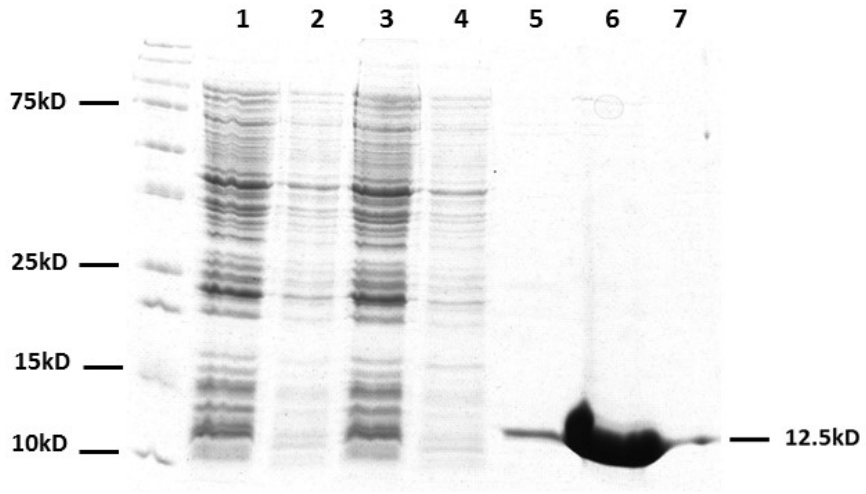


Figure 3. 11 Example SDS PAGE gel of protein fractions after AKTA purification. Proteins were resolved on a 15% SDS PAGE gel and stained with Coomassie Brilliant Blue. W = wash, E = elution. 1 – W1, 2 – W2, 3 – W3, 4 – W4, 5 – E1, 6 – E2, 7 – E3.

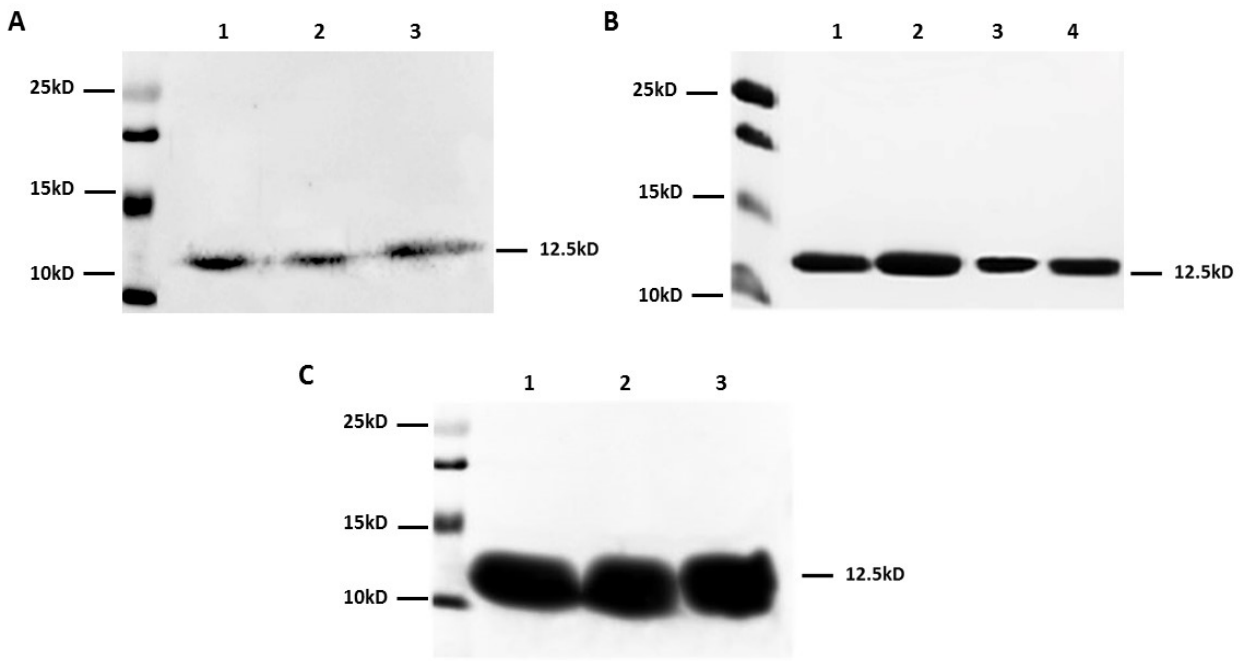


Figure 3. 12 Fully purified MIF proteins. Proteins were resolved on a 15% SDS PAGE gel, transferred to nitrocellulose membrane and recombinant proteins detected by Western blot using a HRP-conjugated anti-His-tag probe. A) Mm-MIF-1, Mm-MIF-1 P2G, Mm-DDT-1; B) Hs-MIF-1, Hs-MIF-1 P2G, Hs-MIF-1 C57S/C60S, Hs-DDT-1; C) Ts-MIF-1, Ts-MIF-1 P2G, Ts-MIF-2.

3.5. Recombinant MIFs retain critical enzyme activities.

To confirm whether the purified recombinant MIF homologues were enzymatically active and to assess what differences there were in the enzymatic activities of the MIFs produced by mammals and *T. spiralis* a series of assays were employed to measure the previously characterized MIF tautomerase activity using pseudo-biological substrate *L*-dopachrome methyl ester. *L*-dopachrome methyl ester is chromogenic, forming an orange colour when added to the enzyme buffer. MIF-tautomerisation of *L*-dopachrome methyl ester generates indole derivatives that are colourless; the loss of colour is detected spectrophotometrically and used to measure specific enzyme activity.

Initial studies revealed that a commercially available recombinant human MIF protein lacked both the tautomerase and oxidoreductase activity (figure 3.13) associated with many of MIF's immunomodulatory roles which resulted in generation of recombinant MIF proteins for this study. Commercially purchased rHMIF may lack enzymatic activities due to lyophilisation disrupting the structure of the protein.

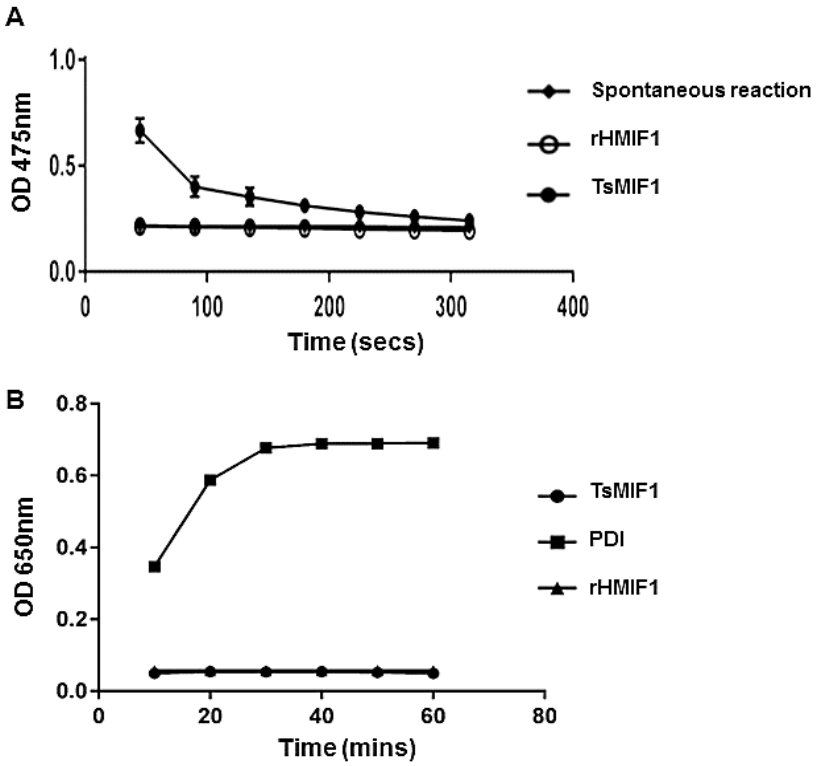


Figure 3. 13 Commercially available rHMIF1 lacks enzyme activity. A) MIF proteins were added to an enzyme solution containing L-dopachrome methyl ester and sodium periodate and absorbance values were recorded every 30 seconds for approximately five minutes. Graph depicts enzyme activity as OD^{475} versus Time (secs). Spontaneous reaction is the natural tautomerization of L-dopachrome methyl ester and contains all reagents excluding MIF. B) Graph depicts increase in absorbance at 650nm due to an accumulation of the Insulin-β chain in the presence of MIF. The data represents the mean ±SEM (n=2).

Figure 3.14 shows that all generated, purified WT MIF recombinants have tautomerase activity demonstrating that these proteins are folding efficaciously in our selected expression system. In accordance with published data from

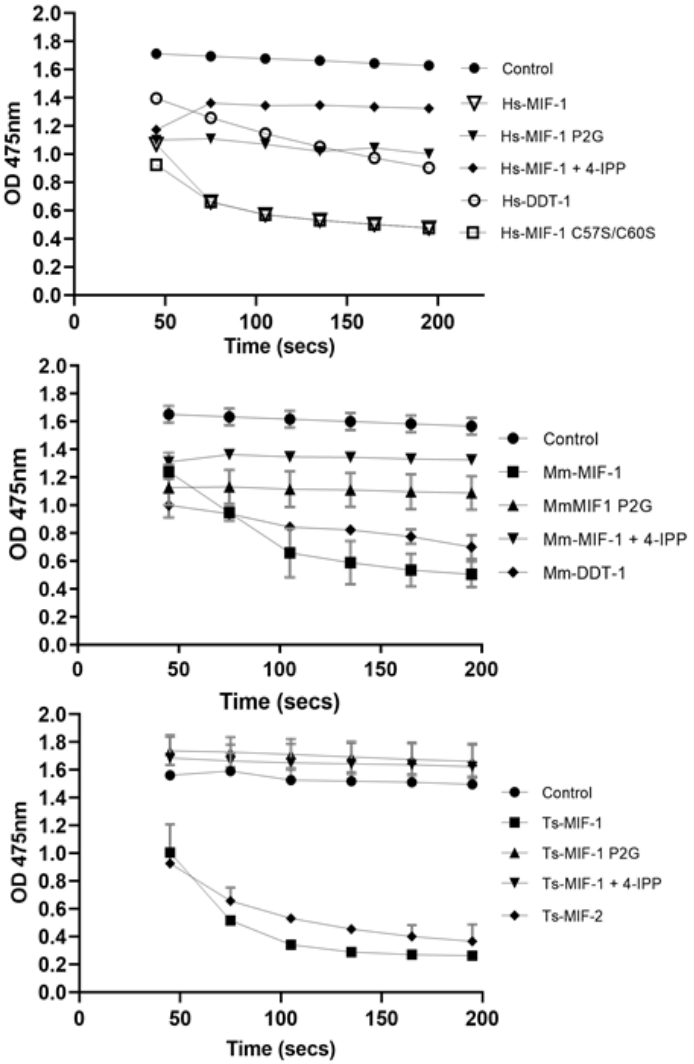
Pennock *et al* (1998) and Tan *et al* (2001), purified Ts-MIF-1 has a specific enzyme activity (359.69 μmol.min^{-1}.mg^{-1}) much greater than that of Hs-MIF-1 and Mm-MIF-1 proteins at 178.01 μmol.min^{-1}.mg^{-1} and 194.33 μmol.min^{-1}.mg^{-1}, respectively. However, the previous studies examining Ts-MIF's tautomerase activities proposed that the critical enzyme activity was six-fold higher than that of Hs-MIF-1, whereas in this study it was found that Ts-MIF-1 tautomerase activity is two-fold greater. Additionally, we corroborate, along with previous findings, that the MIF paralogues Hs-DDT-1 and Mm-DDT-1 also have tautomerase activity, 74.83 μmol.min^{-1}.mg^{-1} and 52.17 μmol.min^{-1}.mg^{-1}, albeit with lower activities than MIF-1 proteins. An interesting point to note is that there is a 39.73% decrease in specific tautomerase activity when comparing Hs-MIF-1 C57S/C60S (107.28 μmol.min^{-1}.mg^{-1}) to Hs-MIF-1 (178.01 μmol.min^{-1}.mg^{-1}) despite the *N*-terminal Proline being intact. Though some studies hypothesise that this is a result of a conformational change in the three-dimensional structure due to an inability to form disulphide bridges, this seems unlikely as Ts-MIF-1, which also lacks any cysteine residues, retains significant tautomerase activity.

Ts-MIF-2 is a novel *T. spiralis* MIF paralogue identified by Dr David Guiliano (UEL), it has not been previously characterised, and like all other MIF proteins, has the conserved proline residue. Enzymatic analysis revealed that Ts-MIF-2 has a specific enzyme activity of 131.83 μmol.min^{-1}.mg^{-1} approximately three-fold lower than that of Ts-MIF-1. The differences in catalytic efficiencies between MIF's suggest that additional amino acid sequences assist in conferring activity despite the catalytic centre being the *N*-terminal Proline.

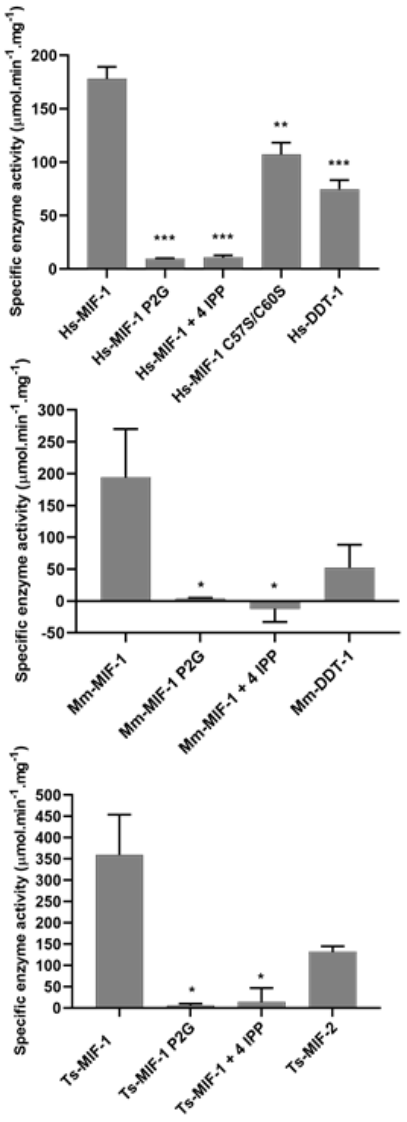
In addition to the WT MIF proteins, we confirm that MIF P2G mutants lack the ability to tautomerise *L*-dopachrome methyl ester decreasing enzyme activity by 94.66% - 98.33% from the WT MIFs. Inhibition of MIF's tautomerase site with a

commercial inhibitor 4-IPP, which covalently binds to MIF's *N*-terminal Proline reside, led to similar results decreasing enzyme activity by 94% - 100%.

A



B



C

Homologue	Specific enzyme activity (µmol.min^{-1}.mg^{-1})
H. Sapiens	
HsMIF1	178.01
HsMIF1 P2G	9.51
HsMIF1 + 4 IPP	10.66
HsMIF1 C57S/C60S	107.28
HsDDT1	74.83

Homologue	Specific enzyme activity (µmol.min^{-1}.mg^{-1})
M. musculus	
MmMIF1	194.33
MmMIF1 P2G	3.83
MmMIF1 + 4 IPP	†
MmDDT1	52.17

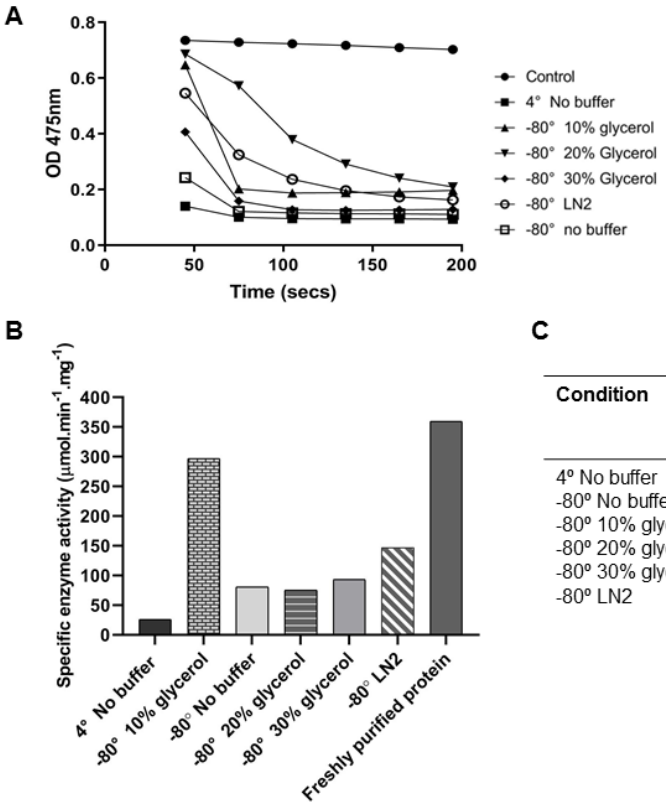
Homologue	Specific enzyme activity (µmol.min^{-1}.mg^{-1})
T. Spiralis	
TsMIF1	359.67
TsMIF1 P2G	6.00
TsMIF1 + 4 IPP	14.33
TsMIF2	131.83

† Negligible enzyme activity

Figure 3. 14 Expressed and purified mammalian and parasite MIFs possess tautomerase activity. MIF proteins were added to an enzyme solution containing L-dopachrome methyl ester and sodium periodate and absorbance values were recorded every 30 seconds for approximately five minutes. A) Graph depicts enzyme activity as OD^{475} versus Time (secs). B) Bar graph shows specific enzyme activity of recombinant MIF proteins. C) Table showing absolute values for specific enzyme activity. The data represents the mean ±SEM (n=3). (*) *p*-value ≤ 0.05, (**) *p*-value ≤ 0.01, (***) as verified by ANOVA with Dunnett's correction for multiple tests.

With the intention of reducing batch to batch variability in enzyme activity, including the detrimental effects of freezing and thawing on protein structure, optimisation of storage solutions was critical to prevent precipitation of recombinant proteins and loss of enzyme activity. Employing Ts-MIF-1 as a model recombinant protein, solutions were resuspended in varying concentrations of cryo-preservative, glycerol, at a final concentration of 1mg/ml and stored at the specified conditions. After a 12-week period, recombinant proteins were assessed for tautomerase activity as an indicator of protein stability and compared to freshly purified Ts-MIF-1. As expected, Ts-MIF-1 stored in glycerol (10%) at -80º retained the highest level of specific enzyme activity at 297.33 $\mu mol.min^{-1}.mg^{-1}$ when compared to the newly purified Ts-MIF-1 which displayed a specific enzyme activity of 359.67 $\mu mol.min^{-1}.mg^{-1}$ (figure 3.15). Although this is a decrease of 17.33%, loss of activity is expected to some degree due to the freeze-thaw method. Unexpectedly, Ts-MIF-1, which was stored minus glycerol, flash frozen in LN2 and subsequently stored at -80º, retained a

substantial amount of specific enzyme activity (147.93 μmol.min^{-1}.mg^{-1}) when compared to the specific enzyme activity of Ts-MIF-1 solutions which contain levels of glycerol at 20% and 30%, 75.33 μmol.min^{-1}.mg^{-1} and 93.24 μmol.min^{-1}.mg^{-1}, respectively. This suggests that levels of glycerol above 10% inhibit enzyme activity, nonetheless, an investigation into the effects of glycerol of tautomerase activity was not within the remits of this study.



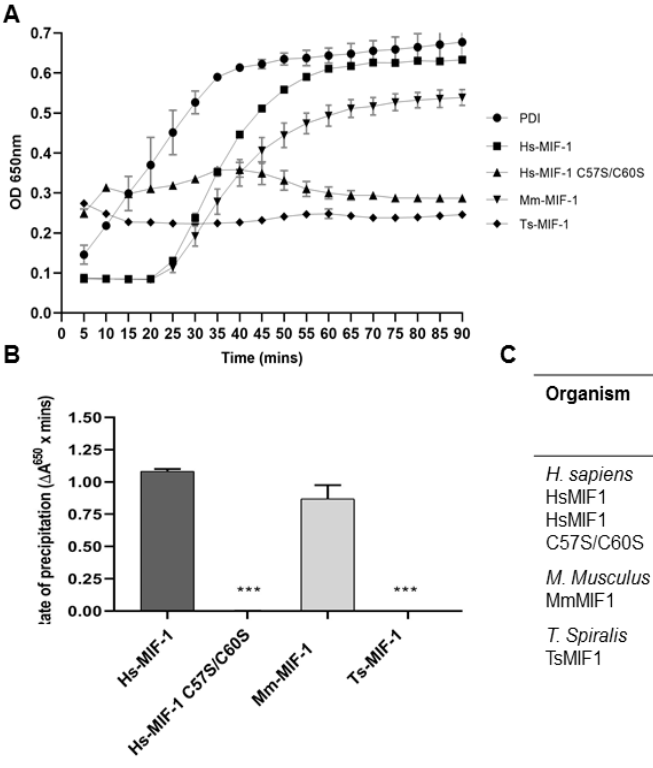
C

Condition	Specific enzyme activity (μmol.min^{-1}.mg^{-1})
4º No buffer	26.00
-80º No buffer	80.66
-80º 10% glycerol	297.33
-80º 20% glycerol	75.33
-80º 30% glycerol	93.24
-80º LN2	147.93

Figure 3. 15 Optimisation of storage conditions for recombinant Ts-MIF-1. Ts-MIF-1 was stored under varying conditions for 12 weeks and assessed for tautomerase activity thereafter. A) Enzyme activity depicted as OD^{475} versus Time (secs). Graph depicts tautomerase activity of Ts-MIF-1 under varying storage conditions. B) Bar chart shows specific enzyme activity in μmol.min^{-1}.mg^{-1}. C) Table illustrating values for specific enzyme activity. N =1.

Finally, to confirm the presence of oxidoreductase activity in MIF proteins an assay utilising reduction of the insulin β-chain was employed. Oxidation of the cystine residues in insulin result in precipitation of the β-chain as aggregates that can be detected spectrophotometrically. As expected, figure 3.16 shows that Hs-MIF-1 and Mm-MIF-1, have high oxidoreductase activity with a rate of precipitation at 1.08 and 0.87 (ΔA^{650} x min), respectively. In contrast, Ts-MIF-1 conferred no oxidoreductase activity, as expected, due to the absence of cysteine residues and catalytic CALC domain. Similarly, Hs-MIF-1 C57S/C60S which has cysteine substituted for serine residues at location 57 and 60 lacks any notable oxidoreductase activity. Comparative analysis revealed that the rates of reaction for Ts-MIF-1 and Hs-MIF-1 C57S/C60S were significantly lower ($p \leq 0.001$) than Hs-MIF-1.

The presence of these critical enzyme activities within WT MIF proteins and the absence of activity in MIF mutants confirmed they were suitable for use in further bioassays.



C

Organism	Time to precipitation (min)	Rate of precipitation (ΔA^{650} x min)
H. sapiens		
HsMIF1	60	1.08
HsMIF1 C57S/C60S	0	0
M. Musculus		
MmMIF1	65	0.87
T. Spiralis		
TsMIF1	0	0

Figure 3. 16 Purified Hs-MIF-1 and Mm-MIF-1 exhibit oxidoreductase activity. A) Graph depicts increase in absorbance at 650nm due to an accumulation of the Insulin-β chain in response to MIF. B) Bar graph showing rate of precipitation. C) Table illustrates values representing time taken to precipitation and rate of precipitation. The data represents the mean ±SEM (n=3). (***) *p*-value ≤ 0.001 as verified by ANOVA with Dunnett's correction for multiple tests.

3.6. Discussion of cloning and characterisation of MIF and MIF homologues.

Previous research examining MIF's immunomodulatory mechanisms utilised commercial recombinant MIF proteins, however, in this study we show that some commercially purchased MIF's lack the critical enzymatic activities associated with many of its classical inflammatory features. Additionally, companies that sell recombinant lyophilised MIF proteins neglect to assess the enzyme activity of MIF as part of the validation and quality control process (Bank *et al*., 2012; Beswick and Reyes, 2008; Figueiredo *et al*., 2018; Ko *et al*., 2019; Letta *et al*., 2018; Lourenco *et al*., 2015; Rossello *et al*., 2016; Stephenson *et al*., 2019; Voss *et al*., 2019; Zhang *et al*., 2015). Consequently, it was vital for the purposes of this research program to clone, express and purify recombinant MIF proteins in order to retain the enzymatic characteristics.

MIF homologues were successfully cloned and expressed as ^{6}His-tag fusion proteins in pET29b and subsequently isolated utilising an Ni-NTA system. Although we initially encountered issues expressing soluble protein from Ts-MIF-2, Hs-DDT-1, Hs-MIF-1 C57S/C60S and Mm-DDT-1, this was overcome by optimising the induction conditions; reducing the incubation time and temperature

post-induction greatly increased the amount of protein within the soluble extract. Nonetheless, the final MIF protein yield was considerably lower in these recombinants than Hs-MIF-1, Ts-MIF-1, Mm-MIF-1 and their associated P2G mutants.

A common feature of the sequences that are liable to misfold is that they lack Cys-60; a cysteine residue which has previously been identified as the controller of redox activities in MIF (Fan *et al*., 2013; Kleemann *et al*., 1998b, 1998a; Robert Kleemann *et al*., 2000b). However, a caveat to this theory is that the Ts-MIF-1 protein sequence lacks any cysteine residues and is uncomplicated in terms of expression and purification on the basis that the protein undergoes precise folding. One explanation for this includes the possibility that single cysteine residues within the amino acid sequences of Ts-MIF-2, Hs-DDT-1 and Mm-DDT-1 have greater reactivity and, for example, may interact with exogenous factors within the bacterial cell during expression, although this is likely to be contingent on the locality of residues and the proximity to charged amino acids such as asparagine and lysine; single cysteine residues closer to the *N*-terminus are more likely to undergo protonation whilst residues at the *C*-terminus tend to be concealed within the protein structure (Haase-Pettingell *et al*., 2001; Miseta and Csutora, 2000; Netto *et al.*, 2007). To determine whether this is conclusively the case, additional studies will need to be performed.

In this study, having enzymatically active MIF's were essential for attempting to interpret their role in MIF's well-documented immunomodulatory mechanisms. Here, we illustrate that MIF proteins expressed and purified in the pET29b/BL21-CodonPlus system maintain the correct three-dimensional structure by use of assays which assess tautomerase and oxidoreductase activity. There were several discrepancies between Ts-MIF-1 tautomerase activity in our study and

that of published work by Tan *et al* (2001), namely that the specific enzyme activity during this study was three-fold lower. This variation may occur because of a number or reasons. Throughout this project the detergent TWEEN was utilised in the tautomerase assay to act as a molecular crowding agent and prevent binding of the MIF protein to plasticware (Kudrin *et al*., 2006). However, there is a possibility that TWEEN may interact with the active site thus reducing activity levels (Acker and Auld, 2014). Substrate concentrations vary largely between published data, after an initial period of optimisation we used concentrations of *L*-dopachrome methyl ester ten-fold lower than Tan *et al* (2001). Finally, as the specific enzyme activity is a representation of enzyme purity there is the possibility that these values will fluctuate widely between purification methods and published work.

In this study, along with others, we noted that the specific tautomerase activities of Hs-DDT-1, Mm-DDT-1 and Ts-MIF-2 were significantly lower than the related MIF-1 counterparts and is likely to be a consequence of different amino acids surrounding the proline residue. Merk *et al* (2011) identified that, while the active site of both human MIF-1 and DDT-1 is positively charged, the surrounding area has a positive charge in MIF-1 and a negative charge in DDT-1 likely modifying the binding capacity of the active site. On the other hand, as no physiological substrate has been identified for the tautomerase activity of MIF-1 and DDT-1, there is the possibility that they act on different biological substrates. Merk *et al* (2012) previously discovered that DDT-1 undergoes an additional de-carboxylation step to form 5,6-dihydroxyindole whereas MIF-1 catalyses a bona fide tautomerisation to produce 5,6-dihydroxyindole-2-carboxylic acid. Aside from this, we successfully produced MIF P2G mutants in which all tautomerase activity was abrogated.

The enzymatic profile of Ts-MIF-2 is a novel finding and from the specific enzyme activity it would appear that it is, as expected, a homologue of mammalian DDT-1. Although Ts-MIF-2 confers tautomerase activity two-fold higher than Hs-DDT-1, this is also two-fold lower than Ts-MIF-1 which follows a similar pattern to HsMIF1 and Hs-DDT-1. Further work will be required to address more complicated questions regarding Ts-MIF-2 enzyme activity including kinetic studies to determine the exact behaviour and compare to Hs-DDT-1.

To ensure that MIF proteins were attaining the correct structural confirmation, oxidoreductase activity was also evaluated. Hs-MIF-1 and Mm-MIF-1 displayed clear redox capacities while Hs-MIF-1 C57S/C60S lacked any notable activity alongside Ts-MIF-1; as Ts-MIF-1 and Hs-MIF-1 C57S/C60S were Cys-57 and Cys-60 deficient this was anticipated.

The biological relevance of MIF enzyme activity has been subject to considerable scrutiny with frequent contradictory results in different studies in terms of whether tautomerase or oxidoreductase deficient MIF retain the pro-inflammatory qualities of WT MIFs. Further work in this thesis examines the effects of catalytic domain mutants on intestinal immune responses.

In summary, this chapter has demonstrated that MIF and DDT homologues can be purified using a Ni NTA method while avoiding the time-consuming and inaccurate refolding procedure. Additionally, we have characterised the enzyme characteristics of MIF and DDT, and identified a novel *T. spiralis* tautomerase, Ts-MIF-2.

Chapter 4: The effects of MIF on intestinal epithelial immune signalling

4.1. Introduction.

4.1.1. The Intestinal Immune system.

The intestinal immune system has been extensively explored and is known to play a key regulatory role in both local and systemic immune responses. Many of the immune cells within the GT are well characterised and their functions understood. Antigen presenting cells such as dendritic cells and macrophages sample antigens from the intestinal lumen and go on to release chemotactic signals and present the processed antigens to adaptive immune cells (Bain and Schridde, 2018; Stagg, 2018). Epithelial cells, which form the mucosal barrier surface, were once considered to be passive in their immune functions, however, studies have revealed that these are an integral point for the initiation and regulation of immune responses. Epithelial cells maintain barrier function by secreting IL-6 and TGF-beta into the local environment which are involved in regulating the T-reg/Th17 axis (Walia *et al*., 2003). Dysfunction of these cytokines has been shown to lead to local inflammation and many chronic inflammatory diseases such as inflammatory bowel disease (IBD) are attributed to issues with these key cytokines (Andrews *et al*., 2018; Neurath, 2014). Moreover, chemical inhibition or mutation of the IL-6 and TGF-beta genes can induce IBD-like symptoms in mice (Jeffery et al., 2017). Expression of IL-6 and TGF-β, like other cytokines involved in mucosal immunity, are modulated by the protein, NF-kB, a pivotal regulator that is activated by many of the innate immune receptors.

4.1.2. The role of Pathogen Recognition Receptors such as TLR4 in regulating immune responses in Intestinal Epithelial Cells.

The role of innate immune receptors (Pathogen Recognition Receptors, PRRs) in the GT must not be underestimated. TLR's expressed on the surface of epithelial cells are responsible for distinguishing between commensal and pathogenic microorganisms and eliciting appropriate responses. It has demonstrated that TLR4 plays a significant role in regulating local immune responses and IECs have been shown to express TLR4 on the basolateral surface of IECs and within the cytoplasmic compartment, restricting reactions to its canonical ligand LPS to circumstances where barrier function is compromised. This represents a critical control mechanism as LPS is constantly present in the GT due to a large number of Gram-negative organisms found in the intestinal lumen. LPS activation of TLR4 requires two co-receptors for efficient signalling, CD-14 and MD-2. The co-factors form a heterotrimer when TLR4 is activated forming part of this critical immune signalling complex.

LPS is a large glycolipid with that forms a structural component of the outer membrane of selective Gram-negative bacteria. Three structural domains make up the glycolipid: lipid A, the core oligosaccharide, and the O antigen (Raetz and Whitfield, 2002). Whilst the lipid A portion and the core oligosaccharide tend to be conserved between species, the O antigen is highly variable. LPS from different organisms vary in their ability to elicit immune responses by differences within the O antigen and steric arrangements of the protein (Bertani and Ruiz, 2018). Regardless, it is the lipid A domain that binds to the LPS canonical receptor, TLR4 (Scott *et al*., 2017). Additionally, many LPS moieties have been

shown to co-signal concurrently through TLR2 and TLR4. LPS from *Salmonella enterica* is specific for TLR4 and studies have shown that this particular LPS lacks the capacity to bind TLR2. TLR 4 is regulated by a complex array of growth factors and soluble mediators within the GT. Two of the most common regulators of TLR4 expression within IEC's are IFN-γ and TGF-β and numerous studies have revealed a critical role for these in epithelial driven innate immune regulation. Priming of IEC's with 10ng/mL – 40 ng/mL recombinant INF-γ for over 12 hours significantly increases TLR4 expression and induces LPS responsiveness in HT29 cells (Abreu *et al*., 2002; Suzuki *et al*., 2003a). Conversely, expression of TLR4 and TGF-β appear to be part of a reciprocal feedback loop: while TLR4 supresses TGF-β signalling in RAW264.7 macrophages (Liu *et al*., 2008), mutation of the TGF-β type II receptor in mice with DSS-induced colitis led to a significant increase in TLR4 expression and LPS hyperresponsiveness in IEC's when compared to WT mice (Hahm *et al*., 2001).

4.1.3. MIF and TLR4

MIF is constitutively expressed and secreted by epithelial cells within the GT at low levels. However, the precise role of MIF in this context is currently unknown. Several studies have confirmed that there is a dramatic increase production of MIF in many pathological conditions. Examples include IBD and the colonic tumour microenvironment where MIF is believed to promote inflammation and in the context of cancer increase cell survival promoting tumorigenesis and angiogenic responses. Currently, studies exploring the interplay between MIF and TLR4 in the GT in both normal and pathological conditions are lacking. The few studies to date indicate that responses to LPS are dampened or impeded in

conditions where macrophages are MIF-deficient (Roger *et al*., 2003). These studies also have shown MIF enhances LPS signalling in a fibroblast cell line (Xi *et al*., 2016), and that the overall expression pattern of TLR4 is reduced in cells lacking the MIF (Roger *et al*., 2001). Taken together, this suggests that MIF may modulate TLR4-mediated signalling in GT cells and potentially be critical for IECs immune activation by LPS.

4.1.4. MIF and TGF-β.

As described earlier in this chapter, TGF-β is a well-established regulator of epithelial-mediated immune responses in the GT and plays a number of key roles in the polarisation of immune cell subsets. TGF-β is most commonly reported to confer regulatory properties and is associated with counter-regulation of inflammatory response and the induction of regulatory immune cell subsets such as M2 macrophage phenotypes and $Foxp3^+$ T-reg cells. However, more recently TGF-β has been implicated in the development of several potential pathogenic immune cell subsets such ILC3, Th17 and Th22 cells (Bauché and Marie, 2017b); all of which are known to exacerbate colonic inflammation during the onset of intestinal disorders such as Ulcerative Colitis and Crohn's disease.

4.2. Chapter aims and objectives.

In order to examine the effects of MIF on intestinal epithelial immune signalling a set of aims were employed, and these are described below:

1. Assess if MIF can modulate responses to LPS in a commercially available TLR4 reporter cell line HEK-Blue™-hTLR4.

2. Develop a derivative of HEK-Blue™-hTLR4 within which the NF-kB transcriptional reporter drives the expression of the fluorescent protein mCherry

and also over express the MIF receptors CD74 and CD44. Assess the activity of MIF on the responses of these cells after LPS treatment.

3. Generate a colonic epithelial transcriptional reporter cell line using HT29 cells which expresses the fluorescent protein mCherry under the control of NF-kB.

4. Identify conditions within which HT29 reporter NF- κB cells are responsive to LPS and assess the activity of MIF on LPS activation of these cells.

5. Generate a TGF-β HEK reporter cell line which expresses the fluorescent protein eGFP under the control of the SMAD binding elements. Assess the activity of MIF on the responses of these cells after LPS treatment.

Table 4. 1 Summary of the genetic background and origins of the cell lines used to complete these objectives.

Cell name	Cell type	Method	Vector	Selection Marker	References
HEK-Blue™-hTLR4	HEK 293	Transfection	pNifty2-SEAP	Zeocin	https://www.invivogen.com/hek-blue-htlr4#about
HEK-hTLR4-NF-kB-mCherry	HEK 293	Transduction	pHRSIGN-NF-kB-mCherry	mCherry	(Breckpot *et al*., 2010b)
HT29-NF-kB-mCherry	HT29	Transduction	pHRSIGN-NF-kB-mCherry	mCherry	(Breckpot *et al*., 2010)
HEK-SBE-eGFP	HEK 293	Transduction	pHRSIGN-SBE - eGFP	eGFP	

4.3. MIF inhibits TLR4 mediated NF-kB activation of HEK-Blue™-hTLR4 cells.

Despite MIF's role in immune modulation being well studied, it's precise mechanisms within the context of immune signalling is poorly understood. Recently, MIF has been shown to activate the master regulator NF-kB in a CD74-dependent fashion in murine splenocyte-derived B-cells (Gore *et al*., 2008), and upregulate genes associated with cancer progression such as ICAM-1, BCL-XL and MMP2 (Kim *et al*., 2017). Conversely, MIF has been shown to inhibit AP-1 activity by binding to its proposed intracellular receptor Jab-1 in HEK 293T cells (Robert Kleemann *et al*., 2000a). However, this particular interaction between MIF and AP-1 did not alter NF-kB activity. NF-kB is a key component of many critical cell processes including those which govern immune responses such as cytokine and chemokine release, cell cycle progression and the upregulation of adhesion molecules. The vast majority of cell surface receptors will have signalling pathways which converge upon the NF-kB family of proteins. TLR4 is a PRR which recognises the endotoxin, LPS, leading to phosphorylation of the intracellular tyrosine kinase associated motifs (ITAMs) triggering downstream NF-kB activation.

In order to explore the role of MIF in TLR4-mediated NF-kB activation we utilised a commercially available HEK 293 cell line HEK-Blue™-hTLR4 (Invivogen) containing an inducible secreted alkaline phosphatase (SEAP) NF-kB reporter gene on a pNifty2 SEAP vector. The pNifty2 vector contains 5 NF-kB transcription factor binding sites under the control of a proximal ELAM promoter which, leads to expression and secretion of SEAP upon binding of NF-kB.

Importantly, the ELAM promoter gene is a truncated version which lacks the typical AP-1/CREB site found in the full-length sequence ensuring NF-kB specificity. The commercially available HEK-Blue™-hTLR4 cell line has been previously co-transfected with TLR4, the LPS coreceptors CD14 and MD-2, and respond to bacterial endotoxin, LPS activating downstream mediator NF-kB which, in turn promote secretion of alkaline phosphatase into the culture media. As HEK 293 cells lack any endogenous TLR receptors they are a useful tool for examining this specific cell signalling pathway. Previous research within our lab has confirmed that HEK 293 pNifty2 SEAP (https://www.invivogen.com/pnifty2-seap) transfected cells, lacking the TLR4 receptor, do not show NF-kB activation when stimulated with LPS (Paralikar., 2017) whilst HEK-Blue™-hTLR4 cells are LPS-responsive from 0.1ng/ml.

Though many studies looking at the interplay between MIF, LPS and NF-kB activation have taken place, the concentrations of endotoxin used to mimic biological interactions often exceed those that would normally be considered relevant *in vivo*. Within a healthy intestinal environment, LPS levels typically range from 0.1pg/ml – 50pg/ml, within the lumen, whilst a diseased intestine, such as that found in many inflammatory bowel diseases, can increase to levels of up to 100ng/mL (Leaphart *et al*., 2007; Sodhi *et al*., 2010). (A. S. Andreasen *et al*., 2008; Hurley, 1995; Marshall *et al*., 2002; Sharma *et al*., 2007; Wellmann *et al*., 1986). Taken together, all experiments within the remits of these investigations utilised LPS concentrations ranging from 10ng/ml – 100ng/ml.

Countless gram-negative bacterial species produce LPS which differ in structure and ability to act as PAMPs. In some circumstances LPS's manipulate the host immune system and each species and often strains of bacteria possess LPS type

which is characterised by a unique structure usually within the O antigen, a repeating unit of oligosaccharides. In a healthy intestine, epithelial integrity is maintained by the cooperative actions of the microbiota and the intestinal immune system whilst intestines in a diseased state fail to preserve barrier function, due to dysbiosis and epithelial dysfunction, allowing LPS to enter the sub-epithelial space. *Salmonella enterica* serotype typhimurium is a significant opportunistic enteropathogenic bacterium expressing a unique LPS pattern containing an extra O-antigen which specifically binds to TLR4. *S. enterica* serotype typhimurium is normally prevented from causing gastroenteropathy by the competing intestinal flora, however, in IBD patients dysbiosis leads to a severely inflamed epithelium with loss of tight junctions enabling *S. enterica* to invade the epithelial cells thereby activating the intracellular TLR4 compartment. Therefore, in this study observing the effects of MIF on TLR-4 signalling, we utilised LPS derived from *S. enterica* serotype typhimurium. Figure 4.1.A. confirms that HEK-Blue™-hTLR4 cells respond to both LPS ($p \leq 0.01$) and the positive control, TNF-α ($p \leq 0.001$), as expected. While there was no significant difference between cells stimulated with LPS alone and those co-incubated with LPS and MIFs (figure 4.1.B.), however, there is a notable difference between cells with LPS and Ts-MIF-1 co-administered and the LPS control. Despite this, further work examining MIF's role in LPS mediated responses in the context of MIF receptors, CD74 and CD44 required additional transfection of HEK cells. To overcome issues with incompatibility in terms of antibiotic resistance and toxicity the decision was made to utilise a transduced NF-kB responsive HEK cell line.

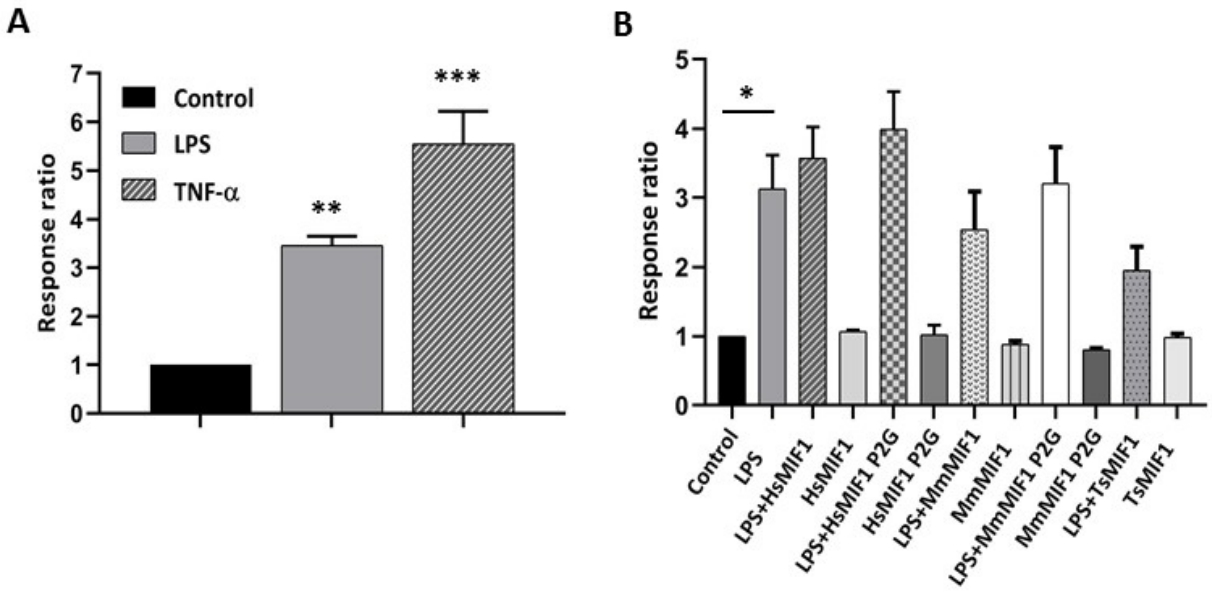


Figure 4. 1 MIFs modulate LPS responsiveness in HEK-Blue™-hTLR4 cells. The graphs show levels of secreted alkaline phosphatase (SEAP) in the media of HEK-Blue™-hTLR4 cells after treatment with different stimulants. The SEAP activity was detected using p-Nitrophenyl Phosphate (pNPP). Response rate is calculated as: OD_{405} experimental sample/OD_{405} control sample. A) HEK-Blue™-hTLR4 cells incubated with 10ng/ml LPS or 50ng/ml TNF-α for 18 hours. B) HEK-Blue™-hTLR4 cells treated with 10ng/ml LPS +/- MIF for 18 hours. The data represents the mean ± SEM (n=3) of the response ratio. (*) *p*-value ≤ 0.05 (**) *p*-value ≤ 0.01, (***) *p*-value ≤ 0.001 as calculated by one-way ANOVA with Dunnett's correction for multiple tests.

To investigate whether the effects of MIF on TLR-4 signalling were influenced by the levels of the canonical MIF receptors, CD74 and CD44, we generated a derivative of the HEK-Blue™-hTLR4 cell line which expresses mCherry in response to TLR-4 stimulation which could be used in a FACs based assay. As

the HEK-Blue™-hTLR4 cell line was already under selection with multiple antibiotics (blasticidin, hygromycin, and zeocin) a viral transduction based selection process that did not require additional antibiotics utilized to introduce the NF-kB mCherry reporter. HEK-Blue™-hTLR4 cells which had previously been transduced with the lentiviral vector pHRSIGN-NF-κB-mCherry (Breckpot *et al*., 2010b) were kindly provided by Dr D.B Guiliano (UEL). Stably transduced cells, termed HEK-hTLR4-NF-κB-mCherry, were initially characterized by FACs and then isogenic clones selected and assessed for presence of the reporter and responsiveness to activation stimuli 10ng/mL LPS and 50ng/mL TNF-α. Figure 4.2.A. shows the results of testing of the two stimulants on three of the isolated isogenic clones. After stimulation IC1 ($p \leq 0.0001$) and IC2 ($p \leq 0.0001$) respond to LPS and TNF-α to a greater extent than IC3 ($p \leq 0.0001$) when assessing the number of mCherry expressing cells compared to non-transduced cells Similarly, the mCherry MFI (fold change) of clones IC1 ($p \leq 0.0001$) and IC2 ($p \leq 0.0001$) is greater than that of IC3 with IC2 being selected for use in further studies and later transfections with pIRES plasmid expressing CD74 and CD44.

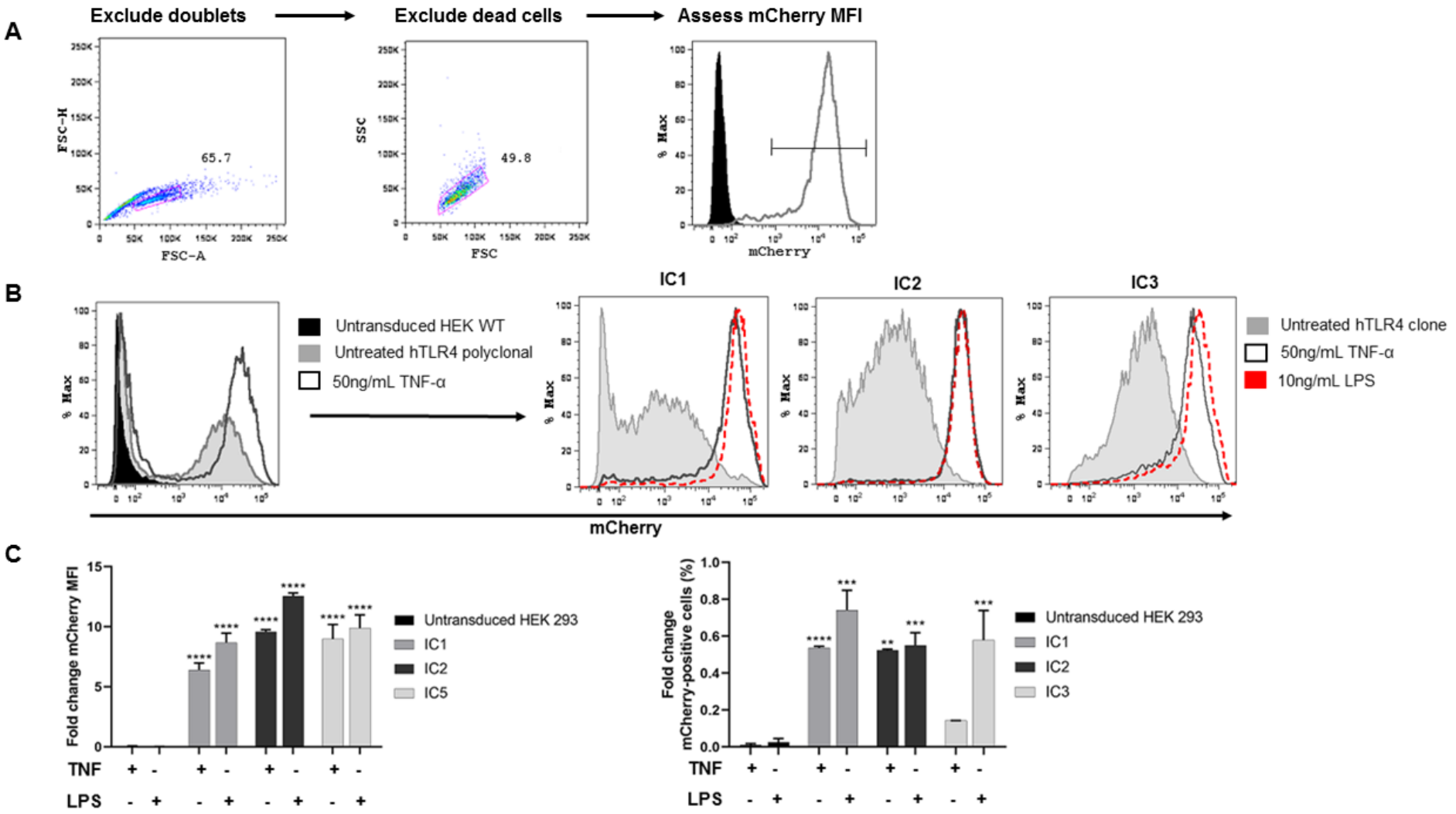

A
Exclude doublets
Exclude dead cells
Assess mCherry MFI
FSC-H
FSC-A
65.7
SSC
FSC
49.8
% Max
mCherry
B
Untransduced HEK WT
Untreated hTLR4 polyclonal
50ng/mL TNF-α
IC1
IC2
IC3
Untreated hTLR4 clone
50ng/mL TNF-α
10ng/mL LPS
mCherry
C
Fold change mCherry MFI
Untransduced HEK 293
IC1
IC2
IC5
TNF
LPS
Fold change mCherry-positive cells (%)
Untransduced HEK 293
IC1
IC2
IC3

Figure 4. 2 Isolation and characterization of an NF-kB responsive isogenic cell line HEK-hTLR4-NF-kB-mCherry. HEK-Blue™-hTLR4 cells previously transduced with the pHRSIN- NF-kB mCherry lentiviral vector were plated at 0.5 cells per well to allow for single cell expansion and the resulting clones assessed for mCherry expression after stimulation with 50ng/ml TNF-α. A) Gating strategy for the assessment of mCherry expression: doublets were excluded using FSC-H versus FSC-A. Within this population dead cells were excluded using SSC-A versus FSC-A; mCherry expression was analysed within this subset of HEK293-hTLR4- NF-kB cells. B) Histograms depict mCherry expression, in response to 50ng/ml TNF-α, within selected isogenic clones. C) Graphical representation of mCherry expressing cells (expressed as a fold change) in selected isogenic clones. D) Graphical representation of mCherry MFI (expressed as a fold change) in selected isogenic clones. The data represents the mean ± SEM (n=3). (***) *p*-value ≤ 0.001 or (****) *p*-value ≤ 0.0001 as calculated by one-way ANOVA with Dunnett's correction for multiple tests.

Cell surface CD74 and CD44 have been identified as primary (but not exclusive) receptors for MIF signalling and deletion of CD74 in mice has been shown to exhibit similar effects as that of some MIF knockout models (Mun *et al*., 2013, p. 74) indicating that CD74 is critical for MIF signalling to occur in some systems. To this end, we transiently transfected the HEK293-hTLR4-NF-kB clone 1C1 cells with a bicistronic vector, pIRES (Eurofins), which contains the cDNAs for CD74 and CD44 (pIRES-CD74-C44) or CD74 cDNA alone (pIRES-CD74). The presence of the expected cDNA inserts was confirmed using endpoint PCR with gene specific primers and are shown in figure 4.3. Figure 4.4.B-D shows that the

cell line (and HEK cells generally) have a high basal expression of CD44 as seen by staining in the controls. However, after transfection the MFI of CD44 PE-CY7 (figure 4.4.E) increased in a linear fashion as the concentration of pIRES-CD74-CD44 increased confirming that the transfection was successful and that transfected cells are incorporating numerous pIRES vectors per cell. Unexpectedly, the presence of CD74 detected at the cell surface was minimal regardless of carrier DNA:experimental vector ratio suggesting after translation the receptor is prevented from being trafficked to the cell surface. This supports earlier evidence to suggest that CD74 is rapidly degraded within lysosomes after maturation within the Golgi (Warmerdam *et al*., 1996)

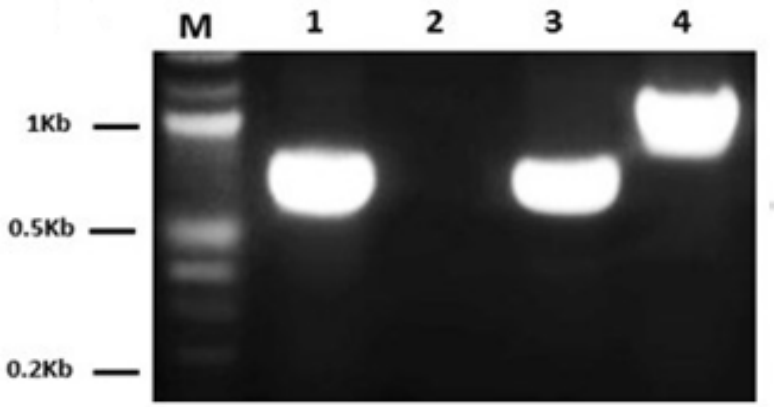


Figure 4. 3 Confirmation of presence of the CD74 and CD44 cDNAs in pIRES vectors. PCR was performed on each pIRES vector using cDNA specific primers and gel electrophoresis of amplicons is shown. 1) CD74 amplified from pIRES-CD74 using CD74 specific primers. 2) No amplicon detected for CD44 from pIRES-CD74, as expected, using CD44 specific primers. 3) CD74 amplified from pIRES-CD74-CD44 using CD74 specific primers. 4) CD44 amplified from pIRES-CD74-CD44 using CD74 specific primers. M, 2-log DNA ladder. PCR products run on a 1.5% gel in TAE buffer and visualised using a Bio-rad ChemiDoc.

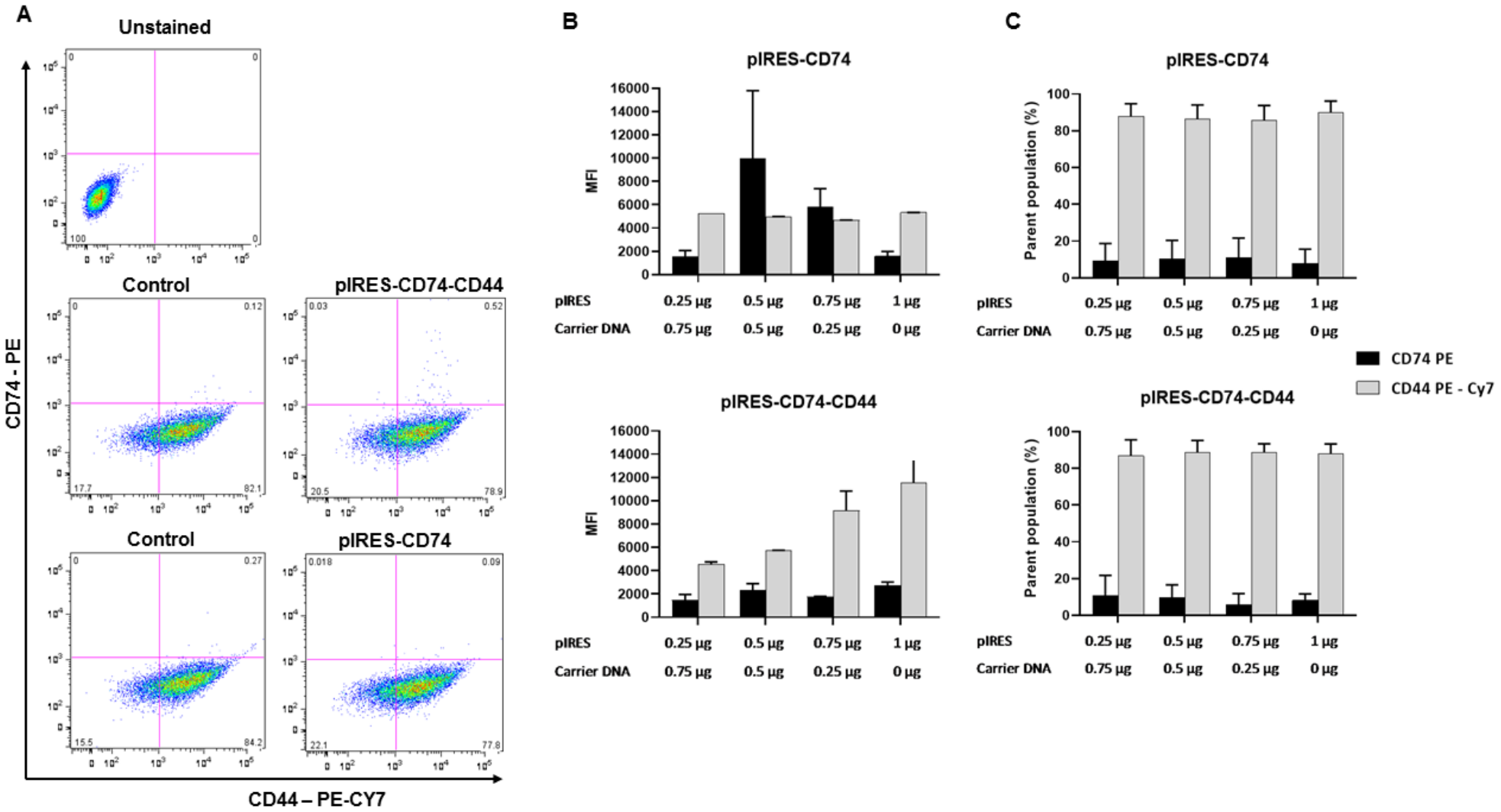

A
Unstained
Control
pIRES-CD74-CD44
Control
pIRES-CD74
CD74 - PE
CD44 – PE-CY7
B
pIRES-CD74
MFI
pIRES 0.25 µg 0.5 µg 0.75 µg 1 µg
Carrier DNA 0.75 µg 0.5 µg 0.25 µg 0 µg
pIRES-CD74-CD44
MFI
pIRES 0.25 µg 0.5 µg 0.75 µg 1 µg
Carrier DNA 0.75 µg 0.5 µg 0.25 µg 0 µg
C
pIRES-CD74
Parent population (%)
pIRES 0.25 µg 0.5 µg 0.75 µg 1 µg
Carrier DNA 0.75 µg 0.5 µg 0.25 µg 0 µg
CD74 PE
CD44 PE - Cy7
pIRES-CD74-CD44
Parent population (%)
pIRES 0.25 µg 0.5 µg 0.75 µg 1 µg
Carrier DNA 0.75 µg 0.5 µg 0.25 µg 0 µg

Figure 4. 4 Optimization of transient transfection of HEK293-hTLR4-NF-kB 1C2 cells with a biscistronic vector expressing CD74 and CD44. HEK293-hTLR4-NF-κB 1C1 were transiently transfected with varying concentrations of the plasmids pIRES-CD74 and pIRES-CD74-CD44 and the surface levels of each protein assessed by FACs. A total of 1 ug of DNA was used in each transfection with carrier DNA being used to supplement the quantity of total DNA used. A) Dot plots depicting transfected cells and analysed using CD74 PE versus CD44 PE-Cy7. B) Bar graphs depicting the MFI of CD74 or CD44 in hTLR cells transfected with pIRES-CD74 or pIRES-CD74-CD44. C) Bar graphs depicting the percentage of positive cells expressing CD74 or CD44 in hTLR4 cells transfected with pIRES-CD74 or pIRES-CD74-CD44. The data represents the mean ± SEM (n=3).

Despite the fact that cell surface expression of CD74 did not markedly increase after transfection with all of the plasmids, we sought to determine whether a small increase enhanced the effect of MIF in the presence of LPS. After transfection of HEK-hTLR4-NF-kB-mCherry 1C2 with pIRES-CD74-CD44 cells were incubated with 10ng/ml LPS or LPS and 100ng/mL MIF for 18. In this instance, Hs-MIF-1 and Hs-MIF-1 P2G were capable of decreasing LPS-driven NF-kB signalling which agrees with earlier results using the SEAP reporter which suggested that Hs-MIF-1 and Hs-MIF-1 P2G might modestly enhance NF-kB activation after LPS treatment (Figure 4.5). Multiple studies have found that MIF does not behave consistently in one bioassay depending on the cell line used and specific output. Examined data, although preliminary, suggests that the expression of CD74 within these cells greatly increases responsiveness to MIF

and that MIF may exert its effects via numerous receptors and pathways including the intracellular signal pathway via Jab1 and/or the chemokine receptor CXCR4.

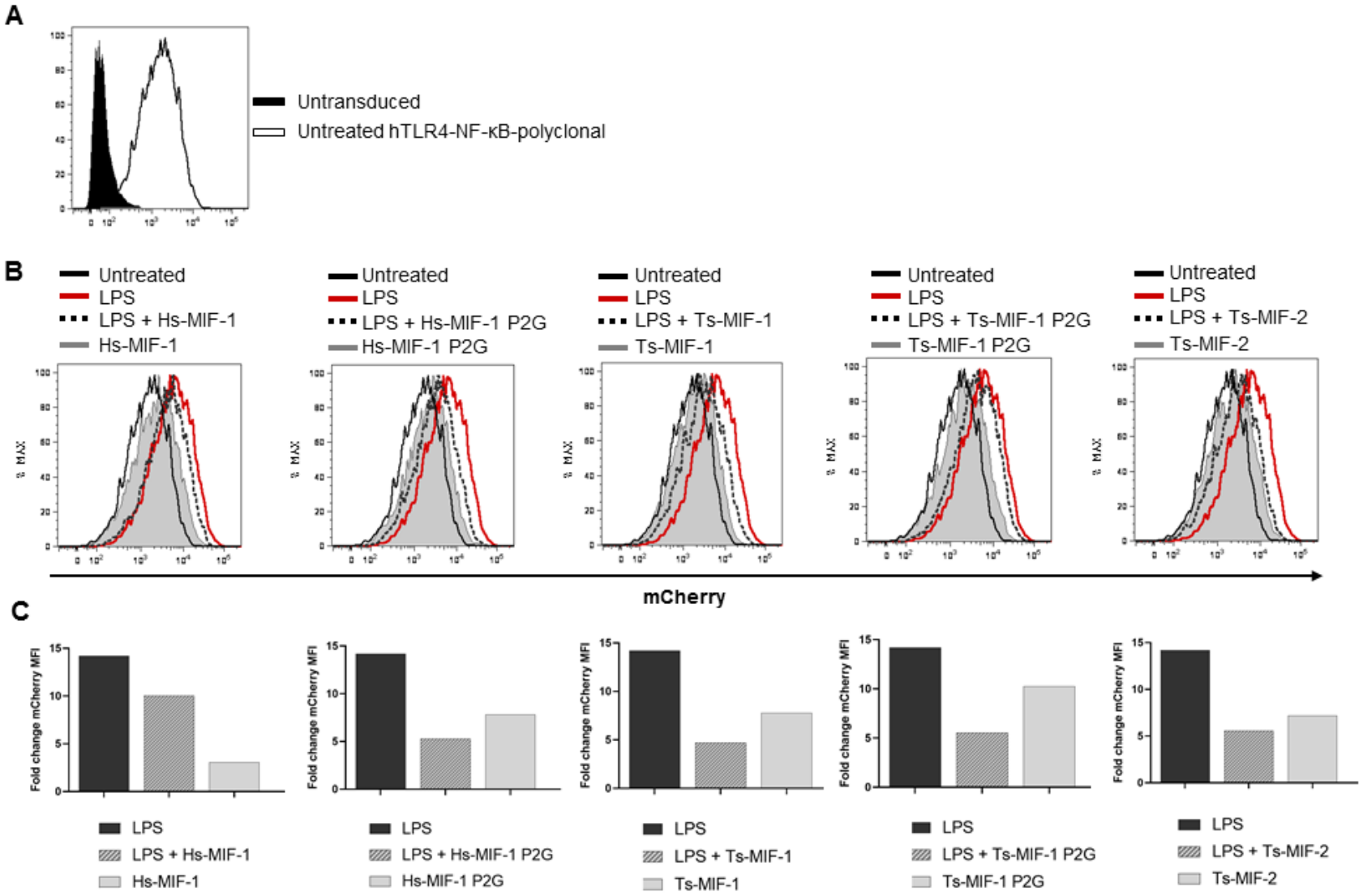

A
Untransduced
Untreated hTLR4-NF-κB-polyclonal
B
Untreated
LPS
LPS + Hs-MIF-1
Hs-MIF-1
Untreated
LPS
LPS + Hs-MIF-1 P2G
Hs-MIF-1 P2G
Untreated
LPS
LPS + Ts-MIF-1
Ts-MIF-1
Untreated
LPS
LPS + Ts-MIF-1 P2G
Ts-MIF-1 P2G
Untreated
LPS
LPS + Ts-MIF-2
Ts-MIF-2
% MAX
mCherry
C
Fold change mCherry MFI
LPS
LPS + Hs-MIF-1
Hs-MIF-1
LPS
LPS + Hs-MIF-1 P2G
Hs-MIF-1 P2G
LPS
LPS + Ts-MIF-1
Ts-MIF-1
LPS
LPS + Ts-MIF-1 P2G
Ts-MIF-1 P2G
LPS
LPS + Ts-MIF-2
Ts-MIF-2

Figure 4. 5 MIF treatment inhibits NF-kB activation by LPS in HEK-hTLR4-NF-kB-mCherry 1C2 cells transfected with pIRES-CD74-CD44. After transfection with pIRES-CD74-CD44 HEK293-hTLR4-NF-kB 1C2 were treated with LPS and or MIF homologues and the level of mCherry assessed by FACs after 18 hrs. A) Histogram depicts mCherry expression of untransduced and untreated hTLR4-NF-kB-polyclonal cells. B) Histograms depict mCherry expression after administration of transfected HEK293-hTLR4-NF-kB 1C2 cells with 10ng/ml LPS and or 100ng/ml MIF. C) Bar graphs represent fold change of mCherry MFI expression after administration of transfected hTLR4-NF-kB cells with 10ng/ml LPS +/- 100ng/ml MIF. (N=1).

4.4. Isolation of a HT29 NF-κB mCherry reporter cell line.

As our previous work indicated that MIF may play a role in TLR-4 mediated LPS signalling within model cell line HEK 293, we wanted to investigate whether this could be replicated within an intestinal epithelial cell line, such as HT29. HT29 cells are mucus-producing adenocarcinoma cells which have been shown previously to respond to LPS (Angrisano *et al*., 2010; Guo *et al*., 2013, p. 29; Mastropietro *et al*., 2015, p. 29). Studies, optimising the growth of another commonly used IEC line, Caco2, showed that these cells were not responsive to LPS in any of the conditions tested (Appendix) so all experiments focused us of the HT29 cell lines. Prior to commencing any experiments examining MIF's role in signalling by LPS, an isogenic HT29 NF-kB reporter cell line was isolated from a polyclonal HT29 cell line which had been transduced with pHRSIN-NF-kB-mCherry (provided by D.B Guiliano, UEL). Figure 4.6.A illustrates the gating strategy employed for mCherry analysis of HT29-NF-kB-mCherry clones. Our

initial findings, shown in figure 4.6.B, indicate that polyclonal HT29-NF-kB-mCherry cells treated with 50ng/ml TNF-α show an increased bimodal mCherry expression when compared to unstimulated cells. To increase the sensitivity and reliability of the mCherry reporter assay six single cell isogenic clones of HT29-NF-kB-mCherry were isolated (figure 4.6.B.). After subsequent characterisation the clone IC5 (p-value ≤ 0.0001) was selected for use in all further studies as this clone responded to TNF-α to a greater extent and lacked the bimodal histogram pattern observed in other clones (figure 4.6.B) indicating that there may be a subpopulation of cells that are either not responding to TNF-α or they are very low expressors of mCherry.

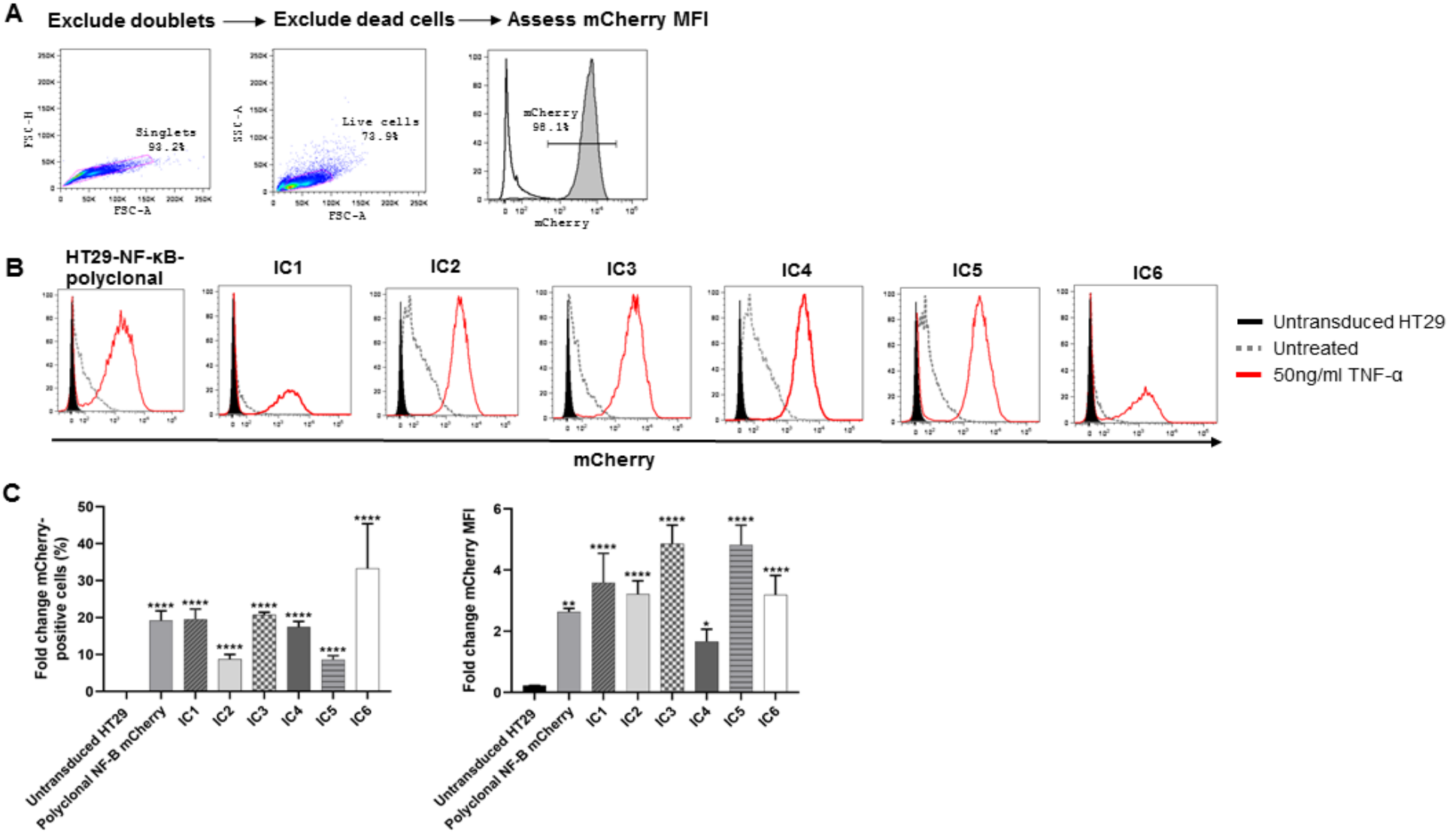

A
Exclude doublets
Exclude dead cells
Assess mCherry MFI
Singlets
93.2%
Live cells
73.9%
mCherry
98.1%
FSC-H
FSC-A
SSC-A
mCherry
B
HT29-NF-κB-polyclonal
IC1
IC2
IC3
IC4
IC5
IC6
mCherry
Untransduced HT29
Untreated
50ng/ml TNF-α
C
Fold change mCherry-positive cells (%)
Fold change mCherry MFI
Untransduced HT29
Polyclonal NF-B mCherry
IC1
IC2
IC3
IC4
IC5
IC6

Figure 4. 6 Isolation of an isogenic HT29-NF-kB-mCherry reporter cell line. HT29 cells previously transduced with the pHRSIN-NF-kB mCherry lentiviral vector were plated at 0.5 cells per well to allow for single cell expansion and assessed for mCherry expression after stimulation with 50ng/ml TNF-α. A) Gating strategy employed for mCherry analysis. Firstly, doublets were excluded using FSC-H versus FSC-A, then live cells were gated using FSC-A versus SSC-A and finally assessed for mCherry expression. Wide-type (untransduced) HT29 cells were used as a negative control. B) Histograms depict mCherry levels in selected isogenic clones. C) Number of mCherry expressing cells depicted as a fold change in response to TNF-α administration. D) mCherry MFI depicted as fold change in response to TNF-α stimulation. Cells were analysed for mCherry expression using a BD FACS Celesta. The data represents the mean ± SEM (n=3). (*) *p*-value ≤ 0.05 (**) *p*-value ≤ 0.01, (***) *p*-value ≤ 0.001, (****) *p*-value ≤ 0.0001 as calculated by one-way ANOVA with Dunnett's correction for multiple tests.

4.5. MIF inhibits NF-kB activation in HT29-NF-kB-mCherry reporter cells after LPS treatment.

Following selection of the IC5 HT29-NF-kB-mCherry-clone, cells were examined for LPS responsiveness in order to determine whether activation of NF-kB within the reporter could be observed after TLR4 signalling. Initial studies showed that LPS treatment of IC5 cells did not activate NF-kB (figure 4.6.) in the same way that was observed within the HEK-hTLR4-NF-kB-mCherry cell line. HT29 is refractory to transfection with lipid-based agents we have successfully used on

HEK 293 cells, so it has not been possible to genetically modify them to make them equivalent to the HEK-hTLR4-NF-kB-mCherry.

A study by Suzuki *et al* (2003) showed that priming of HT-29 cells with IFN-γ may augment LPS-mediated signalling by upregulating TLR4 transcripts and transporting internal TLR4 receptors to the cell surface. Our preliminary studies corroborate this finding, showing that priming cells with 10ng/ml IFN-γ for 12 hours leads to induction of NF-kB activation which is significantly potentiated with the addition of 100ng/ml LPS (p-value ≤ 0.05) when assessed using mCherry MFI (Figures 4.7.A and 4.7.B). Interestingly, the number of mCherry expressing cells significantly decreased when cultured under identical conditions. The results imply that a number of cells may undergo cell death when stimulated with both IFN-γ and LPS.

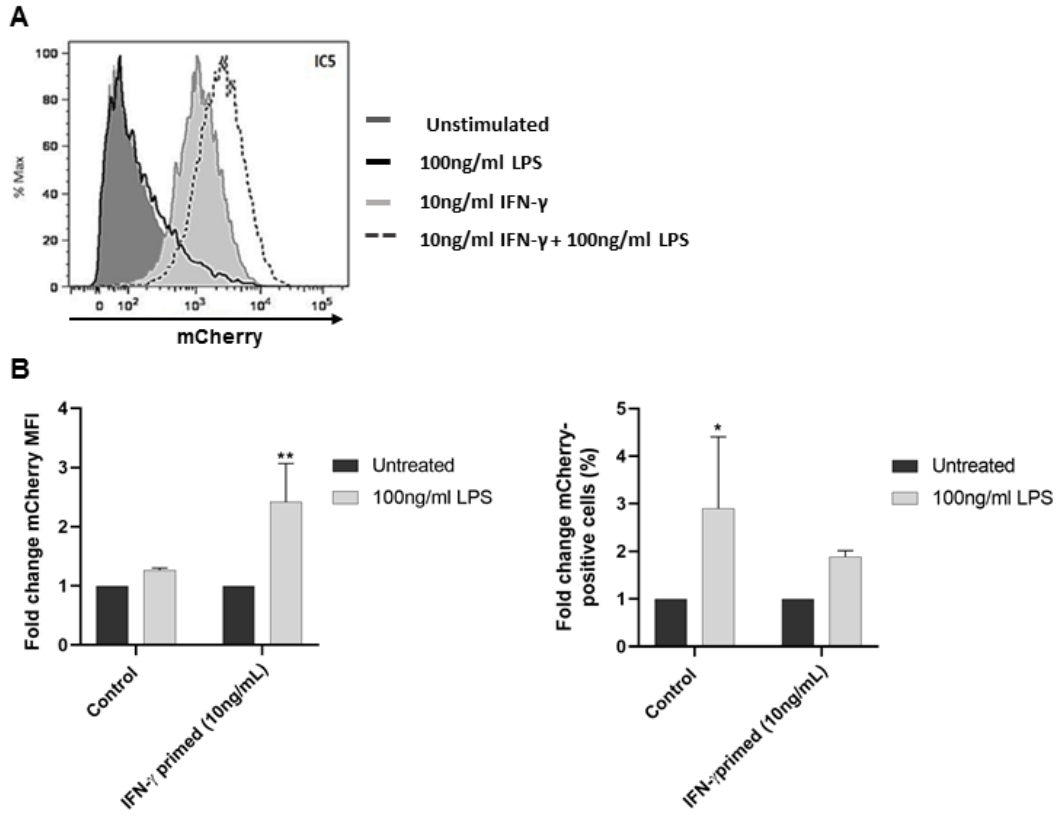


Figure 4. 7 Priming of the HT29-NF-kB-mCherry reporter cell line with IFN-γ is required for LPS responsiveness. IC5 cells were selected and incubated with 10ng/ml rIFN-γ for 12 hours and then stimulated with 100ng/ml LPS. Cells were assessed at 24 hours post-LPS stimulation for mCherry expression. IC5 cells primed with 10ng/ml IFN-γ prior to LPS stimulation led to a substantial increase in mCherry expression when compared to LPS or IFN-γ alone. A) Histogram depicting mCherry levels in IC5 cells. B) Bar graph shows mCherry fold change (MFI) and mCherry positive cells (fold change) in cells primed with +/- 10ng/ml IFN-γ then stimulated with 100ng/ml LPS. Cells were analysed for mCherry expression using a BD FACS Celesta. The data represents the mean ± SEM (n=3). (*) *p*-value ≤ 0.05 as calculated by one-way ANOVA with Dunnett's correction for multiple tests.

As discussed previously, HT29 cells are refractory to typical transfections and this led to numerous issues when handling these cells. Aside from the aforementioned TLR4 vector, these technical constraints prevented the transfection of HT29 cells with the pIRES-CD74-CD44 vector. With this in mind, we performed RT-PCR in order to detect transcriptional levels of MIF signalling proteins after IFN-γ priming as this has also been shown to increase transcriptional levels of MIF signalling receptors CD74 and CD44 in other systems. We confirmed that like other cells, CD74 and CD44 expression is increased (Figure 4.8.A.) after priming with 10ng/ml IFN-γ. After confirming the presence of CD74 and CD44 transcripts in HT29 cells, we co-incubated cells with 10ng/ml LPS and 100ng/ml Hs-MIF-1 as a preliminary test. In IFN-γ primed cells, Hs-MIF-1 dramatically reduced LPS-driven NF-kB signalling, assessed by MFI of

mCherry in those cells. An interesting and unexpected result was that the number of mCherry expressing cells in non-primed samples were generally higher than those primed with 10ng/ml IFN-γ indicating that IFN-γ is selectively targeting a subgroup of HT29-NF-kB-mCherry cells. In addition, these results validate that MIF receptors, CD74 and CD44, are upregulated in response to IFN-γ treatment.

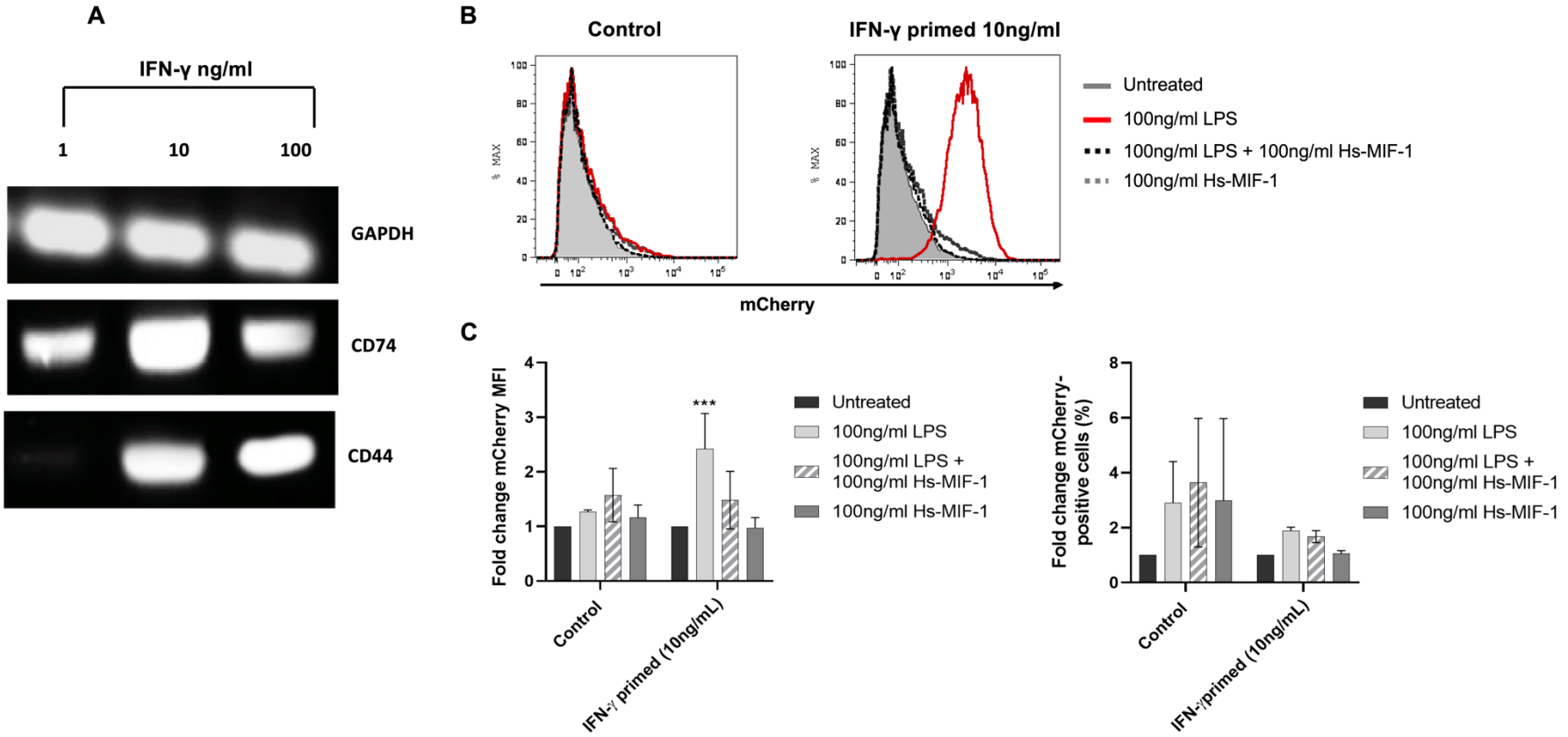

A
IFN-γ ng/ml
1
10
100
GAPDH
CD74
CD44
B
Control
IFN-γ primed 10ng/ml
% MAX
mCherry
Untreated
100ng/ml LPS
100ng/ml LPS + 100ng/ml Hs-MIF-1
100ng/ml Hs-MIF-1
C
Fold change mCherry MFI
***
Control
IFN-γ primed (10ng/mL)
Untreated
100ng/ml LPS
100ng/ml LPS + 100ng/ml Hs-MIF-1
100ng/ml Hs-MIF-1
Fold change mCherry-positive cells (%)
Control
IFN-γ primed (10ng/mL)
Untreated
100ng/ml LPS
100ng/ml LPS + 100ng/ml Hs-MIF-1
100ng/ml Hs-MIF-1

Figure 4. 8 Hs-MIF-1 suppresses TLR4-driven NF-kB signalling in HT29-NF-kB-mCherry IC5 cells. A) IC5 cells were stimulated with increasing concentrations of IFN-γ and RNA collected to assess RT-PCR assessment of transcriptional levels of GAPDH, CD74 and CD44. RT-PCR products were run on a 2% agarose gel and visualised using a Bio-rad ChemiDoc. B) IC5 cells (non-primed or primed) were cultured with 100ng/ml LPS and or +/- 100ng/ml Hs-MIF-1 for 18 hours and mCherry expression assessed using a BD FACS Celesta. Bar graph depicts (left) fold change mCherry MFI and (right) fold change mCherry positive cells. The data represents the mean ± SEM (n=3). (*) *p*-value ≤ 0.05 as calculated by one-way ANOVA with Tukey's correction for multiple tests.

4.6. MIF and TGF-β

Finally, due to its reported varied roles in influencing activation of immune cells and inflammation an examination of the effects of MIF on regulatory immune signalling. Part of this work focussed on TGF-β, a key regulator of intestinal immune homeostasis that is implicated in a variety of GI disorders and pathologies including colorectal cancer (Bellam and Pasche, 2010).

There are several studies that indirectly suggest MIF may interact with TGF-β however this has not been directly tested (Choi *et al*., 2012; Wang *et al*., 2015). To analyse the potential roles of MIF on TGF-β modulation of epithelial-derived immune responses we employed polyclonal HEK cells which have been previously transduced with the lentiviral vector pHRSIN-SBE-eGFP (cells provided by D.B Guiliano). This reporter construct has the fluorescent protein eGFP cloned down stream of set of SMAD binding elements (SBE). EGFP expression in this polyclonal cell line (HEK-SBE-eGFP) increases after treatment

with TGF-β (figure 4.9). Figure 4.9.A indicates that polyclonal HEK-SBE-GFP WT cells treated with 2.5ng/ml TGF-β show an increased bimodal GFP expression when compared to unstimulated cells. To increase the sensitivity and reliability of the eGFP reporter assay five single cell isogenic clones of HEK-SBE-eGFP were isolated. After subsequent characterization, the clone IC3 was selected for use in all further studies (figure 4.9B/C). Initially the kinetics and specificity of the transcriptional reporter was assessed and then the effects of MIF on TGF-β mediated activation of the SBE reporter was assessed.

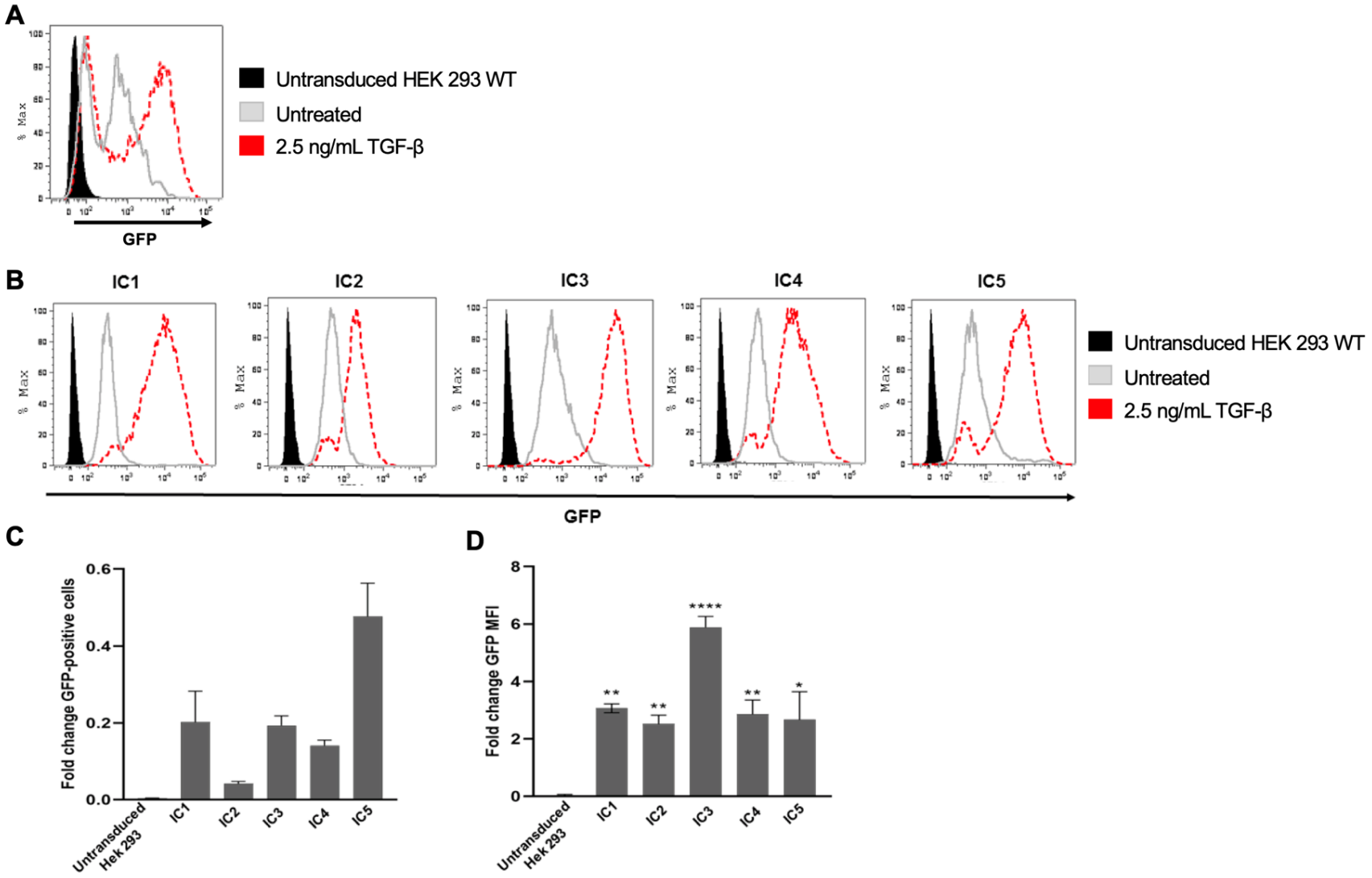

A
% Max
GFP
Untransduced HEK 293 WT
Untreated
2.5 ng/mL TGF-β
B
IC1
IC2
IC3
IC4
IC5
% Max
GFP
Untransduced HEK 293 WT
Untreated
2.5 ng/mL TGF-β
C
Fold change GFP-positive cells
0.0
0.2
0.4
0.6
Untransduced Hek 293
IC1
IC2
IC3
IC4
IC5
D
Fold change GFP MFI
0
2
4
6
8
**
**
****
**
*
Untransduced Hek 293
IC1
IC2
IC3
IC4
IC5

Figure 4. 9 Characterisation of HEK-SBE-eGFP Isogenic cell lines. (A) HEK SBE-eGFP WT cells were seeded at $3x10^3$ cells per well in a 96-well microtitre plate and stimulated with 2.5ng/mL TGF-β for 18 hours. Expression of GFP was assessed using a BD FACS Celeste. (A) Incubation of HEK SBE-eGFP WT cells with 2.5ng/mL TGF-β for 18 hours leads to an increase in eGFP expression in a bi-modal manner. (B) Panel of histograms depicts GFP expression in response to TGF-β administration in isogenic clones. (C) The bar graph shows the increase in mean fluorescence intensity of eGFP depicted as fold change. (D) The bar graph shows the increase in the number of cells showing eGFP-expressing cells as fold change in response to TGF-β administration. Error bars represent +/- SEM. N=3.

Previous studies have elucidated that the SBE is responsive to a common SMAD (Zawel., 1998), SMAD4, implying that both TGF-β and Bone Morphogenetic Protein 4 (BMP-4) should increase eGFP expression in HEK IC3 cells. To further characterize the responses of the IC3 reporter cell to TGF receptor ligands a time course was performed analysing the increase in expression of eGFP after treatment with either TGF-β and BMP-4 over 24 hours. Interestingly, as shown in figure 4.10, incubation with 2.5ng/ml TGF-β induces a gradual increase in eGFP expression which begins to reach plateau at 12 hours. Conversely, treatment with 2.5ng/ml BMP-4, had little effect on eGFP expression until 12 hours post-stimulus and this remained constant. The results obtained demonstrate that the responses of isogenic HEK-SBE-eGFP clones to TGFβ and BMP4 vary in signal strength and duration and that IC3 cells may respond preferentially to TGF-β ligand binding.

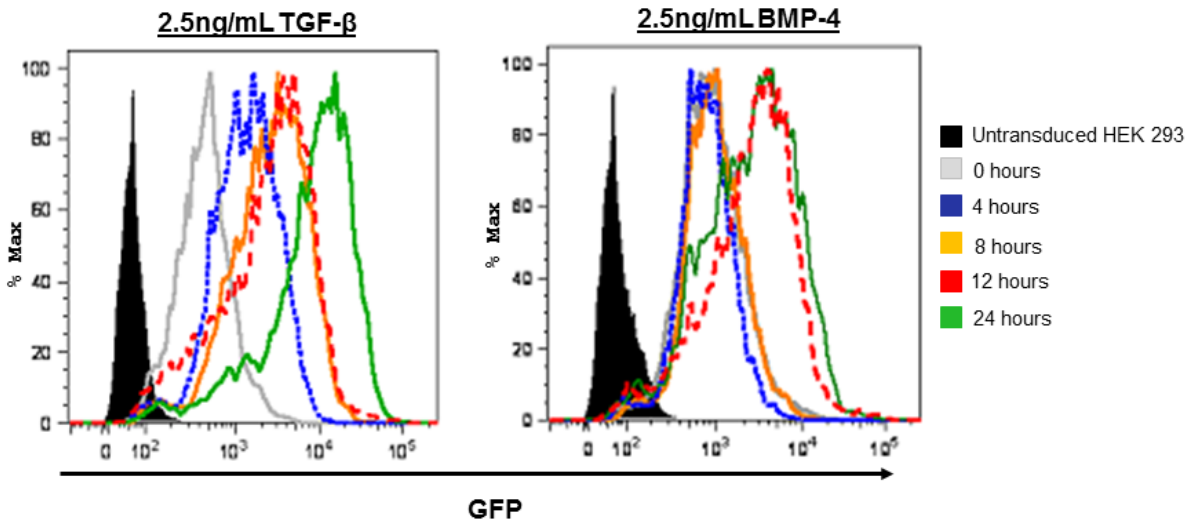


Figure 4. 10 Analysis of the temporal dynamics of TGFβ and BMP4 signalling and the expression of eGFP in the HEK-SBE-eGFP isogenic cell line, IC3. IC3 cells were selected and incubated with 2.5ng/ml TGFβ or 2.5ng/ml BMP4 for 24 hours. Cells were assessed periodically at 4, 8, 12 and 24 hours for GFP expression. (A) GFP expression increases progressively over time, from 4 hours to 24 hours, in response to 2.5ng/ml TGFβ. (B) GFP expression increases at 12 hours post-incubation with 2.5ng/ml BMP4.

After defining the dynamics of TGF-β signalling in IC3 HEK-SBE-eGFP cells, preliminary experiments were performed to assess the role of MIF on the TGF-β signalling pathway. Figure 4.11 shows that initial experiments did not show any MIF-dependent modulation of TGF-β signalling at 24 hours post-treatment, in the presence or absence of recombinant TGF-β, even at concentrations as high as 20μg/mL recombinant human or *T. spiralis*-derived MIF protein. Further experiments will be required to increase the *n* number to confirm whether the

absence of a MIF affect in this case is an outlier or whether MIF does not effect TGF-b signalling in the context of HEK 293 cells.

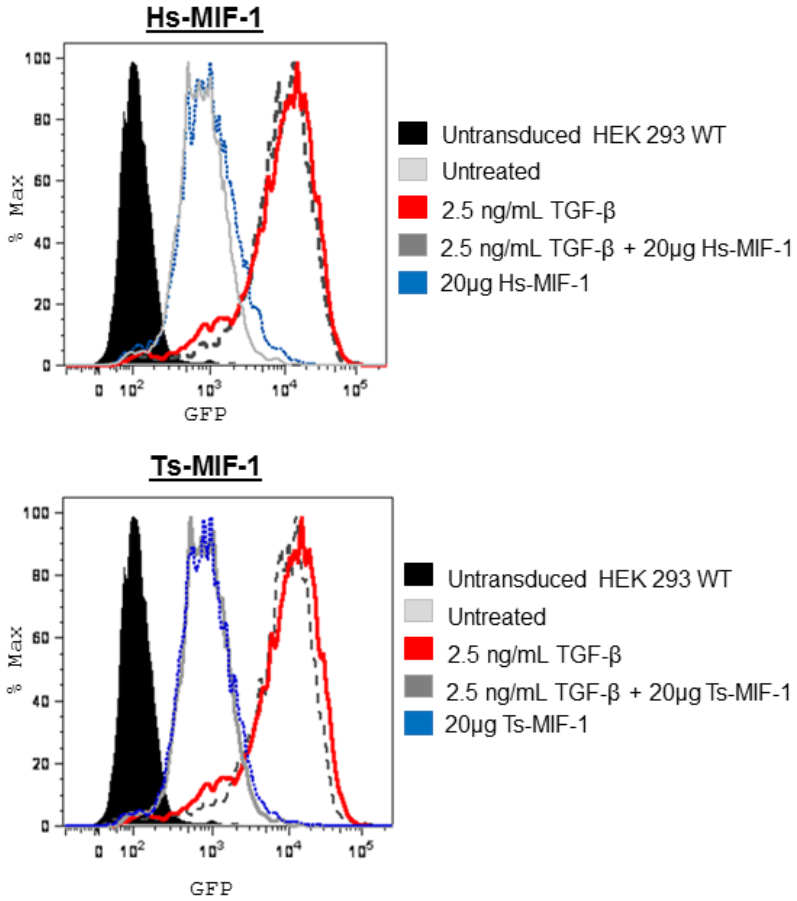


Figure 4. 11 MIF does not modulate TGF-β signalling in the HEK-SBE-eGFP isogenic cell line, IC3. IC3 cells were co-incubated with either Hs-MIF-1 or Ts-MIF-1 in the presence or absence of 2.5ng/ml TGF-β for 24 hours. GFP expression increases after treatment of IC3 cells with TGF-b. Neither Hs-MIF-1 or Ts-MIF-1 modulate this effect in HEK 293 cells.

4.7. Discussion of the effects of MIF on intestinal epithelial immune signalling.

The work encompassed within this chapter demonstrates that, in both HEK 293 and HT29 cells, MIF may modulate responses to LPS via TLR-4 thus potentially playing a critical role in intestinal immune responses. In both epithelial cell line models, if the canonical MIF receptor complex (CD74, CD44) is present and or enhanced in its levels either via transfection or via upregulation after IFN-γ treatment this appears to affect responsiveness to MIF and mediate its effects on TLR-4 activity. In addition to this, we successfully show in our preliminary data that MIF modulates TLR-4-driven NF-kB activation, in epithelial cell lines, HEK-hTLR4-NF-kB-mCherry and HT29-NF-kB-mCherry, which is demonstrated using a colorimetric reporter assay measuring alkaline phosphatase activity and utilising a fluorescent mCherry reporter assay both of which are detected upon NF-kB activation.

As in studies by Roger *et al* (2001; 2003), our data initially indicated that Hs-MIF may augment TLR-4 signalling in HEK Blue™ hTLR4 cells when co-cultured with LPS. In comparison to this, we show that Ts-MIF-1 partially inhibits TLR4 signalling within the same conditions and this may indicate that MIF's evolutionarily conserved tautomerase site plays a role in the modulation of TLR4 signalling when considering that Ts-MIF-1 has a tautomerase activity which is six times greater than that of Hs-MIF-1. However, the fact that there is a complete absence of the oxidoreductase conferring CXXC site in Ts-MIF-1 proteins may also provide some clues as to the mechanisms required for TLR-4 modulation. Further studies will be required, which focus on utilising the MIF tautomerase mutants (P2G) or other MIF mutants (CXXC) generated earlier

within this study, to determine whether the tautomerase activity or other parts of the protein are responsible for the observed results.

One caveat to this study is that, MIF appears to opposingly modulate TLR-4 signalling with respect to the SEAP versus mCherry reporter assays. Nevertheless, there are several potential reasons for this: MIF may modulate secretion of alkaline phosphatase in a mechanism that is independent of NF-κB a theory that has been previously tested and evidenced in a study by Kleemann *et al* (2000) which demonstrated MIF inhibits AP-1 activity via Jab1 bypassing NF-κB. Use of a selective NF-κB inhibitor such as Sulfasalazine (Yeligar *et al*., 2009) or Bay 11-7085 (Clark *et al*., 2015) would confirm whether the SEAP activity observed is being modulated independently of NF-κB. Furthermore, SEAP is particularly susceptible to changes in ER stress (Kitamura and Hiramatsu, 2011; Lenin *et al*., 2015) and as MIF targets Jab1 which is involved in regulation of protein degradation it is possible that the variations observed within this study are, in part, a result of induced ER stress. Whilst this can be tested in several ways, an examination of MIF's ability to modulate ER stress is not within the remit of this study.

As studies have indicated that MIF signals via CD74 and CD44 (Gore *et al*., 2008; Mun *et al*., 2013; Schwartz *et al*., 2009; Shi *et al*., 2006) we transfected cells with a bicistronic vector expressing both sequences to observe whether overexpression of the MIF receptor affected MIF's response to LPS. Remarkably, the results showed that, in these conditions and those of IFN-γ primed HT29 cells, Hs-MIF-1 could inhibit TLR4-driven NF-kB activation in the presence of LPS. Disparities between MIF responses in HEK Blue™hTLR4 cells, as assessed by SEAP secretion, and HEK-hTLR4-NF-kB-mCherry transfected with pIRES-

CD74-CD44 and IFN-γ-primed HT29 cells may be due to the increase in MIF receptors, however, there may be additional factors relating to the significant inherent differences within the genomes of each cell line.

The lack of well-researched CD74 antibodies for the study of MIF signalling limited the analysis of cell surface expression of this key receptor in this study. Therefore, future optimisation of several commercially available CD74 antibodies against a positive control such as a CD74 expressing cell line like Raji or Daudi lymphoma cells, or a recombinant CD74 protein would be of significant benefit. Increasing the reliability of CD74 antibody detection will allow for rapid determination of cell surface CD74 in all cells.

Several possibilities exist for future work in order to determine how MIF regulates TLR-4 signalling. To determine whether MIF co-localises with TLR-4 *in vitro*, MIF proteins should be fluorescently tagged using a commercially available Alexa Fluor antibody whilst simultaneously staining the TLR4 receptor. Studies that focus on whether MIF binds directly to the TLR-4 receptor thereby competing with LPS will enable further studies to investigate the differences in binding affinity between WT and mutant mammalian and Trichinella derived MIF's. As the effect observed in this study was partial inhibition or potentiation of the TLR-4 signal pathway this may suggest that MIF interacts with downstream components of the pathway such as the earlier mentioned AP-1 transcription factor. Selective inhibition of AP-1 using a commercially available inhibitor, SR11032 (Ye *et al*., 2014), which importantly does not affect the levels of NF-kB, would provide evidence as to which part of the pathway MIF targets. In addition to our work examining MIF's role on TLR-4 signalling in epithelial cell lines, preliminary studies investigating the role of MIF on TGF-b signalling was

also undertaken. TGF-b is a critical regulator of intestinal barrier function and, like MIF, is produced in significant amounts by the intestinal epithelial cells. Though the data presented here did not show that MIF regulated TGF-b signalling in HEK-SBE-eGFP cells, the results are representative of one experiment so future work should firstly replicate our earlier experiments. Moreover, it is entirely possible that MIF modulation of TGF-b is cell specific and cannot be observed in HEK cells or that, as our TLR-4 experiments showed, CD74 is not trafficked to the cell surface preventing MIF from transducing a signal effectively. With this in mind, transient transfection of HEK-SBE-eGFP IC3 cells with the pIRES CD74 or pIRES CD74/CD44 vector would provide further evidence for reliance on a cell surface receptor for MIF to efficiently mediate its effects.

As we showed that HEK-SBE-eGFP IC3 cells also respond to members of the TGF-b family such as BMP-4, further studies could investigate MIF's role in BMP signalling alongside TGF-b experiments. This would determine whether MIF regulates the common SMAD, SMAD4 but by an alternative receptor or SMAD protein as TGF-b and BMP's have very distinct mediators of signalling. Aside from this, the data in this chapter clearly evidences that MIF plays a partial role in epithelial immune signalling.

Chapter 5: Evaluation of MIF's role in murine bone-marrow-derived-macrophages.

5.1. Introduction.

5.1.1. The history of Macrophages.

Macrophages are widely recognised as being the original responders to secreted MIF, elucidated in 1966, *in vitro* studies examining delayed-type hypersensitivity discovered that lymphocyte-derived MIF inhibited the random migration of macrophages (Deshmane *et al*., 2009). Macrophages were first discovered by Elie Metchnikoff in 1883 though the existence of phagocytes had been revealed some 20 years earlier by the presence of coal dust in the alveolar macrophages from miners (Cavaillon, 2013; Merien, 2016). Since that time, countless studies have taken place and a multitude of critical roles have been elucidated for macrophages including classical pro-inflammatory responses to upkeep of homeostasis by mechanisms comprising of clearance of cellular debris and tissue remodelling. Therefore, these cells are often termed 'janitorial' cells as they are complicit surveyors of their local area. Though macrophages are part of the innate immune system, they are a key player in directing adaptive immune responses utilising both contact-dependent mechanisms and chemical signals such as cytokine and chemokines.

The macrophage repertoire is extensive and many of the associated characteristics decidedly intricate; most circulating macrophages are derived from bone-marrow and begin their journey as monocytic cells, maturing as they exit the bone marrow after receiving chemical signals from the bone marrow stroma. On the other hand, tissue resident macrophages express markers associated with a more mature macrophage phenotype, many of which are

thought to develop prenatally, and this is largely linked to their anatomical niche (Haldar and Murphy, 2014). A well-documented theory for why this may occur is that tissue resident macrophages are primed and act as sentinels ready to respond to any incoming threat in a timely manner. Of interest to this study is that intestinal macrophages, despite having unique characteristics such as high IL-10 expression, are derived from bone marrow precursors and are consistently undergoing replenishment in order to facilitate efficient responses to infection. Several studies which have characterised intestinal macrophages have noted that, despite expressing elevated levels of IL-10, TLR stimulation via bacterial PAMPs leads to a classical inflammatory response (Bain *et al*., 2013; Bernardo *et al*., 2018)

Macrophages are known to secrete a vast selection of cytokines such as TNF-α (Parameswaran and Patial, 2010; Young *et al*., 2001), IL-6 (Braune *et al*., 2017; Chen *et al*., 2018; Gubernatorova *et al*., 2018; Luckett-Chastain *et al*., 2016), IL-8 (MCP-1 in mice) (Moore *et al*., 2015; Takada *et al*., 2010b; Takahashi *et al.*, 2009) and IL-10 (Elcombe *et al*., 2013; Sanin *et al*., 2015) but this is largely dependent on the chemical and mechanical signals they receive during their lifespan.

A distinguishing feature of macrophages is their ability to engulf surrounding pathogens (phagocytose) and kill them with cytotoxic particles within the phagosome. Though phagocytosis is a key feature of all macrophages, prior work suggests that alternately activated M2 polarised macrophages have an increased ability to phagocytose. Aside from phagocytosing pathogens, macrophages can ingest and clear cellular debris from dying cells preventing further unnecessary inflammation and studies have shown that this type of

internalisation can induce immune-suppressive cytokines such as IL-10 and TGF-β.

Though it has long been known that parasitic helminths can modulate local immune response via numerus mechanisms, studies investigating innate immune cells responses to *T. spiralis* infection or *T. spiralis* crude excretory/secretory (ES) products have demonstrated that, firstly, macrophages dominate the *T.spiralis* site of infection (Beiting *et al*., 2004) and secondly, they undergo alternative activation becoming regulatory M2 macrophages. ES perturbs NF-kB signalling in BMDMs and RAW 264.7 cells, inhibiting LPS-driven expression of pro-inflammatory cytokines IL-6 and TNF-α while increasing IL-10 production *in vitro* (Bai *et al.*, 2012; Du *et al*., 2014; Han *et al*., 2018). Additionally, a more recent study by Kang *et al* (2019) showed that, not only do peritoneal macrophages from *T. spiralis* infected mice produce high levels of Arginase-1 and CD206, but that adoptive transfer of bone marrow derived macrophages, cultured in the presence of ES, into DSS-induced colitis mice significantly reduce clinical scores. This study also confirmed that transferred macrophages had migrated to the site of inflammation verifying that it is specifically macrophages which are directly responsible for reducing DSS-scores.

A critical point of interest is that, although *T. spiralis* produces MIF at all stages of its life cycle, after embedding into the intestinal epithelium, molting and maturing into an adult worm, MIF expression is greatly increased and the amount of tautomerase activity increases 7-fold from the larvae stage (D.B. Guiliano, unpublished). This might suggest that *T. spiralis* utilises MIF to modulate intestinal immune responses as part of the adaption to the enteral niche.

In this study, we propose that Ts-MIF proteins modulate macrophage activation using mechanisms that contrast with mammalian MIF and that this may be conferred by MIF's conserved tautomerase site.

5.2. MIF in macrophage studies.

Studies investigating macrophage subsets such as the pro-inflammatory M1 and alternatively activated M2 'healing' types have revealed that endogenous MIF expression is essential for M1 polarisation and this has been demonstrated in murine MIF$^{-/-}$ models of obesity and tumourigenesis. In obesity studies, MIF was shown to be an essential upstream regulator of the M1 cytokine profile as inhibiting expression of MIF led to complete absence of M1 macrophages whilst M2 macrophages were significantly increased. Intriguingly, within the context of tumourigenesis, MIF was found to promote M2 polarisation indicating that the modulatory actions of MIF are, in part, contextually governed by the local environment. Sánchez-Zamora *et al* (2016) demonstrated, in a type 1 Diabetes mouse model, that macrophages in *MIF*$^{-/-}$ mice have lower levels of antigen-presenting and co-stimulatory molecules such as MHC-II, CD80, CD86 and TLR-4 than that of WT mice and these deficits can be recovered by the addition of exogenous recombinant MIF. Additionally, studies utilising mouse models of obesity indicated that *MIF*-deficient mice have improved glucose metabolism and significantly decreased levels of adipose tissue while also inhibiting pro-inflammatory cytokine, TNF-α, which was also associated with a decrease in adipose tissue macrophage infiltration (Finucane *et al*., 2014). In contrast, within the tumour microenvironment MIF plays a protective role by preventing the expansion of tumour growth. Two pivotal studies demonstrate this: Castro *et al* (2017) revealed that Bevacizumab resistance in glioblastoma patients is a consequence of increased M2 tumour-associated-macrophages (TAMs) at the

tumour periphery. Microarrays performed within the study indicated that this is most likely attributable to a decrease in MIF expression in patients receiving Bevacizumab. Additionally, *MIF*$^{-/-}$ mice with colitis-associated-cancer have significantly decreased levels of TAMs and increased tumour burden as a result (Rodriguez-Sosa *et al*., 2017). Conversely, Yaddanapudi *et al* (2013) show that TAMs from *MIF*$^{-/-}$ melanoma tumour bearing mice secrete vast levels of inflammatory markers such as TNF-α, IL-12 and iNOS when compared to their WT counterparts. Moreover, cytokines associated with the resolution of inflammation were significantly decreased in MIF-deficient TAMs suggesting that, in this context, MIF is required for the polarisation and expansion of an M2 TAM phenotype. Taken together, these studies indicate that MIF's mechanisms of action are highly context dependant and may modulate both inflammatory and regulatory responses.

In addition to the aforementioned research, additional studies have revealed, utilising *MIF*$^{-/-}$ mice, that MIF is essential for the proper induction of TNF-α (Bozza *et al*., 1999; Thierry Roger *et al*., 2001), IL-6 (Roger *et al*., 2016), MCP-1 (Barnes *et al*., 2013; Gregory *et al*., 2006a; Xie *et al*., 2016) and IL-10 (Rodriguez-Sosa *et al*., 2017) and that loss of cytokine production disrupts macrophage function by altering polarisation or preventing the recruitment of monocytes to the inflammatory location.

While numerous studies have elucidated a role for MIF in both regulatory and proinflammatory immune responses, to date, none have characterised the effect of exogenous MIF and tautomerase-null MIF mutants on macrophage cytokine secretion and its phagocytic capabilities.

5.3. Chapter aims and objectives.

To examine the role of MIF on macrophage activation, including whether MIF and MIF P2G mutants modulate inflammatory responses by regulating IL-6, MCP-1, IL-10 and TNF-α, the following aims were proposed:

1. Utilising qPCR assess transcriptional changes in three key macrophage derived cytokines IL-6, MCP-1 and IL-10 after exposure to LPS +/- MIF homologues and mutants.
2. Assess secretion of the proinflammatory cytokines IL-6 and TNF-α, in media from MIF treated macrophage cultures, utilising ELISA.
3. Ascertain whether MIF and tautomerase-null MIF can modulate phagocytosis capabilities in primary murine BMDMs.

5.4. MIF modulates the macrophage cytokine transcriptome in response to LPS.

To determine whether administration of exogenous MIF or MIF P2G tautomerase mutants induced a specific cytokine profile in macrophages, murine bone marrow derived macrophages from C57BL/6 female mice aged six to eight weeks old were generated. Figure 5.1. depicts the gating strategy used to assess F4/80 expression in M-CSF matured macrophages after a seven-day incubation period and demonstrates the successful generation of macrophages with >90% expressing F4/80, a glycoprotein found exclusively on the surface of murine macrophages.

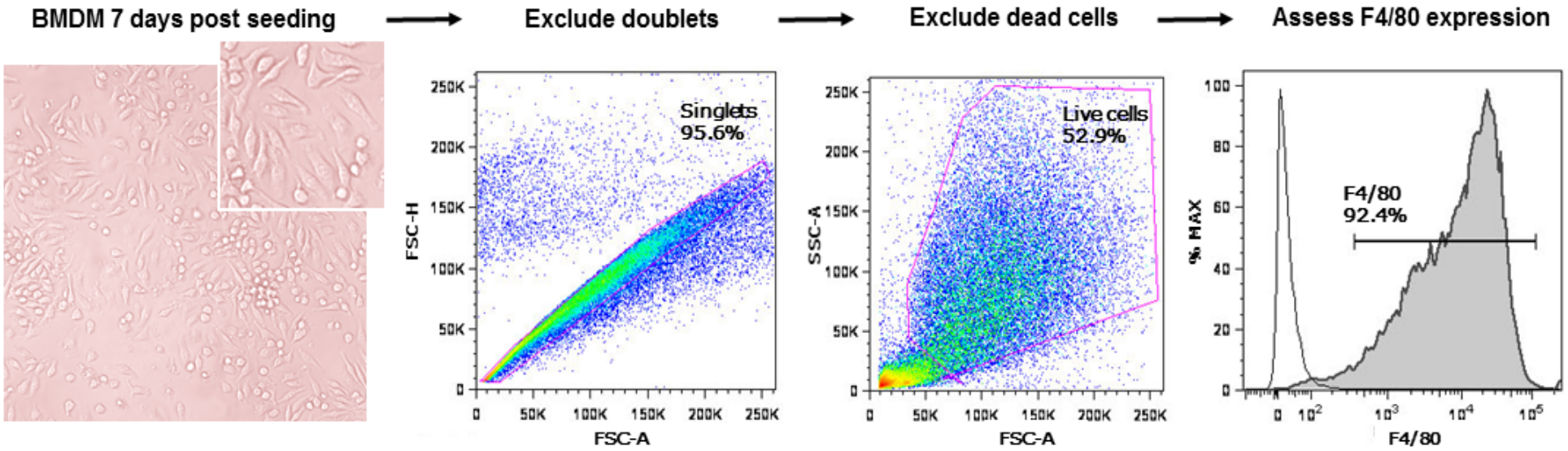


Figure 5. 1 Successful generation of bone marrow derived macrophages from C57BL/6 female mice. Bone marrow from the femurs of 6-8-week female C57BL/6 mice was seeded at 1 x 10^6/mL, at a total volume of 10mL, in a sterile petri dish. Cells were grown in the presence of 30% L292-cell conditioned media for a total period of 7 days with 10mL fresh media added at day 3 and 5 after which cells were assessed for the expression of pan-macrophage marker, F4/80. From left to right: figure shows the successful development of macrophages (as shown by light microscopy); during FACs assessment, doublets were excluded to prevent any false signal being included in analysis; dead cells are omitted to ensure detected F4/80 expression is in live cells; histogram depicting F4/80 expression (grey filled) versus unstained cells (No fill) and the gating strategy used for F4/80 analysis. Macrophages yielded from routine cultures were >90% F4/80.

To test whether exogenous MIF or MIF P2G mutants modulate macrophage activation, murine BMDMs were incubated with 10ng/ml LPS +/- 100ng/ml MIF for 20 hours before performing transcriptomic analysis to examine the relative levels of IL-6, MCP-1 and IL-10 using GAPDH as the reference housekeeping control gene. Representative results for IL-6 expression are depicted in figure 5.2 which shows that Mm-MIF-1 significantly ($p \leq 0.05$) enhances IL-6 mRNA levels when incubated with LPS in comparison to LPS alone. Additionally, Mm-MIF-1 P2G failed to increase IL-6 expression when co-incubated with LPS suggesting that the tautomerase site is required for the rise in IL-6 transcription. However, in contrast to this, Ts-MIF-1 and Ts-MIF-1 P2G marginally increased IL-6 expression in response to LPS which was a surprising result considering that the tautomerase activity of Ts-MIF-1 is three times higher than that of Mm-MIF-1, as shown in chapter 3.

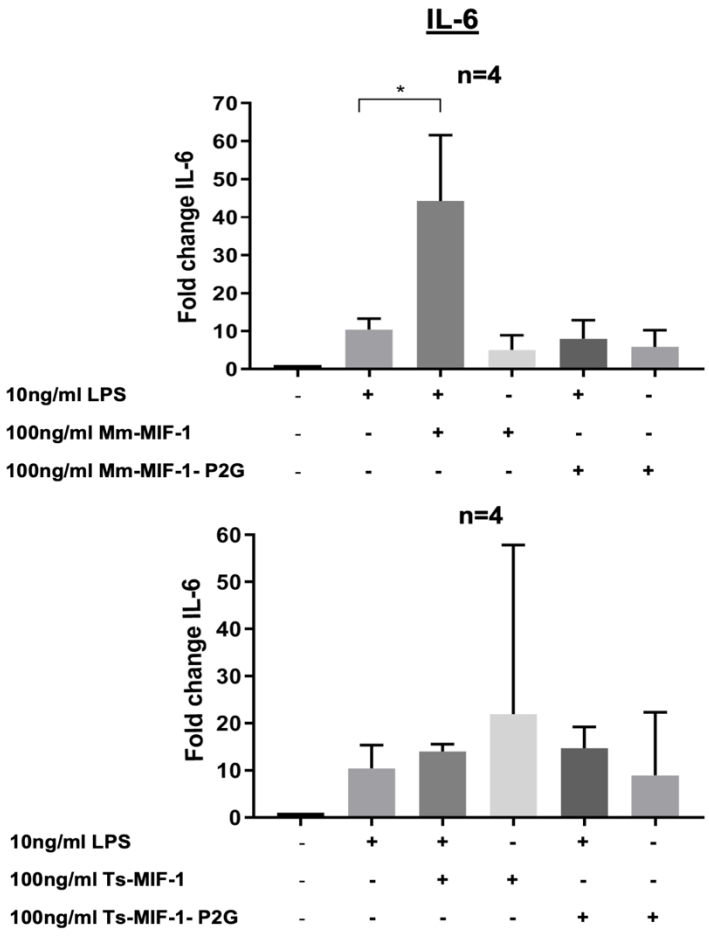


Figure 5. 2 MIF homologues modulate the IL-6 transcriptional responses to LPS in BMDMs. The graphs above show the qPCR analysis of BMDMs in response to 10ng/ml LPS +/- 100ng/ml MIF. Data represents the mean ±SEM (n=4) of the fold change. All values were assessed utilising the Pfaffl equation prior to statistical testing. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

MCP-1, the murine equivalent to human IL-8, is a chemokine which is highly expressed by activated macrophages and chemoattracts additional monocytes and macrophages to the site of inflammation (Deshmane *et al*., 2009). MIF has previously been shown to upregulate MCP-1 and the absence of MIF leads to abrogated MCP-1 responses including decreased leukocyte and monocyte recruitment. To this end, we predicted that MIF's tautomerase site may play a role in macrophage-derived MCP-1-driven chemotaxis. Therefore, we assessed the mRNA expression levels of MCP-1 in response to LPS in the presence or absence of MIF homologues.

Figure 5.3 shows that, in the presence of LPS, Mm-MIF-1 partially inhibits the expression of MCP-1 (no significance) and this response is not affected by the absence of MIF's tautomerase site. On the other hand, Ts-MIF-1 and Ts-MIF-1 P2G appear to act in concert with LPS to increase MCP-1 transcripts indicating that mammalian MIF elicits different responses to helminth-derived MIF that are independent of the tautomerase activities.

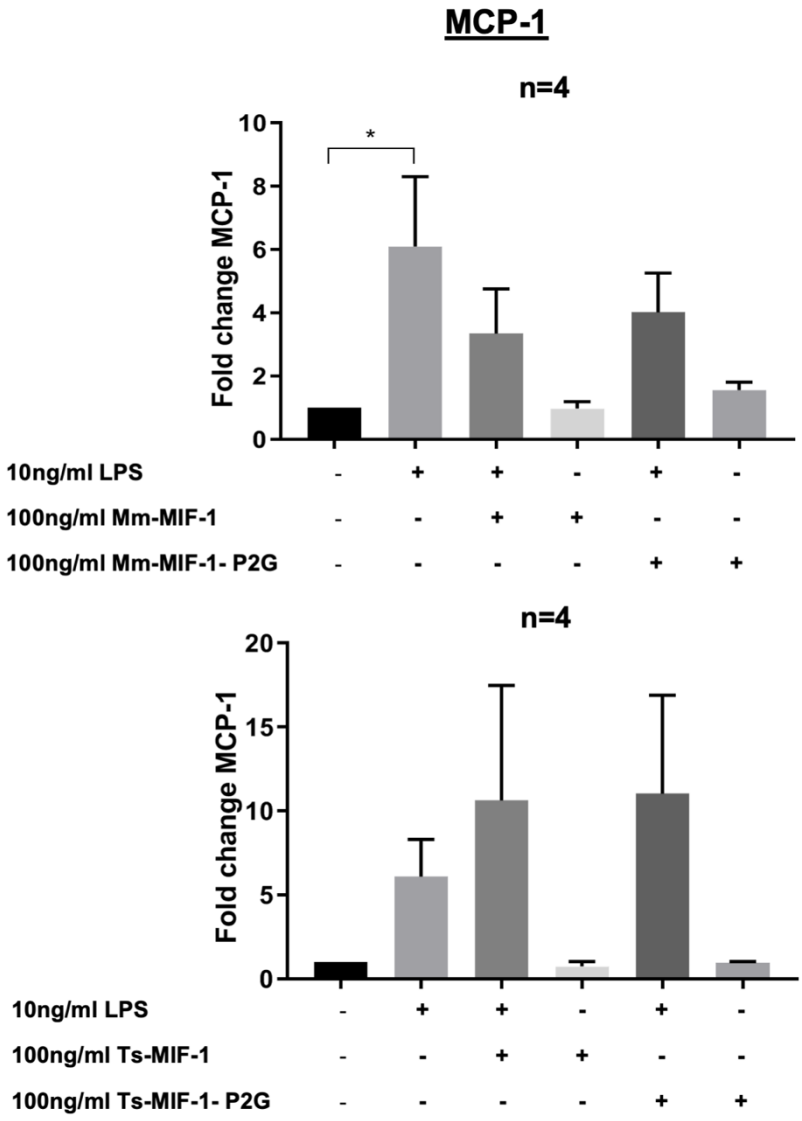


Figure 5. 3 MIF homologues modulate the MCP-1 transcriptional responses to LPS in BMDMs. The graphs above show the qPCR analysis of BMDMs in response to 10ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=4) of the fold change. All values were assessed utilising the Pfaffl equation prior to statistical testing. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

When we examined the effects of LPS and MIF homologues on IL-10 expression in macrophages. Aside from a small decrease in samples cultured with LPS and Mm-MIF-1 P2G, there were no significant differences between groups (Figure 5.4). This likely means that MIF does not modulate LPS-driven IL-10 responses in BMDM's *in vitro*.

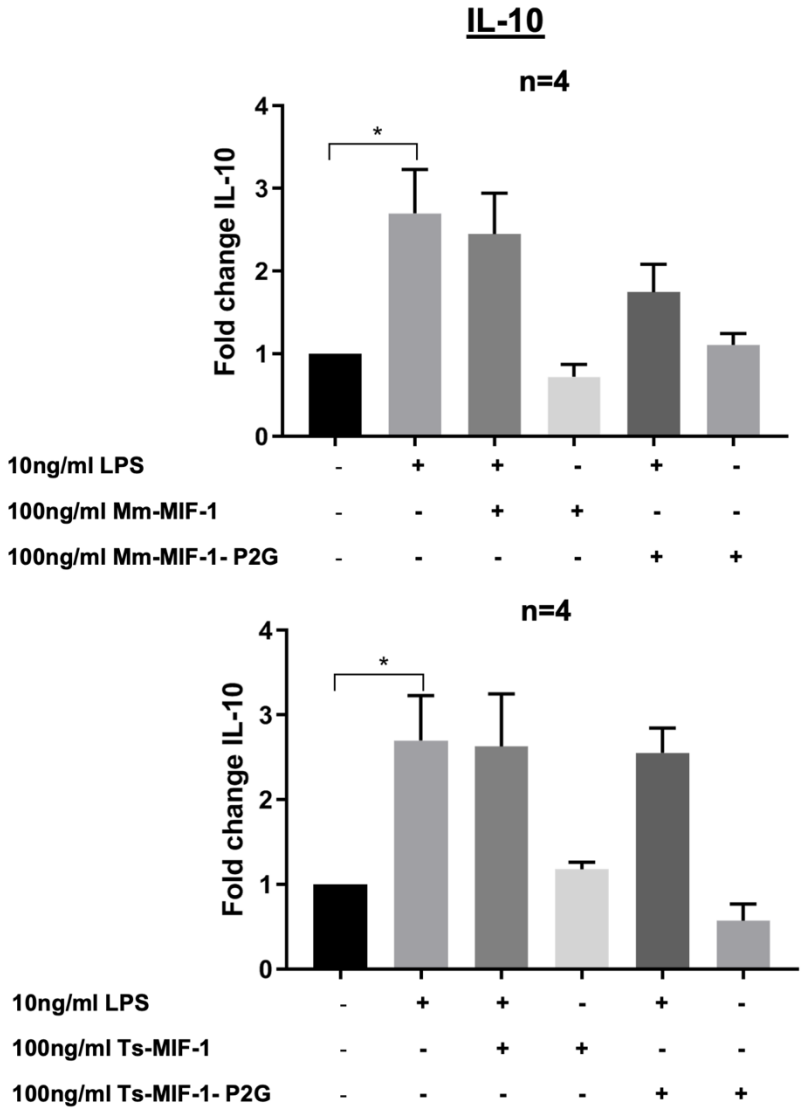


Figure 5. 4 MIF homologues do not modulate the IL-10 transcriptional responses to LPS in BMDMs. The graphs above show the qPCR analysis of BMDMs in response to 10ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=3) of the fold change. All values were assessed utilising the Pfaffl equation prior to statistical testing. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

To further expand our transcriptome studies levels of two proinflammatory cytokines IL-6 and TNF-α were assessed in the culture supernatants of MIF and LPS stimulated BMDMs by ELISA. Figure 5.5. shows that, whilst the amount of IL-6 and TNF-α increased considerably in the culture supernatants of LPS stimulated macrophages, there were no notable differences in samples derived from cells co-incubated with LPS and MIF or in samples incubated with MIF alone.

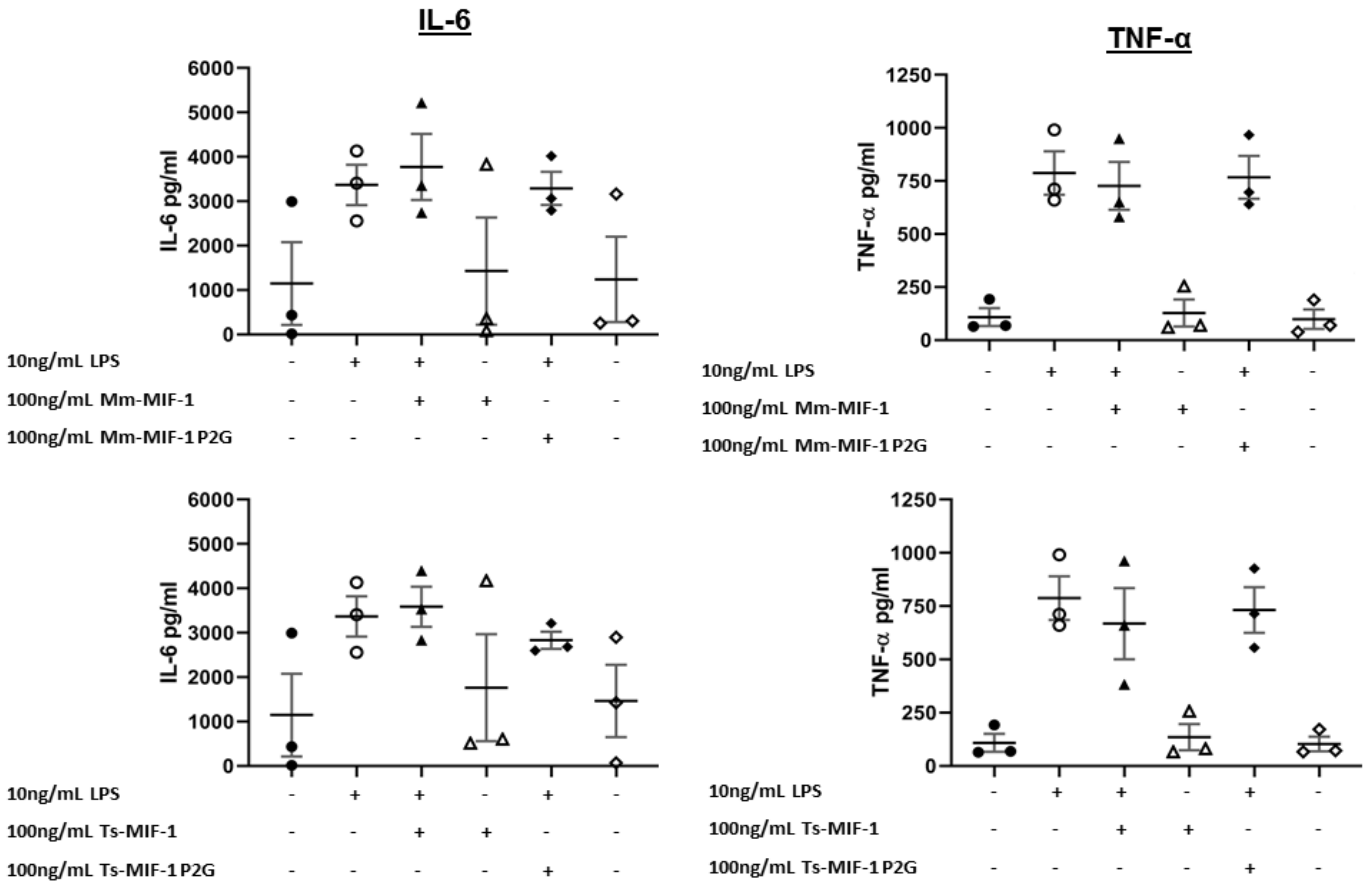


Figure 5. 5 MIF homologues do not modulate the secretion of IL-6 and TNF-α from BMDMs after LPS stimulation. The graphs above show the secreted cytokine (pg/ml) levels of IL-6 and TNF-α within BMDMs in response to 10ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=3) of the fold change. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

5.5. Discussion of MIF's role in modulation of cytokine and phagocytic responses to LPS in BMDM's

Whilst MIF's role in macrophage development and activation has been extensively explored, only limited studies have investigated the role that MIF's tautomerase activity in MIF's immunomodulatory actions. Nor have these studies examined the differences between mammalian and helminth-derived MIF homologues.

In this chapter, the successful generation of F4/80 positive macrophages was achieved by differentiating bone marrow derived monocytes using conditioned media from L929 murine fibroblast cells. L929 cells secrete large quantities of active M-CSF which is essential for the propagation and differentiation of macrophages. Using these BMDMs the relative transcriptional levels of IL-6, MCP-1 and IL-10 were assessed after stimulation with LPS in the presence or absence of different MIF recombinants. These experiments yielded clear instances where differential activities between mammalian wild-type MIFs and mutants lacking tautomerase activity or murine and Trichinella MIF homologues could be observed.

In experiments focussing on IL-6, Mm-MIF-1 significantly augmented the LPS-mediated increase in IL-6 transcripts whilst in macrophages incubated with Mm-MIF-1 alone there were no observable effects. This data is consistent with results found by Kudrin *et al* (2006) showing that treatment of murine macrophages with MIF alone is not capable of inducing IL-6 expression. However, we show here that Mm-MIF-1 acts in concert with LPS to potentiate the transcriptional levels of LPS-mediated IL-6. Contrastingly, Mm-MIF-1 P2G, did not augment IL-6 responses in the presence of LPS suggesting that the tautomerase enzymatic

activities of Mm-MIF-1 may be responsible for modulating the IL-6 transcriptional responses. The mechanism that this occurs through remains to be determined although one possible mechanism is that the tautomerase activity is required for receptor binding. Disruption of Pro2 and the subsequent tautomerase activity may prevent binding of murine MIF to the canonical MIF receptor CD74. Evidence for this is demonstrated in a study by Senter *et al* (2002) showing that MIF tautomerase inhibition by *N*-acetyl-*p*-benzoquinone imine induces a conformational change in the protein structure effecting MIF's ability to override glucocorticoid suppression by dexamethasone.

In terms of cytokine modulation by *Ts* derived MIF, the transcriptional levels of IL-6 were largely unaffected in the presence of LPS regardless of whether the tautomerase site was present demonstrating that; 1) the conserved tautomerase activity of *Ts* MIF is dispensable for the enhancement of LPS-mediated IL-6 transcriptional responses in BMDMs and, 2) Ts-MIF-1 appears to act differently to Mm-MIF-1 in terms of the transcriptional regulation of IL-6 which may be an immune-subversion mechanism utilised by *T. spiralis* to prevent immune recognition. This finding indicates that factors beside the tautomerase activity are responsible for the modulation of LPS-directed IL-6 responses and this may include the oxidoreductase activity, nevertheless, future work utilising oxidoreductase mutant proteins will be required to elucidate if the CALC motif plays a role in directing macrophage development.

IL-6 is a key player in driving intestinal immune responses such as ILC3, Th17 and Th22 polarisation and dysregulation of IL-6 is associated with several intestinal immune disorders such as colitis. Several studies have demonstrated that macrophages are the principal innate immune cell surrounding the site of

T.spiralis infection in muscle from C57Bl/6 mice (Beiting *et al*., 2004). In addition, another study has shown ES from *T. spiralis* is capable of abrogating LPS-mediated IL-6 production in J774A.1 macrophages (Bai *et al*., 2012), this data may provide clues as to the identity of the ES protein components responsible for immune-subversion in macrophages which is a novel finding. IL-6 expression in macrophages is generally accepted to be associated with a classical inflammatory M1 macrophage phenotype. Our data demonstrates that whilst Mm-MIF-1 augments this response to LPS, Ts-MIF-1 has no effect on it's expression. Though the oxidoreductase activity may provide some clues as to what is conferring IL-6 modulating activities, this is unlikely considering that Mm-MIF-1 P2G retains the oxidoreductase motif yet does not modulate IL-6 transcriptional responses. Furthermore, as discussed in chapter 3, studies analysing the protein structure and enzymatic activities of MIF have shown that mutation of the proline site to an alternative amino acid like glycine does not alter the oxidoreductase activities of MIF when assessed within the insulin reduction enzyme assay.

In addition to the above, we also found evidence that Mm-MIF-1 inhibited LPS-mediated transcription of MCP-1 but this was not influenced by substituting the tautomerase-conferring proline residue for an inactive glycine residue in Mm-MIF-1 P2G demonstrating that the tautomerase activities of MIF may only be partially responsible for cytokine regulation and additional factors like the oxioreductase activity may be a requirement for full enzyme activity *in vitro* and *in vivo*. Contrastingly, Ts-MIF-1 increased LPS–driven MCP-1 mRNA levels and like the murine MIF this was not dependant on the associated tautomerase activity.

MCP-1, otherwise known as CCL2, is a monocyte chemoattractant protein expressed by macrophages amongst other immune cells. Although MCP-1 recruits' monocytes to the site of infection, several studies have shown that MCP-1 expression drives the development of AAMs by increasing IL-6 which, in turn, re-amplifies MCP-1 expression in a loop mechanism (Roca *et al*., 2009). Conversely, Sierra-Filardi *et al* (2014) utilised a $CCR2^{-/-}$ murine model to demonstrate that abrogation of MCP-1 signalling increased IL-6 expression in LPS-treated macrophages. In addition to this, numerous studies have elucidated contrasting roles for MCP-1 in the polarisation of Th1 and Th2 responses (Helmby and Grencis, 2003; Huang *et al*., 2001; Lu *et al*., 1998; Omata *et al*., 2002). These studies suggest that MCP-1, like many other cytokines and chemokines, acts accordingly with the environment in a temporal and contextual manner. In this regard, we show that both Mm-MIF-1 and Ts-MIF-1 may act directly or indirectly, via MCP-1, to modulate IL-6 expression using distinct mechanisms although what these might be remain unclear. Future work in this study should focus on determining whether the oxidoreductase site plays a role in macrophage modulation due to the fact that Ts-MIF-1 lacks the oxidoreductase-conferring CXXC motif and thus in many respects might mimic the activities of murine MIF CXXC mutant.

Despite being unable to demonstrate that mammalian MIF or *Ts*-derived MIF modulated IL-10 expression in response to LPS, it is important to note that studies eluding to MIF modulation of IL-10 responses frequently report correlative data patterns in clinical models of inflammatory disorders such as sepsis. Additional work is required to determine whether there is a causative relationship between MIF signalling and IL-10 production. MIF mediated modulation of cytokine transcriptional responses are summarised in figure 5.7.

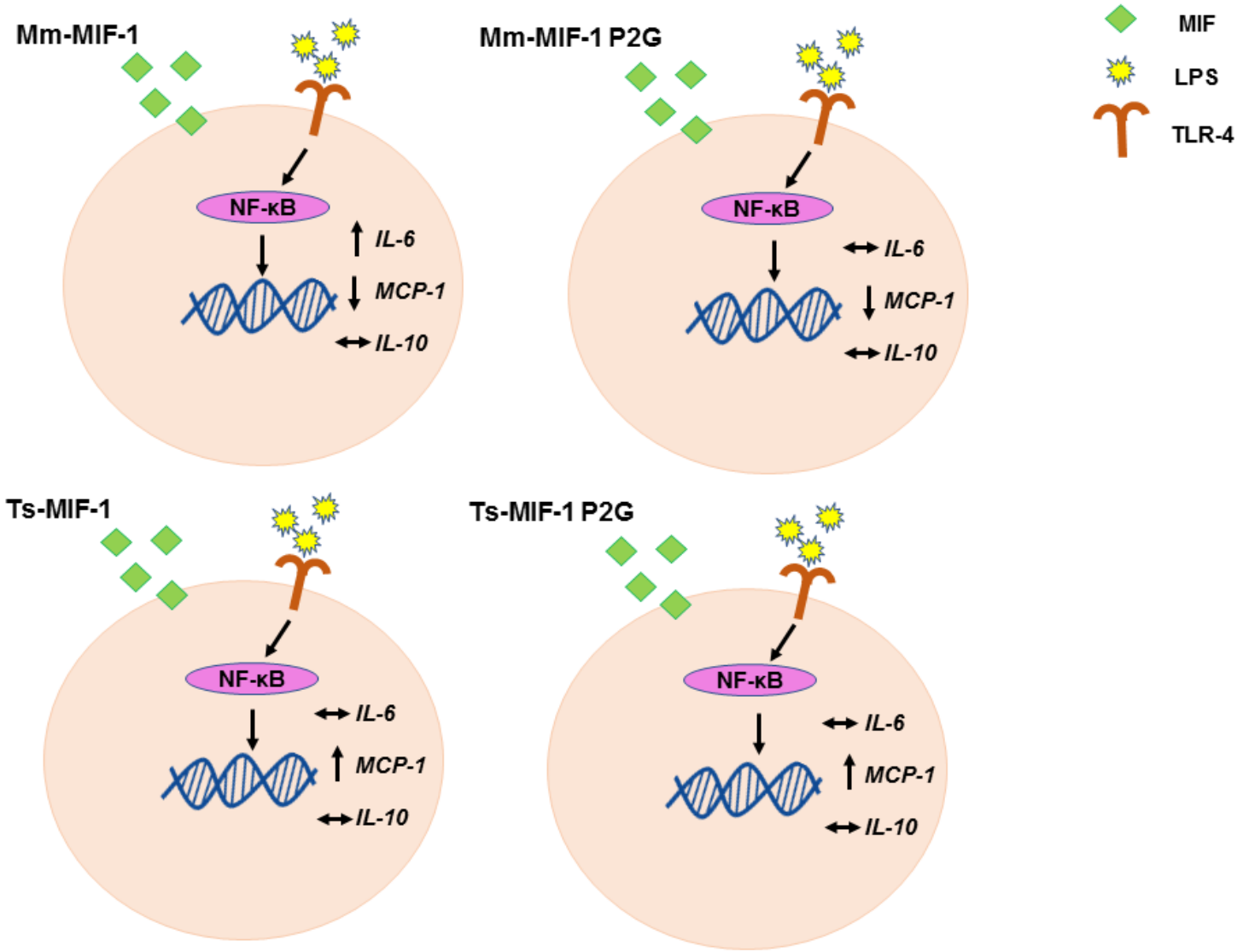

Mm-MIF-1
NF-κB
IL-6
MCP-1
IL-10
Mm-MIF-1 P2G
NF-κB
IL-6
MCP-1
IL-10
MIF
LPS
TLR-4
Ts-MIF-1
NF-κB
IL-6
MCP-1
IL-10
Ts-MIF-1 P2G
NF-κB
IL-6
MCP-1
IL-10

Figure 5. 6 Summary of MIF modulation of LPS-driven cytokine transcriptional responses in BMDMs. Murine MIF potentiates the macrophage IL-6 transcriptional response to LPS and mutation of the tautomerase-conferring Proline site abolishes this effect. Transcription of the chemotactic MCP-1 cytokine, in the presence of LPS, is inhibited by murine MIF regardless of the tautomerase site. LPS-mediated IL-6 transcription is unaltered by *Trichinella spiralis* derived MIF whilst MCP-1 levels are augmented. The tautomerase site of MIF appears to play a critical role in murine MIF-modulation of IL-6 transcription. MIF does not play a role in the modulation of IL-10 in response to LPS in BMDMs.

In this study there were no observable differences in the levels of IL-6 or TNF-α found in macrophage culture supernatants in response to LPS and MIF homologues or in samples incubated with MIF alone despite the results of our transcriptional assessment and other previously published studies. Regardless of this there are notable differences between this study and in studies that allude to MIF modulation of IL-6 and TNF-α secretion: Kudrin *et al* (2006) successfully demonstrated that recombinant human MIF enhanced LPS driven TNF-α secretion in human macrophages derived from peripheral monocytes. However, the concentration of LPS used to stimulate macrophages was 1ng/mL which is 10-fold lower than the LPS concentration utilised within this study; consequently, additional works investigating the effect of MIF on pro-inflammatory cytokine secretion in macrophages should introduce a titration of LPS and MIF to determine the precise point of interaction. Within the same study it was also demonstrated that MIF, up to concentrations of 10μg/mL, had no effect on the

secretion of several additional pro-inflammatory cytokines typically associated with MIF, such as IL-6. Contrastingly, a study by Prieto-Lafuente *et al* (2009) established that recombinant murine MIF significantly induces IL-6 secretion in BMDMs from C57BL/6 mice. Upon further examination, the concentrations of MIF used were found to be excessively high, from 1µg/mL - 10µg/mL, suggesting that any result would have limited biological relevance. Furthermore, recombinant MIF proteins used within the Prieto-Lafuente study were not purified with an additional 'polishing' stage to ensure the complete removal of endotoxin. Instead the study utilised the antibiotic Polymixin B (PmB) derived from *Bacillus polymyxa*, to try and inhibit any effects from endotoxin contamination. In spite of this, the effectiveness of PmB as an inhibitor of LPS in cell based assays using antigen-presenting cells is somewhat disputed with one study demonstrating that PmB has limited efficacy as an inhibitor of LPS when assessing the secretion of cytokines IL-6 and TNF-α in dendritic cells *(*Tynan *et al*., 2012). As the purification protocol of recombinant MIF proteins used within this study were based on methods used by Kudrin *et al* (2006) it is perhaps unsurprising that our data failed to show any response to MIF in ELISA assays. This is likely to be a consequence of numerous contributing factors including the kinetics of protein turnover of these cytokines. Introducing a time course study in future macrophage assays may allow for the detection of cytokines at earlier or later timepoints.

Further explanations for the lack of translatability from transcriptome to proteome exist and include the inherent stochastic and dynamic nature of gene expression. Regardless of the fact that the work encompassed within this chapter did not show that MIF regulates the secretion of cytokines IL-6 and TNF-α at 20 hours after incubation, the possibility that MIF transcriptionally regulates cytokines, as show in our data, may indicate that alternative targets are activated as result.

This is investigated in detail in chapter 6 whereby RNA sequencing technology is utilised to assess MIF's transcriptional profile in macrophages.

Nonetheless, future work examining MIF's role as a regulator of macrophage function will be required to characterise macrophage phenotypes by looking at cell-surface markers and transcription factors in order to understand the precise differences in macrophage responses to mammalian and Trichinella -derived MIF proteins. Additionally, knock-out of endogenous MIF using siRNA techniques would be a rapid and efficient way of assessing whether observed traits are a result of exogenous recombinant MIF or the subsequent upregulation of endogenous MIF.

Chapter 6: Transcriptomic analysis of MIF's role using RNA Sequencing.

6.1. Introduction.

6.1.1. Transcriptomic modulation by MIF homologues.

Despite the fact that many aspects of MIF effects on different cell types or contribution to diseases has been extensively explored, information regarding transcriptional responses to exogenous MIF protein treatment is lacking. The few studies which have attempted to unravel transcriptional responses to MIF have been focused on identifying specific markers of immune regulation which are modulated including cytokines IL-6 and IL-8 and chemokine, CCL-2 (Chuang *et al*., 2010a; Gregory *et al*., 2006b). These studies have primarily relied on using targeted techniques such as RT-PCR and qPCR and, whilst there is the suggestion that MIF may directly or indirectly modulate these specific targets, its precise mechanism of action on the cellular transcriptome remains elusive.

Several studies have revealed an essential role for MIF in the regulation of immune responses by counter-regulating glucocorticoid suppression of inflammatory mediators such as TNF-α or modulating the expression and localization of innate immune receptors such as TLR-4 (Bernhagen *et al*., 1998; T. Calandra *et al*., 1995; Calandra and Bucala, 1997; Roger *et al*., 2003b; T. Roger *et al*., 2001), maintenance of barrier function (Maaser *et al*., 2002b; Man *et al*., 2008b; Vujicic *et al*., 2018b) and cell cycle progression (Fingerle-Rowson *et al*., 2003; Fingerle-Rowson and Petrenko, 2007; Welford *et al*., 2006). The majority of these studies utilise murine MIF KO models to elucidate the effect of MIF on transcription. A study investigating MIF's role in maintaining barrier

function in isolated colonic epithelial cells revealed that global KO of MIF increased levels of transcripts associated with the formation of cell-to-cell junctions such as *zonula occludens-1* and *claudin 2* whilst causing a decrease in the transcriptional levels of *E-cadherin* and *occludin* suggesting that MIF is essential for the maintenance of barrier integrity. Interestingly, the same study also noted significantly increased levels of the Th1-associated cytokine, *IL-18*, a by-product of microbial receptor activation which may account for the dysregulation of the previously mentioned transcripts. However, a recent study by (Pacheco-Fernández *et al*., 2019) (2019) investigating the effect of MIF KO in dextran sodium sulfate (DSS)-treated mice revealed that, within this environment, IL-18 is decreased whilst iNOS, Arg-1 and IL-17 are all increased compared to their WT counterparts indicating that the absence of MIF leads to the development of inflammatory macrophages, and potentially the suppression of Th2 phenotypes, driven by intestinal epithelial cells. MIF has also been shown to be a critical negative regulator of pro-inflammatory cytokine production in some cancer models where tumour associated macrophages from $MIF^{-/-}$ mice could not fully supress T-cell activation and enhanced transcription levels of TNF-α, COX-2, IL-12 and iNOS in peritoneal exudate cells which typically include high levels of macrophages (Kavitha Yaddanapudi *et al*., 2013) The observation that MIF may also promote an 'anti-inflammatory' macrophage phenotype is further examined in a study using primary bone marrow derived macrophages stimulated with recombinant MIF in which long-term (72 hours) culture of cells incubated with MIF increased the transcription of TGF-β, IL-10, PD-L1, and Arg-1 significantly in comparison to unstimulated macrophages. Another study utilising microarray and qPCR to assess the effect of siRNA-mediated MIF knockdown in HEK293 cells uncovered a number of novel transcriptional targets associated with NF-kB

signalling (GADD45β, IKBα) and transcription factors linked to cell cycle progression (c-Myc, FOXO4) suggesting that HEK cells deprived of MIF were locked in the G_0/G_1 phase (Liu *et al*., 2012). While these studies have revealed numerous vital findings, particularly in respect to MIF's role as a modulator of immune responses, how MIF treatment alters the global transcriptional response in macrophages has not been directly examined.

While MIF's tautomerase site is highly conserved across species, few attempts have been made to understand its role in MIF function or the target of its enzymatic activity. Studies that have investigated the effect of tautomerase mutants *in vitro* have revealed conflicting results. Swope *et al* (1998) demonstrated that substitution of the tautomerase conferring proline to glycine reduced superoxide production in human neutrophils by seventy-five per cent. Another study revealed that covalent modification of the proline site by the acetaminophen metabolite *N*-acetyl-*p*-benzoquinone imine (NAPQI) reduced cell surface binding in human microvascular endothelial cells (Senter *et al*., 2002b). Additionally, with respect to MIF's distinctive ability to counter-regulate glucocorticoid suppression, the importance of the tautomerase conferring Pro2 site is in dispute. This biological activity appears to be dependent on the chosen substituted amino acid. In monocytes, substitution of proline to serine maintains MIF's counter regulation of glucocorticoid suppression despite abolishing the tautomerase enzyme activity (Bendrat *et al*., 1997). Conversely, a catalytically inactive MIF mutant, whereby an alanine residue is inserted between Pro2 and Met3, does not override glucocorticoid suppression in LPS-stimulated human monocytes. Despite this, the significance of MIF and MIF's conserved tautomerase site remains ambiguous, particularly in respect to macrophage

function and with this is mind, this study utilised RNA sequencing technologies to fully explore the macrophage transcriptional landscape in response to MIF and MIF P2G recombinant proteins.

In parallel with the studies of endogenous murine and human MIFs, homologues derived from pathogens have also been extensively studied revealing distinct and context-dependent transcriptomic responses. MIF is commonly expressed and secreted by protozoan parasites and has been shown to drive pathogenesis and modulate host immunity. For instance, MIF derived from *Plasmodium berghei* supressed PMA induced AP-1 transcription in HEK 293 cells pointing towards a potential role for Pb-MIF in cell cycle regulation (Augustijn *et al*., 2007) whilst *Trypanosoma cruzi* infected hearts from MIF$^{-/-}$mice displayed significantly increased expression of IFN-γ but reduced IL-12p35, IL-12p40 and IL-23 expression relative to WT MIF mice (Reyes *et al*., 2006). Moreover, IL-22 expression in ileal explants from *Toxoplasma gondii* infected MIF$^{-/-}$ is significantly increased compared to WT MIF though, surprisingly, TNF-α and IL-12 transcripts were decreased. In addition to the aforementioned parasites *Trichinella spiralis*, a well characterised intestinal nematode, has been shown to produce several MIF homologues (Tan *et al*., 2001, Guiliano. D.B. unpublished). The protein sequence of Ts-MIF-1 contains the conserved tautomerase-conferring Pro2 site but, remarkably, the activity of this is 6-fold greater than that of mammalian MIF suggesting that the tautomerase site may be of greater importance for infection purposes. This study utilises WT and tautomerase mutant Ts-MIF-1 proteins to assess their role on the macrophage transcriptome.

6.1.2. Utilising RNA sequencing technologies to assess the transcriptome.

Assessing a cell or tissue's transcriptome allows for quantification of varying types of transcript including mRNA and small RNA and the ability to comparatively deduce the relative expression of each transcript. RNA-sequencing profiles the transcriptome utilising deep sequencing technologies and the advantages of RNA sequencing over microarray assays is discussed below.

RNA sequencing was elected as an alternative to microarray for transcriptomic analysis in this study for several carefully considered reasons. RNA sequencing lacks the need for transcript-specific probes like many microarray-based assays, providing an unbiased view into the transcriptome, including novel transcripts, splice variants and nucleotide variants. Moreover, RNA-sequencing has a larger dynamic range allowing greater sensitivity for transcripts expressed at very low or more accurate quantification of very high-level transcripts. In addition to the above, several studies have undertaken comparative studies investigating whether one type of transcriptomic platform offers significant advantages over another with the general consensus being that RNA sequencing is far superior to microarray for transcriptomic analysis (Rai *et al*., 2018; Rao *et al*., 2018; Zhao *et al*., 2014)

6.2. Research aims and objectives.

To examine the bone marrow derived macrophage transcriptome in response to MIF and MIF P2G mutants the following aims were proposed:

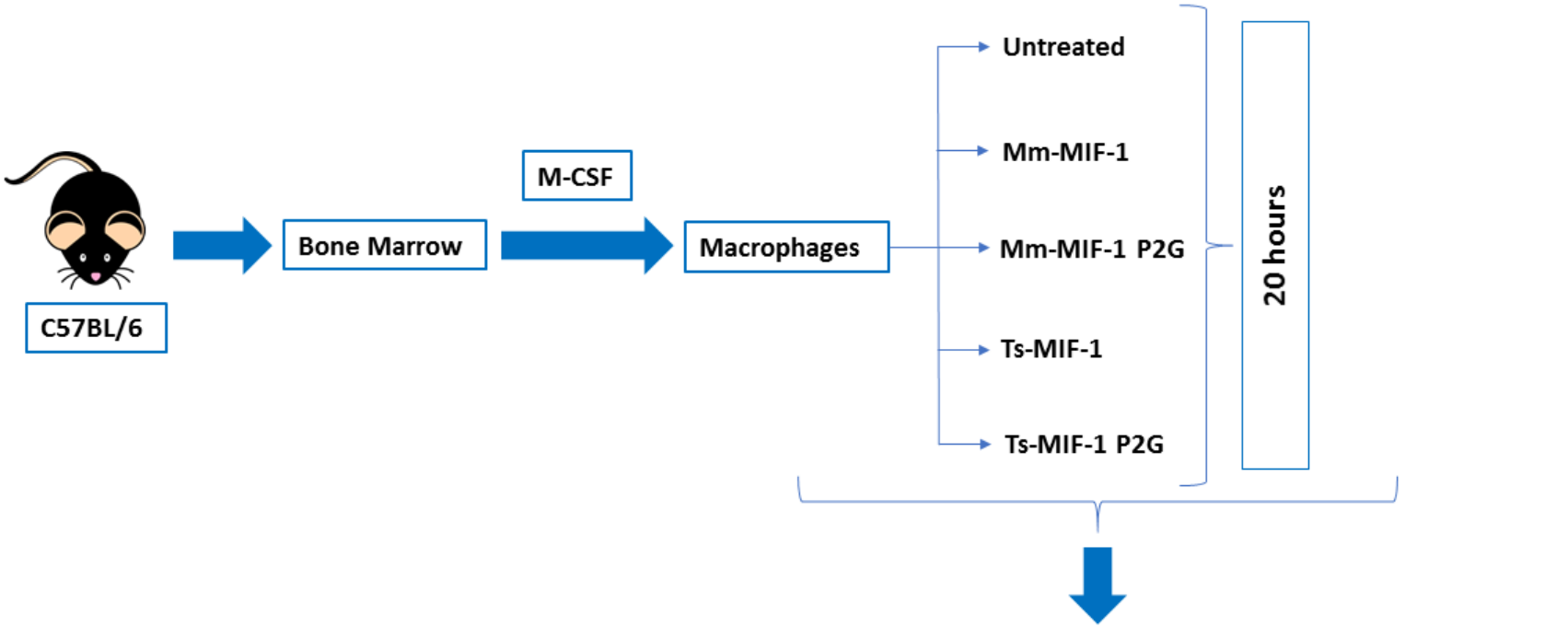
1. To compare the transcriptional response to murine and *Trichinella spiralis* MIFs and tautomerase deficient MIF mutants in macrophages, including the

relative expression levels normalised to the untreated macrophages, using RNA sequencing technology.

2. Perform gene ontology analysis to establish potential relationships between MIF targets and cellular process and pathways.

6.3. Quality control of RNA sequencing.

RNA sequencing was carried out at the Genomics facility (UCL) under the guidance of Dr Paola Niola and basic RNA seq analysis including the associated quality control which was performed by Tony Brooks (UCL). The RNA seq workflow from the cellular assay to final analysis is summarised in figure 6.1. Analysis included aligning reads to the reference genome for *Mus musculus* were generated using the STAR (Spliced Transcripts Alignment to a Reference) algorithm and low-quality, incorrectly called raw reads were trimmed prior to analysis. A mean of 20.2 million reads per sample was acquired. RNA seq quality control metrics are summarised in table 6.1.



- Total RNA isolation, rRNA depletion and library construction.
- RNA seq.
- Raw reads trimming, mapping to genome.
- Gene Ontology analysis.
- Identification of DEGs.

Figure 6. 1 Schematic representing RNA seq workflow. Bone marrow from C57BL/6 female mice aged 6-8 weeks was isolation and cultured in the presence of M - CSF for 7 days. Macrophages were treated with 100ng/ml recombinant MIF proteins for 20 hours before harvesting for RNA isolation. rRNA was depleted to ensure reads were derived from mRNA and the sequencing library constructed. Single end read RNA seq was performed prior to analysis in R.

Table 6. 1 RNA seq quality control metrics. Quality control results for RNA sequencing showing the percentage of mRNA, percent of uniquely mapped sequences aligned to the reference genome, duplication rate in filtered reads, percentage of reads passing filter (PF), percentage of adapter trimmed reads. All values are depicted as percentages.

Sample Name	mRNA	Aligned	Duplication	PF	Adapter
Experiment 1					
Untreated Control	75.10	87.70	4.50	99.40	2.30
Mm-MIF-1	75.50	87.70	5.00	99.30	1.70
Mm-MIF-1-P2G	77.00	88.80	10.60	99.40	1.20
Ts-MIF-1	79.30	89.00	9.80	99.40	1.50
Ts-MIF-1 P2G	75.50	87.80	14.50	99.40	0.90
Experiment 2					
Untreated Control	64.50	82.70	5.20	99.30	2.50
Mm-MIF-1	79.30	88.20	3.80	98.90	3.80
Mm-MIF-1 P2G	76.80	87.10	5.40	99.30	2.30
Ts-MIF-I	76.50	87.70	5.60	99.30	2.40
Ts-MIF-1 P2G	80.70	88.80	5.20	99.30	2.20
Experiment 3					
Untreated Control	80.80	87.80	6.20	98.80	3.90
Mm-MIF-1	83.70	89.20	15.50	99.30	1.90
Mm-MIF-1 P2G	80.80	88.60	7.30	99.40	1.70
Ts-MIF-1	76.20	87.30	16.50	99.30	
Ts-MIF-1 P2G	82.60	88.60	15.60	99.30	1.80

6.4. Differential gene analysis.

To determine the differentially expressed genes (DEGs), transcripts from Mm-MIF-1, Mm-MIF-1-P2G, Ts-MIF-1 and Ts-MIF-1-P2G treated macrophages were analysed against a control of untreated bone-marrow-derived-macrophages. Analysis was performed using DEseq2 and EdgeR in R studio with a batch parameter defined in order to take the pre-determined batch variation into account. This includes modelling of the dataset using surrogate variables to estimate the coefficients of the model (Leek, 2014, 2014) prior to statistical inference tests. DEGs were primarily identified using the adjusted *P* value. For this analysis, a Benjamini-Hochberg p-value adjustment was performed using the formula $(\mathrm{i}/\mathrm{m})\mathrm{Q}$ where i = *P*-value rank, m = the total number of tests and *Q* = false discovery rate, to ensure that *P* values below 0.05 were not a consequence of Type I error (Benjamini, 2010; Benjamini and Hochberg, 1995). In this instance, the level of controlled false positive rate was set to 0.05. To extract the most differentially expressed genes between MIF groups versus the control, genes were filtered to include only those genes with a log2 fold change and *q* value of $\leq$ - 1.5 or $\geq$ 1.5 and $\leq$ 0.05, respectively.

As depicted in figure 6.2 and figure 6.3, DEseq2 analysis revealed that, using the stringent criteria mentioned previously, there were 114 key genes differentially regulated between the sample groups. Specifically, there were 13 DEGs between Mm-MIF-1 and the relative untreated control, 8 of which were downregulated and 5 was upregulated. In macrophages incubated with the tautomerase-deficient Mm-MIF-1 P2G the number of DEGs increased to 27 with all DEGs being upregulated compared to the control; 5 of which were in common with genes upregulated in Mm-MIF-1.

Analysis of Ts-MIF-1 and Ts-MIF-1 P2G DEGs revealed that there were only 19 differentially regulated transcripts in samples with Ts-MIF-1 when compared to the control,18 upregulated and 1 downregulated. Additionally, in macrophages cultured with tautomerase-deficient Ts-MIF-1 P2G, 8 genes were found to be differentially regulated as compared to control cells, 5 downregulated, 3 upregulated. In Ts-MIF-1 treated samples, 2 upregulated transcripts and 1 downregulated transcript overlapped with Ts-MIF-1 P2G samples. Full gene lists are in table A.4.

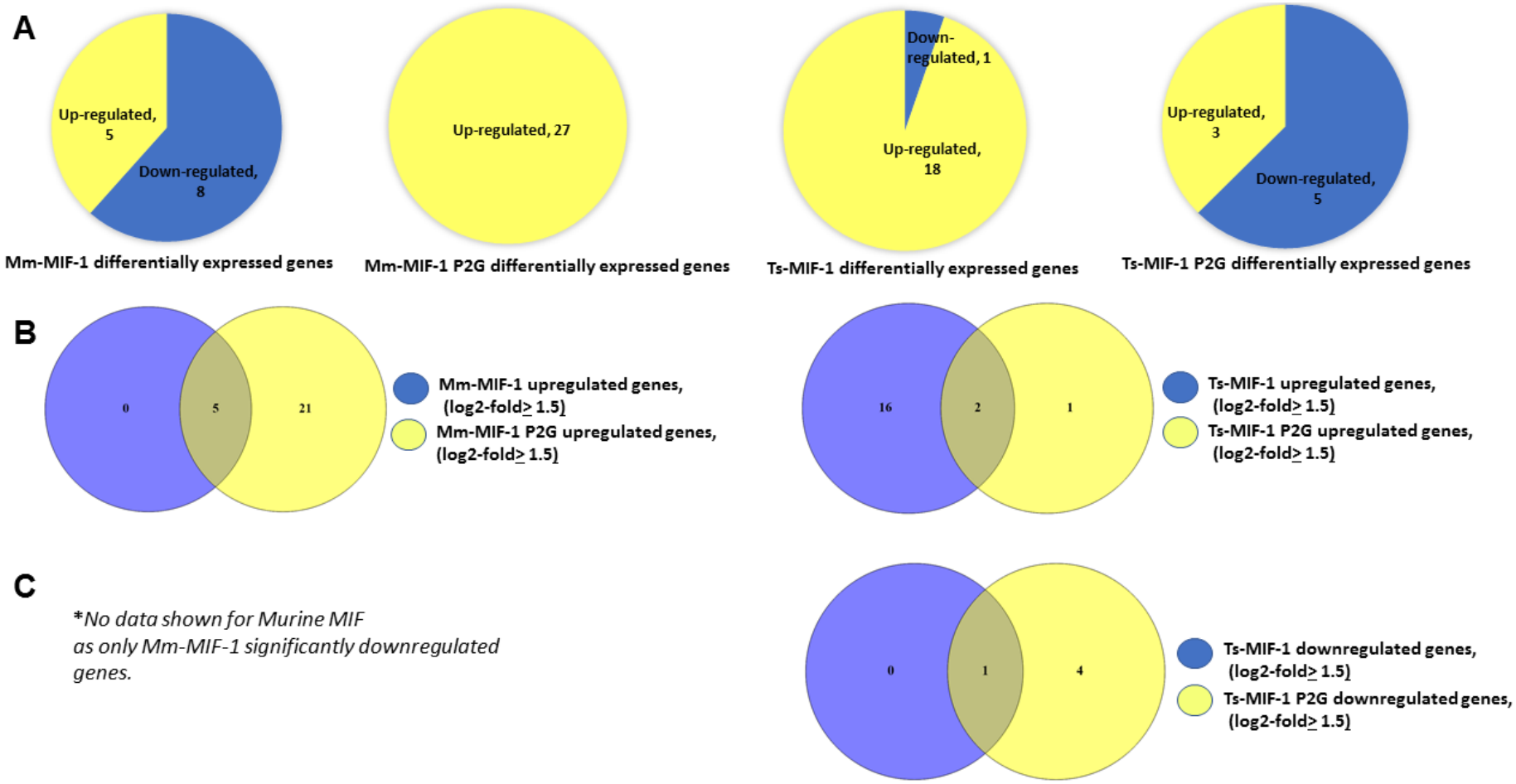

A
Up-regulated, 5
Down-regulated, 8
Mm-MIF-1 differentially expressed genes
Up-regulated, 27
Mm-MIF-1 P2G differentially expressed genes
Down-regulated, 1
Up-regulated, 18
Ts-MIF-1 differentially expressed genes
Up-regulated, 3
Down-regulated, 5
Ts-MIF-1 P2G differentially expressed genes
B
0
5
21
Mm-MIF-1 upregulated genes, (log2-fold≥ 1.5)
Mm-MIF-1 P2G upregulated genes, (log2-fold≥ 1.5)
16
2
1
Ts-MIF-1 upregulated genes, (log2-fold≥ 1.5)
Ts-MIF-1 P2G upregulated genes, (log2-fold≥ 1.5)
C
*No data shown for Murine MIF as only Mm-MIF-1 significantly downregulated genes.
0
1
4
Ts-MIF-1 downregulated genes, (log2-fold≥ 1.5)
Ts-MIF-1 P2G downregulated genes, (log2-fold≥ 1.5)

Figure 6. 2 Number of differentially expressed genes identified in MIF-treated macrophages. Transcriptional variation in Mm-MIF-1, Mm-MIF-1 P2G, Ts-MIF-1 and Ts-MIF-1 P2G treated BMDMs. (A) Pie charts depicting the number of regulated transcripts in Mm-MIF-1, Mm-MIF-1 P2G, Ts-MIF-1 and Ts-MIF-1 P2G treated BMDMs. (B) Venn diagram depicting the number of overlapping or exclusively up-regulated genes in MIF or MIF P2G treated BMDMs. (C) Venn diagram depicting the number of overlapping or exclusively down-regulated genes in MIF or MIF P2G treated BMDMs.

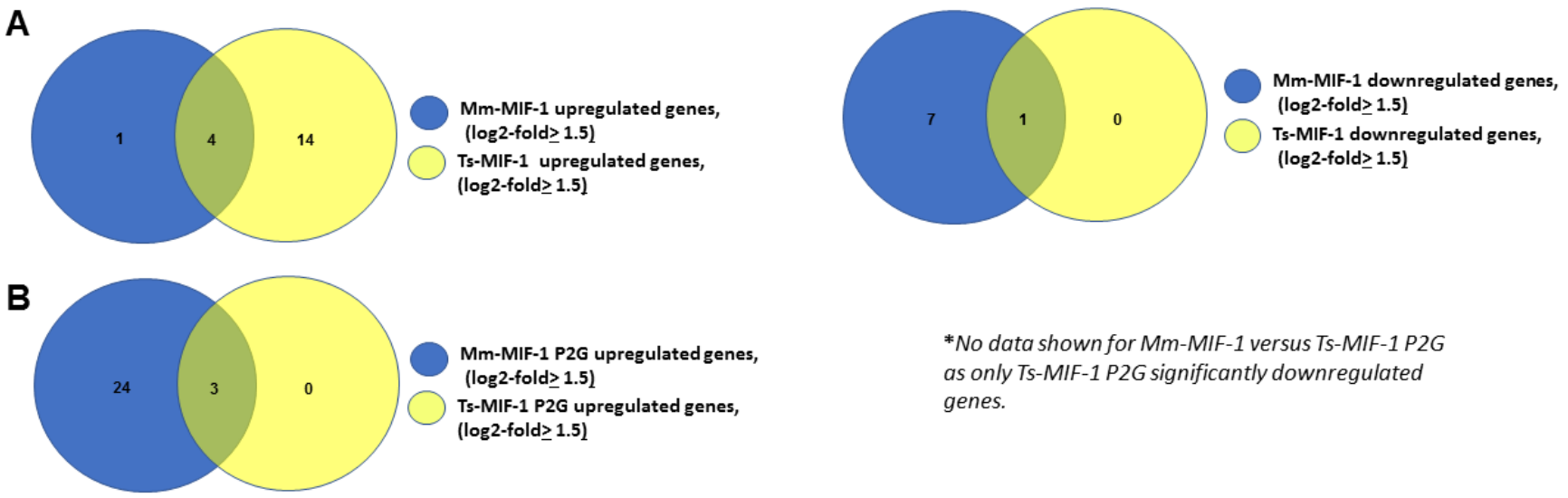


Figure 6. 3 Number of differentially expressed genes identified in MIF-treated macrophages. Transcriptional variation in Mm-MIF-1, Mm-MIF-1 P2G, Ts-MIF-1 and Ts-MIF-1 P2G treated BMDMs. (A) Venn diagram depicting the number of overlapping or exclusively up-regulated or downregulated genes in Mm-MIF-1 or Ts-MIF-1 treated BMDMs. (B) Venn diagram depicting the number of overlapping or exclusively up-regulated or downregulated genes in Mm-MIF-1 P2G or Ts-MIF-1 P2G treated BMDMs.

To determine whether specific gene groups were differentially regulated in response to MIF homologues, gene ontology analysis was performed using The Database for Annotation, Visualization and Integrated Discovery (DAVID). DAVID is an established classification tool which clusters genes into functional groups utilising a Fuzzy Heuristic Partition (FHP) algorithm allowing genes to cluster into several functional groups. FHP automatically determines the optimum number of clusters (K) and excludes genes that have weak relationships to other gene groups (Dennis *et al*., 2003; Tjhi and Chen, 2008). DAVID was utilised to identify key gene clusters using fold enrichment score to highlight the most differentially expressed groups. Figure 6.4.A shows the DEG's related to their functional groups with the most significantly enriched functional groups being BMP receptor binding (*BMP3*, *BMP7*), TGF-β receptor binding (*BMP3, BMP7*), regulation of SMAD phosphorylation (*BMP3, BMP7*), regulation of MAPK cascade (*BMP3, BMP7*), Structural molecule activity (*Cldn8, Krt19, Krt5, Krt7, Krt15, Krt8, Krt4, Sprr1a, Sprr2a3*), anatomical morphogenesis (*Trp63, BMP7*) and pattern specification processes (*Trp63, BMP7, Rab27b*). In addition to gene ontology analysis, using the top 114 genes, the most differentially expressed are summarised in a heatmap in figure 6.4.B.

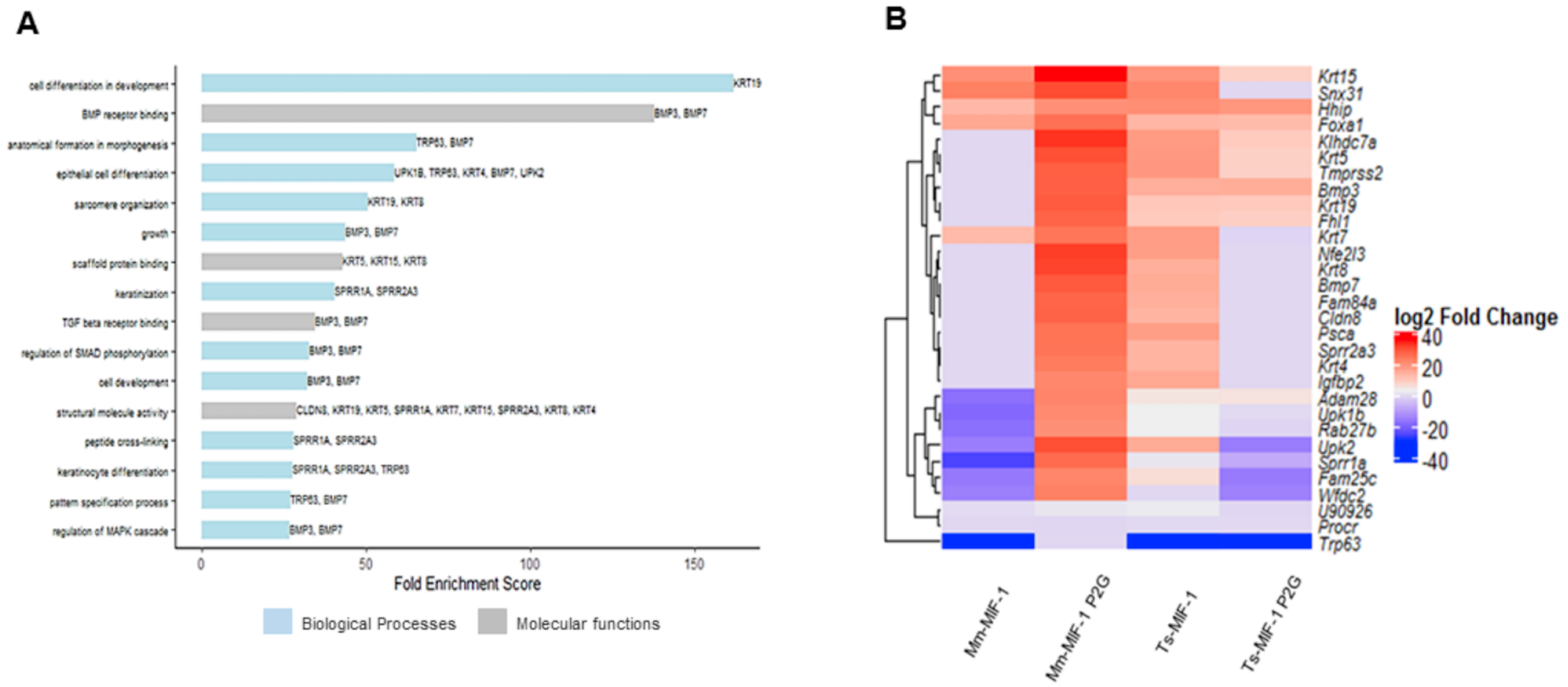


Figure 6. 4 Comparison of DEG's in Mm-MIF-1, Mm-MIF-1 P2G, Ts-MIF-1 and Ts-MIF-1 P2G treated BMDMs. (A) Enrichment analysis showing terms associated with modulation of the BMDM transcriptome by MIF homologues. (B) Heatmap representing DEG's, determined by DEseq2, in MIF-treated BMDMs. Heatmap was generated using the complex heatmap package (ggplot2) in R. Transcripts were considered significantly modulated with a log2 fold change $\geq$ 1.5 and adjusted *p* value $\leq$ 0.05. Blocks are represented in a colour range with red indicating higher expression and blue lower expression.

6.4.1. Transcriptional divergence of TNF-α modulating genes in MIF and MIF P2G treated BMDMs.

In this study, the expression profile of BMDMs cultured with Mm-MIF-1 homologues and Mm-MIF-1 P2G mutants revealed that the tautomerase site of murine MIF is essential for the MIF's overall modulatory actions on the macrophage transcriptome. BMDMs cultured with Mm-MIF-1 P2G exhibited widespread upregulation of the transcriptome targets down-regulated by MIF. Despite this, there were a number of clear divergences in samples treated with Mm-MIF-1 and Mm-MIF-1 P2G with a notable difference being that several transcripts associated with the modulation of TNF-α signalling being dysregulated in the absence of the tautomerase activity including *ADAM28*, *Trp63* and *Rab27b*.

ADAM28 is a metalloproteinase and a member of the disintegrin family which is found at both the cell surface and as a soluble protein. Involved in numerous biological functions including cell adhesion, proteolytic processes and cell signalling, *ADAM28* has been implicated in several pathologies such as colorectal and lung cancer, Crohn's disease and systemic lupus erythematosus (Seals and Courtneidge, 2003). More recently, studies investigating the potential role of *ADAM28* as a sheddase have revealed, utilising siRNA silencing of *ADAM28* in human THP-1 cells, that levels of soluble, active TNF-α are significantly reduced in cells in which endogenous *ADAM28* is inhibited indicating that expression of *ADAM28* is an essential prerequisite for the cleavage and activation of TNF-α (Jowett *et al*., 2012; Worley *et al*., 2003). Despite growing evidence that ADAM28 may modulate TNF-α, to date, there has been little indication to suggest that MIF is responsible for this.

Results from RNA-seq of MIF treated BMDMs in this study highlighted that *ADAM28* transcripts were downregulated in Mm-MIF-1 treated macrophages whilst in cells treated with the tautomerase deficient Mm-MIF-1 P2G, *ADAM28* was significantly upregulated. In macrophages, the biological activity of TNF-α is generally regulated by processing at the protein level rather than transcription and these results suggest that the tautomerase site may be crucial for the indirect regulation of TNF-α processing.

Regardless of there being clear differences between WT Mm-MIF-1 and the tautomerase deficient Mm-MIF-1 P2G in the modulation of *ADAM28,* when transcriptional differences in Ts-MIF-1 and Ts-MIF-1 P2G BMDMs were examined, there was no notable difference found in macrophages treated with either of the two homologues indicating that *Ts*-derived MIFs (with or without the tautomerase activity) do not modulate the levels of TNF-α via *ADAM28.* Interestingly, a distinct difference between mammalian MIF and Ts-derived MIF proteins is an oxidoreductase site which is conferred by a CXXC motif present in murine MIF which has previously been shown to regulate several innate immune pathways, as discussed in chapter 3.

In addition to the aforementioned TNF-α sheddase *ADAM28,* a second gene involved in TNF-α activity *Rab27b* was also modulated in MIF treated macrophages. *Rab27b* is a member of the RAS oncogene protein family and currently, knowledge regarding the role of *Rab27b* in macrophage function is limited. Previous studies investigating the role of *Rab27b* in immune cell behaviour have shown that it is highly expressed by macrophages at sites of wound healing (Mori *et al*., 2011), that it is responsible for the secretion of exosomes in various cancer cell types leading to metastasis (Gomi *et al*., 2007;

Ostrowski *et al*., 2010), and is implicated in the induction of mast cell degranulation (Mizuno *et al*., 2007).

Though *Rab27b* has been demonstrated to be essential for a number of secretory pathways, more recently, a role for *Rab27b* in the regulation of macrophage exosomal biogenesis and secretion has been revealed (Ostrowski *et al*., 2010). In addition, Li *et al* (2018) demonstrated that blockade of Rab27b-mediated exosome release from MΦ resulted in a significant downregulation of TNF-α after treatment with LPS suggesting that Rab27b is required for efficient TNF-α release. Exosome release from primary macrophages plays a significant role in the cellular communication between macrophage and responding cells such as neutrophils and effector lymphocytes. Rab27b induces exosomal secretion in LPS stimulated macrophages causing an increase in proinflammatory cytokines (Alexander *et al*., 2017) whilst in the cancer microenvironment Rab27b - driven vesicle trafficking has been shown to contribute to tumour growth and metastasis (Tzeng and Wang, 2016).

Data from our study indicates that BMDM's treated with Mm-MIF-1 for 20 hours have significantly decreased transcriptional levels of *Rab27b* as compared to the untreated samples. Contrastingly, *Rab27b* transcripts were significantly increased in the tautomerase deficient, Mm-MIF-1 P2G treated macrophages confirming earlier results that highlighted the significance of the tautomerase site in the modulatory capabilities of mammalian MIF.

Notably, earlier studies have demonstrated that matrix metalloproteinases, such as *ADAM28*, are expressed in abundance in exosomes from tumour-associated macrophages suggesting that *Rab27b* and *ADAM28* modulation may be linked in some way (Mochizuki *et al*., 2020; Solinas *et al*., 2010; Zhu *et al*., 2015).

An additional key MIF-modulated gene involved in transcriptionally mediating TNF-α driven responses is *Trp63.* *Trp63*, also known as *p63* in humans, is a member of the p53 tumour suppressor family and shares approximately 63% amino acid sequence similarity with p53's N-terminal transcription activation domain, a DNA binding domain and a C-terminal oligomerization domain. Though a large number of residues are homologous, the fact that 37% are dissimilar suggests that the functions of the p63 homologue diverge somewhat from that its counterpart, p53 (Levrero *et al*., 2000). Moreover, it is well-documented that p63 and a second p53 homolog p73 are principally involved in cellular development and are not involved in maintaining genomic stability like their counterpart p53 (Inoue and Fry, 2014). Similarly, numerous studies have evidenced that while p53 is always mutated in cancer cells, p63 and p73, are typically unaltered (Dötsch *et al*., 2010). Early studies using murine *p63* knockout models proved largely unsuccessful in revealing the specific roles for *p63* due to mice acquiring severe developmental abnormalities leading to early death (De Laurenzi and Melino, 2000).

Further probing into the functions of *p63* have revealed that there are several isoforms driven by two distinct promotors: TAp63 and ΔNp63, both of which have been implicated as inducers and targets of the NF-kB signalling pathway. The promoter regions for TAp63 and ΔNp63 contain NF-kB binding sites and mutation of these bases abolishes TNF-α driven NF-kB activation and the subsequent expression of *p63* (Wu *et al*., 2010). Of particular note, TAp63 appears to be modulated by the dimerization of the canonical MIF receptor, CD74. Binsky *et al* (2010) noted a relationship between MIF and TAp63 in CLL cells, with CD74-dependant MIF signalling increasing cellular survival via the upregulation of *p63*.

This was also demonstrated in primary *ex vivo* B-cells whereby MIF treatment led to the expression of several anti-apoptosis genes such as *Bcl-2* and this was shown to be dependent on the CD74/NF-kB/TAp63 axis (Lantner *et al*., 2007). Conversely, several studies have shown that *p63* expression increases cell surface expression of death receptors such as the TNF-α receptor, TNF-R, thereby inducing a subset of genes associated with apoptosis (Gressner *et al*., 2005). Apoptosis is typically associated with p63's more widely researched counterpart, p53, nevertheless few studies have also shown that p53 can increase cellular survival under specific conditions such as cellular starvation by downregulating autophagic mediators (Scherz-Shouval *et al*., 2010).

In this study, *Trp63* was significantly downregulated in Mm-MIF-1 treated macrophages when compared to the untreated cells. Conversely, treatment of BMDMs with Mm-MIF-1 P2G had no effect on the expression of *Trp63* demonstrating, firstly, that mammalian MIF in the setting of primary macrophages acts to decrease *Trp63* which contradicts previous studies that noted MIF increased *Trp63* through the CD74 receptor in leukemic cell lines and murine B cells Secondly, we show here that the Mm-MIF-driven reduction in *Trp63* transcripts are dependent on MIFs tautomerase activity. Future work should focus on identifying how the tautomerase site or its activity is linked for the modulation of *Trp63* or whether it is a consequence of reduced receptor binding. This may be achieved by co-treating BMBMs with MIF homologues and commercially available tautomerase inhibitors to confirm whether the observed effect is a direct effect of MIF's tautomerase enzymatic activity. An additional objective would be to determine which isoform of Trp63 is being expressed within the context of MIF treatment as the data retrieved within this study does not

differentiate between Np63 or Tap63. However, several studies suggest that Np63 is exclusive to epithelial cells whilst Tap63 is found in immune cells of haematological origin .

Much like the mammalian MIFs, Ts-MIF-1 also represses *Trp63* however in contrast to the murine MIF, this repression is maintained after treatment with the tautomerase deficient mutant Ts-MIF-1 P2G. This suggests that the tautomerase site is not completely required for MIF driven modulation of *Trp63.* However, this may also be a consequence of differences in transducing signals between mammalian and *Trichinella*-derived MIFs. A summary of the transcriptional modulatory mechanisms of MIF in BMDMs is provided in figure 6.5.

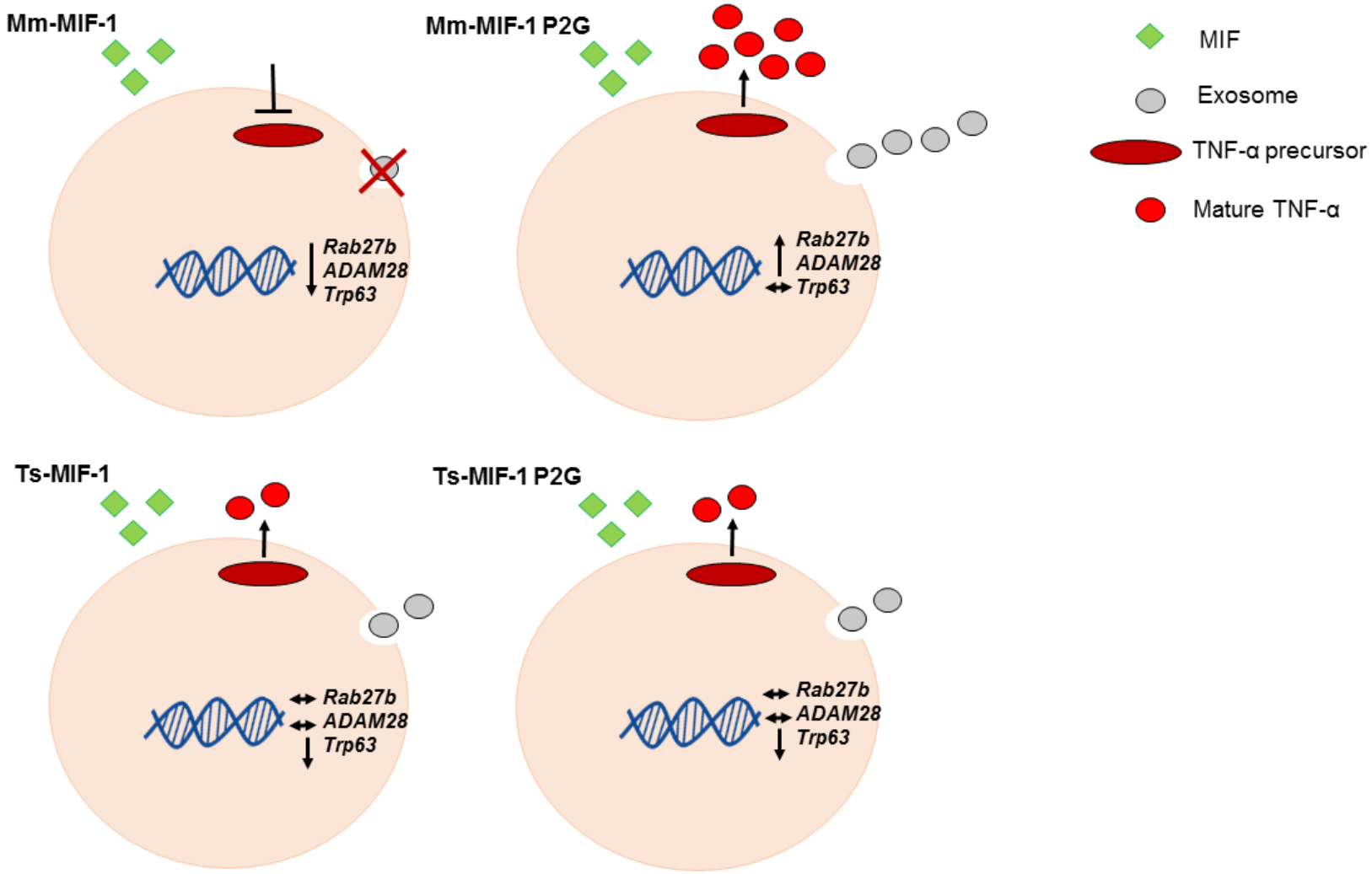

Mm-MIF-1
Rab27b
ADAM28
Trp63
Mm-MIF-1 P2G
Rab27b
ADAM28
Trp63
Ts-MIF-1
Rab27b
ADAM28
Trp63
Ts-MIF-1 P2G
Rab27b
ADAM28
Trp63
MIF
Exosome
TNF-α precursor
Mature TNF-α

Figure 6. 5 Schematic of MIF's TNF-α modulating capabilities. Treatment of BMDMs with Mm-MIF-1 leads to an indirect downregulation of TNF-α processing and secretion by inhibition of TNF-α sheddase, *ADAM28,* preventing processing of TNF-α precursors to the active, mature state. Mm-MIF-1 also inhibits TNF-α containing exosome release via *Rab27b*. Mammalian modulation of these two key genes is highly dependent on the Pro2 tautomerase-conferring site.

6.4.2. Ts-MIF-1 primes BMDM's for polarization inducing transcripts involved in cell cycle regulation.

Macrophages play an important role in the development of *Trichinella spiralis* infection and studies investigating the *T. spiralis* infected niche have demonstrated that macrophages, amongst other innate immune cells, dominate the local immune environment serving several key purposes including clearance of debris, caused by *T. spiralis* mechanically burrowing through epithelial cells, and the modulation of immune cells such as neutrophils and T-cells in the local area. The majority of studies that investigate the macrophage profile in response to *T. spiralis* infection or administration of *T. spiralis*-derived excretory/secretory antigens suggest that macrophages undergo polarization to an M2 anti-inflammatory phenotype (Bai *et al*., 2011; Chen *et al*., 2016).

As described in Chapter 3, *T. spiralis* secretes vast amounts of a MIF homologue in response to the harsh acidic stomach environment, however, research exploring the potential role of Ts-MIF-1 in the modulation of the macrophage transcriptional landscape is lacking. Furthermore, the significance of the conserved tautomerase site in driving these responses remains to be elucidated.

The data from this study clearly demonstrates the novel prospect that Ts-MIF-1 is driving a unique transcript profile consistent with structural remodelling and cell cycle regulation with the most upregulated genes being those involved in cell development (*BMP3, BMP7, Hhip, IGFbp2*), scaffold protein binding (*Krt5, Krt15, Krt8*), structural molecule activity (*Cldn8, Krt19, Krt5, Krt7, Krt15, Krt8, Krt4, Sprr1a, Sprr2a3*) and modulation of the TGF-β pathway (*BMP3, BMP7*).

Morphogens, BMP and Hedgehog, are widely known for being critical regulators of embryonic morphogenesis and patterning, with BMP's forming a subset of the TGF-β superfamily of proteins. BMPs have been implicated in numerous diseases and studies have investigated their role in various cell types including B-cells, T-cells, epithelial cells and macrophages. BMPs bind to the canonical receptor BMPRI and, upon phosphorylation of BMPRII form a heterotetrameric complex leading to phosphorylation of the receptors SMADs, SMAD 1, 5 and 8. The complex of SMAD 1, 5 and 8 associates with a common SMAD, SMAD4, whereby translocation across the nuclear membrane occurs leading to the regulation of gene expression. Importantly, both TGF-β and BMP signalling converge via Smad4 and many of the pathways are interlinked. All morphogens act through a series of reciprocal feedback loops and numerous studies have suggested a pattern of crosstalk dynamics between BMP and Hedgehog signalling. Additionally, canonical Hedgehog signalling is inhibited by the induction of Hedgehog-interacting protein (Hhip) (Liao *et al*., 2017) which binds with high affinity directly to Hedgehog ligands to prevent binding to the hedgehog receptor, Patched1 (Chuang *et al*., 2003; Chuang and McMahon, 1999).

Despite the fact that studies investigating the role of BMP and Hedgehog ligands in macrophage function are limited, few studies have hinted that they may be

essential for driving macrophage polarisation. BMP-2 has been shown to inhibit several markers of the inflammatory M1 phenotype including IL-6 and iNOS (Wei *et al*., 2018) in RAW 264.7 cells and induce monocyte chemotaxis (Pardali *et al*., 2018) while BMP-3 appears to antagonise BMP-2 signalling by upregulating components of the TGF-β pathway in a murine osteoblast cell line (Bahamonde and Lyons, 2001; Daluiski *et al*., 2001). Moreover, BMP-7 has been described as a key mediator of macrophage plasticity and drives the differentiation of monocytes into M2 'healing' macrophages expressing key M2 markers arginase-1 and CD206 in THP-1 cells while simultaneously inhibiting iNOS expression (Rocher and Singla, 2013; Shoulders, 2016; Singla *et al*., 2016). In contrast, BMP-7 antagonises TGF-β signalling despite TGF-β being a potent inducer of the M2 macrophage phenotype (Feng *et al*., 2018; Gong *et al*., 2012) highlighting the importance of the environmental context. However, a serious caveat to these studies is that, contextually, all of the systems examine a cancer environment which is likely to bias the direction of macrophage polarisation towards an M2 phenotype.

Notably, a more recent observation of a role in p53 modulation by BMP and Hedgehog proteins has been revealed. BMP7 is a target gene of the tumour suppressor protein p53 with a p53-responsive element located at nucleotide 2,852 to 2,871 of intron 1 Yan and Chen (2007) . Additionally, Hedgehog signalling can override p53 driven tumour suppression by directly activating MDM2 thereby increasing proliferation (Abe *et al*., 2008). Likewise, inhibition of receptor binding by Hhip increased phosphorylation of p53 in a podocyte cell line (Liao *et al*., 2017).

With this in mind, it is interesting to note that MIF has previously been shown to antagonise p53 and has been established as a ligand of the MAPK/ERK/ Jab1 signalling pathway (Mitchell *et al*., 2002). Mammalian MIF physically interacts with the p53 protein to prevent nuclear translocation and inhibit the anti-proliferative effects of p53 *in vitro* and *in vivo*. In the same study, mutation of cysteine 81 to serine abolished the inhibitory actions of MIF on p53 (Jung *et al*., 2008a) indicating that, within the context of HEK 293T cells, MIF cysteine 81 is critical for p53 modulation. In addition to this, p53 dependent inhibition of *ras*-mediated transformation in embryonic fibroblasts was significantly increased in a C57BL/6 MIF-KO model (Fingerle-Rowson *et al*., 2003). Despite this, Brock *et al* (2014) demonstrated that efficient p53 inhibition in A549 alveolar epithelial cells was dependent on the collective action of MIF-1 and the MIF homologue DDT-1/MIF-2.

With this is mind, we demonstrate that Ts-MIF-1 distinctively modulates genes involved in cell cycle regulation. In this study, *BMP-3* and *BMP-7* remained at basal levels in Mm-MIF-1 treated macrophages whilst, substitution of the tautomerase conferring proline residue to inert glycine led to a significant increase in the expression of the transcripts indicating that the enzymatic activity is vital for the regulation of MIF-dependant macrophage function. Crucially, both Ts-MIF-1 and Ts-MIF-1 P2G treated macrophages upregulated *BMP-3* transcripts when compared to the untreated cells whilst *BMP-7* was exclusively upregulated in Ts-MIF-1 cultured macrophages and did not change in response to Ts-MIF-1 P2G. In addition, the hedgehog inhibitory protein, *Hhip,* was upregulated in all MIF treated macrophages.

Interestingly, during nurse cell formation in *Trichinella spiralis* infection BMP's and components of the TGF-β signal pathway that are also common to BMP such as the shared mediator, Smad4, are significantly upregulated contributing to cell cycle arrest and regenerative processes within the infected muscle tissue (Wu *et al*., 2006, 2005). This data may provide evidence to suggest that Ts-MIF-1 modulates the macrophage cell cycle to prevent differentiation into an inflammatory phenotype in the presence of an additional stimulus such as LPS. However, further work should be undertaken to determine macrophage phenotypes in response to *Trichinella* derived MIF's. Additionally, the data presented here may provide novel insight into MIF's p53 regulatory mechanisms and provide further evidence for the idea that mammalian MIF-mediated inhibition of p53 may be dependent on cysteine 81, as previously described, as the protein sequence of Ts-MIF-1 is devoid of any cysteine residues. Nevertheless, as Ts-MIF-1 P2G treated cells failed to exhibit upregulation of many of the genes associated with Ts-MIF-1, it is difficult to rule out the possibility that the conserved tautomerase site may play a direct or indirect partial role in driving this behaviour. This novel finding provides crucial insight into mammalian MIF's role as a p53 antagonist and future work should investigate the p53 status in macrophages after treatment with Ts-derived MIF utilising western blot and immunoprecipitation techniques.

Further validation for the concept that MIF proteins regulate p53 associated pathways was observed in the Ts-MIF-1 but not Mm-MIF-1 driven increase in Insulin growth factor binding protein 2 (*Igfbp2*). *Igfbp2* encodes for a protein involved in the negative regulation of insulin growth factor 1 (IGF-1) and insulin growth factor 2 (IGF-2). IGF's, including IGFBP-2, have previously been shown

to be significantly upregulated within the Trichinella spiralis nurse cell niche (Wu *et al*., 2008) contributing to cell cycle arrest and re-entry. Additionally, IGFBP-2 has been shown to inhibit IGF driven tumorigenesis using several distinct mechanisms: competing for the IGF receptor (Bach, 2018) and binding to IGF-1 or IGF-2 leading to sequestration of the proteins (Pickard and McCance, 2015). Furthermore, IGFBP-2 has been implicated in several processes associated with tumour suppression; Grimberg *et al* (2006) revealed that shRNA knockout of *IGFBP-2* in PC3 cells, a prostatic cancer cell line, resulted in a significant increase in IGF-1 signalling which is known to be inhibited by p53, highlighting the fact that *IGFBP-2* is a transcriptional target of p53.

With the exception of p53 modulatory activities, *IGFBP-2* has been shown to have contradictory roles in macrophage polarisation. Recent work by Du *et al* (2019) demonstrated that IGF-2 primes developing macrophages driving an anti-inflammatory phenotype by reprogramming the metabolic profile towards performing oxidative phosphorylation and increasing the expression of inhibitory ligand, PD-L1. Additionally, IGF-1 is expressed and secreted by IL-4 mediated M2 macrophages derived from bone marrow monocytic cells (Martinez *et al*., 2006) and inhibition of IGF-1 abrogates Akt activation and the upregulation of M2 markers (Barrett *et al*., 2015; Spadaro *et al*., 2017). As IGFBP-2 binds to and inhibits the actions of both IGF-1 and IGF-2 this would suggest that an increase in *IGFBP2* may inhibit the polarization of several macrophage phenotypes to prevent the clearance of infections.

Aside from its putative role in the inhibition of IGF proteins, several studies have established a direct role for IGFBP-2 in the modulation of tumour progression by binding to integrins such as α5β1 inducing migration and invasiveness of

glioblastoma tumour cells (Wang *et al*., 2006). In the context of macrophage development, Liu *et al* (2019) reported that shRNA knockdown of *IGFBP2* in gliomas significantly reduced the overall number of CD163+ M2 macrophages. Importantly, several studies have elucidated a negative regulatory role for IGFBP-2 in phagocytosis with one study demonstrating that it increases phosphorylation of FCYRIIB on macrophages thereby downregulating the phagocytic capabilities to prevent uptake of local debris (Clynes *et al*., 2000).

6.4.3. Ts-MIF-1 drives architectural changes in murine BMDM cells.

Interestingly, a further set of genes in involved in strengthening components of cellular cytoskeleton (*Krt4, Krt5, Krt7, Krt8, Krt15, Krt19, Cldn8, Sprr1a, Sprr2a3*) were found to be upregulated in macrophages treated with Ts-MIF-1 and, to a lesser extent Ts-MIF-1 P2G. Minimal modulation of these genes were noted in Mm-MIF-1 treated samples, however, abolishing the tautomerase activity led to a significant increase in all of the aforementioned transcripts suggesting that the enzymatic activity is crucial for canonical MIF signalling to occur.

Krt genes code for Keratin proteins that act to strengthen the intermediate filaments associated with the cellular cytoskeleton and their function in macrophage development and polarisation is poorly understood. Nevertheless, modulation of *Krt* genes has been noted in several studies that utilise RNA sequencing to analyse the macrophage transcriptome including: expression of *Krt4* and *Krt19* in alveolar macrophages (Mould *et al.*, 2019) and *Krt5* expression in monocyte derived macrophages infected with influenza H5N1 (Zhang *et al*., 2018). An interesting finding is that granuloma macrophages express high levels of *Krt* genes when compared to alternative macrophages subtypes. Additionally, MIF is expressed in large quantities within the granuloma niche (Wang *et al*.,

2012). Granuloma macrophages are a distinct sub-population of macrophages originally designated ‘epithelioid cells’ due to their ability to acquire epithelial-like characteristics. Immunophenotypically, they exist partway along the M1/M2 continuum and are considered intermediate macrophages due to concomitant expression of markers such as iNOS, Arg1 and the *Krt* genes (Adams., 1974; Mattila *et al*., 2013). Additionally, epithelioid macrophages lack the phagocytic capabilities associated with M1 and M2 polarised cells (Turk and Narayanan, 1982; Williams and Williams, 1983) which would confirm our earlier finding that increased expression of *IGFbp2* also negatively regulates phagocytosis.

In addition to *Krt* genes, *Claudin-8* was highly upregulated in Ts-MIF-1 treated macrophages. The paucity of studies investigating the role of *Cldn* genes in macrophages suggests that the expression may be dispensable for macrophage function, however, Bossche *et al* (2012) noted a significant increase in the induction of *Cldn8*, as well as several other *Cldn* genes, in IL-4-driven M2 bone marrow derived macrophages from BALB/c mice. The increase in tight junction proteins in macrophages may be relevant in a number of scenarios such as: the fusion of macrophage and tumour cells; macrophage integration during wound healing processes; and, more crucially in the formation of granuloma structures. Interestingly, granulomatous inflammatory reactions have previously been linked to infection with *T. spiralis* larvae in rats (Etewa *et al*., 2018) and a study by (Li and Ko, 2001) also observed granulomatous inflammation at sites nearby worm invasion, however, it is important to note that these studies were both investigating muscle stage infections. Furthermore, *T.spiralis* undergoes a long period of convalescence following the acute phase of infection within the intestine. In this instance *T.spiralis* larvae hijack the surrounding skeletal muscle

cells inducing dedifferentiation to form a cyst termed a 'nurse cell'. Several studies have revealed that *T.spiralis* secretes a number of proteins that act to regulate the cell cycle and includes the modulation of genes such as IGF's, IGF binding proteins and TGF-β/BMP molecules (Wu *et al*., 2005) A summary of the modulatory actions of Ts-MIF's are described in figure 6.6.

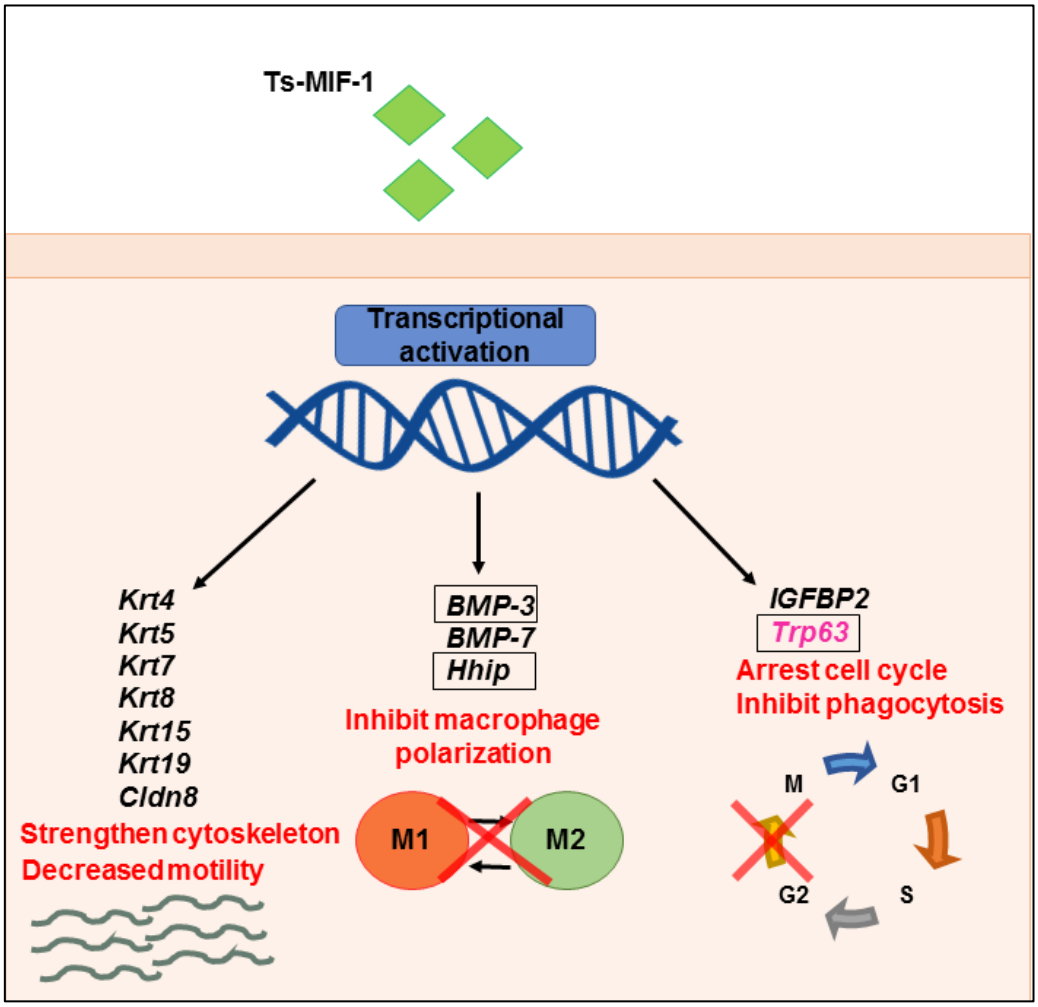


Figure 6. 6 Trichinella derived MIF's halt macrophage polarisation. Treatment of BMDMs with Ts-MIF-1 leads to cell cycle arrest via the downregulation of *Trp63* and the concurrent increase in *IGFBP2*. Ts-MIF-1 increases expression of BMP-3, BMP-7 and Hhip to prevent macrophage polarisation. Transcripts involved in increasing the tensile strength of the cellular architecture such as Krt's and Cldn8 are upregulated to strengthen the cytoskeleton and inhibit motility. Transcripts in black are upregulated. Transcripts in pink are downregulated. Transcripts within a black box are not dependent on the tautomerase activities of Ts-MIF-1.

6.5. Discussion of the transcriptomic analysis of MIF's role using RNA sequencing.

MIF proteins have been widely studied and are implicated in numerous pathologies from responses to exogenous antigens such as the bacterial endotoxin, LPS, through to the development of both solid tissue tumours and haematopoietic cancers. Aside from endogenous mammalian MIFs, a plethora of organisms secrete MIF homologues that are known to modulate host immunity in order to increase their survival and drive pathogenesis. A commonality between all MIF homologues is a highly conserved proline residue that confers its tautomerase activity. The significance of the enzymatic activities of MIF is still unclear despite various studies attempting to demonstrate clear links to its known biological functions.

One of the most critical cell types in the innate response to antigens is the macrophage which can polarise into numerous subsets owing to high plasticity that is dependent on the local environment. Despite the discovery several decades ago that MIF, secreted from T-cells, can inhibit the migration of macrophages, to date there is insufficient evidence to suggest that MIF and MIF's evolutionarily conserved tautomerase site may play a role in the global regulation

of the macrophage transcriptome. Taking this into account, whole transcriptome RNA sequencing was utilised to analyse the global macrophage transcriptome in response to *M. musculus* and *T.spiralis* derived MIFs and tautomerase deficient mutants of these proteins.

Differential gene expression analysis revealed that, in bone marrow derived macrophages after 20 hours of treatment, murine and Trichinella derived MIFs modulate two distinct gene groups. Murine-derived MIF modulated transcripts associated with the processing and secretion of TNF-α whilst Trichinella-derived MIF predominantly modulated genes involved in cell cycle regulation and strengthening of cellular architecture.

Overall, a limited selection of genes were regulated by MIF homologues aside from the tautomerase mutant Mm-MIF-1 P2G which appeared to drive significant dysregulation of the MIF controlled transcriptome indicating that the tautomerase site may be critical for the modulatory actions of MIF. Despite the small number of modulated transcripts, in Mm-MIF-1 treated macrophages, *ADAM28*, *Trp63* and *Rab27b* were significantly downregulated. While these transcripts are not considered to be conventional regulators of the TNF-α pathway there is ample data to suggest that they are involved in the indirect regulation of TNF-α processing for several reasons: (1) knockout of *ADAM28* in mice led to a significant decrease in secreted TNF-α (Jowett *et al*., 2012); (2) Trp63 modulated NF-kB signalling (Si *et al*., 2016) and (3) TNF-a secretion in Rab27b$^{-/-}$ mice is severely dysregulated (Alexander *et al*., 2017). Taking this into account, we propose that MIF may prime macrophages for a particular response ready upon receiving and additional stimulus such as LPS. This is further evidenced by our earlier findings, in chapter 5, that macrophages treated with LPS and Mm-MIF-1

for 20 hours had significantly increased transcriptional levels of IL-6 when assessed by qRT-PCR. Given that a level of reciprocal regulation exists between IL-6 and TNF-α (Lee *et al*., 2017; Sanceau *et al*., 1991; Yimin and Kohanawa, 2006), Mm-MIF-1 may negatively regulate TNF-α at the post-transcriptional level subsequently potentiating an IL-6 response to PAMPs such as LPS. Furthermore, *ADAM28 and Rab27b* transcriptional levels increased after treatment with Mm-MIF-1 P2G whilst *Trp63* is unchanged, which again was supported by results in chapter 5 showing that relative levels of IL-6 transcripts were not potentiated in macrophages co-incubated with LPS and MIF attesting to the idea that the tautomerase site is essential for the regulation of the IL-6/TNF-α axis in murine bone marrow derived macrophages. Despite the apparent role of the tautomerase site in the regulation of these transcripts in Mm-MIF-1, neither Ts-MIF-1 or Ts-MIF-1 P2G mutant treatment significantly modulated *ADAM28* or *Rab27b*. However, the fact that mammalian MIF and Trichinella-derived MIF regulate distinct gene groups is a completely novel finding. As before (in chapter 5), in macrophages treated with the two Trichinella-derived MIF proteins and LPS, there was a negligible increase in transcriptional levels of IL-6 suggesting that the tautomerase enzymatic activities may play a more critical role in mediating the transcriptional responses of mammalian MIF homologues.

The data presented in this chapter also demonstrated that all MIF proteins with the exception of Mm-MIF-1 P2G significantly downregulated *Trp63*. Relatively little is known regarding the role of *Trp63* in macrophage development, however, multiple studies have shown that naturally occurring tumour p53 gain-of-function mutants bind the p63 DNA binding domain with high affinity leading to a significant downregulation of *p63* transcriptional activity (Li and Prives, 2007;

Strano *et al*., 2002). Furthermore, under normal circumstances p53 proteins do not have the ability to hetero-oligomerize with p63 and this is suspected to be a consequence of mutation-driven conformational changes in the DNA binding domain of p53 as demonstrated by Gaiddon *et al* (2001). Interestingly, mammalian MIF has been shown to physically bind with residues 113 – 290 of p53 in a co-immunoprecipitation assay. Mutation of mammalian MIF's cysteine 81 residue limits its ability to for a complex with p53 (Jung *et al*., 2008a) thereby abrogating its ability to inhibit apoptosis. Conversely, MIF has also been shown to stabilise responses to DNA damage as in murine $p53^{-/-}$ $MIF^{-/-}$ models there is increased proliferation of lymphoma cells when compared to $p53^{-/-}$ mice, indicating that MIF may also play a protective role in cell cycle check point process (Nemajerova *et al*., 2007). Our data, taken in concert with the evidence provided above suggests that MIF may indirectly downregulate p63 as a consequence of p53 binding. Additionally, we propose that the mechanism of p63 regulation may be a result of MIF inducing a conformational change within the DNA binding domain of p53 leading to sequestration of p63 and transcriptional inhibition, however, future work looking at utilising co-immunoprecipitation techniques to study protein interactions would be required to confirm this. Moreover, the data from these RNA sequencing results suggest the possibility that the oxidoreductase site may be required for mammalian MIF p53 regulation, which is not conserved in Trichinella-derived MIF's.

Further confirmation of p53 modulation was found in the large number of Trichinella-derived-MIF modulated transcripts that play a key role in cell cycle regulation and cellular architecture including *BMP3*, *BMP7*, *Hhip*, *IGFbp2*, *Cldn8* and numerous *Krt* genes; all of which are recognised as either targets or inducers

of p53. With the exclusion of *Hhip*, none of the other aforementioned transcripts were affected by Mm-MIF-1 suggesting that the modulation of widespread macrophage cell cycle pathways is exclusive to *Trichinella*-derived MIF. Several previous studies have assessed the role of parasite derived MIF proteins on p53 activation as earlier research using mammalian MIF's evidenced that p53 could be inhibited in the presence of MIF proteins. Despite a study by Jung *et al* (2008) noting that the physical interaction of p53 and MIF is abolished by the substitution of cysteine 81 to serine, there is likely to be a more complex mechanism of p53 modulation. This most likely involves the MIF receptor CD74 and the interaction between MIF and Jab1 and we show here that Ts-MIF-1 and Ts-MIF-1 P2G, both of which lack any cysteine residues, also downregulate *Trp63.*

The differences between Mm-MIF-1 and Ts-MIF-1 mediated transcriptional regulation may be a result of differences in the way the two homologues bind receptors and transduce signals. Mammalian MIF's have previously been shown to bind with high affinity to the canonical MIF receptor CD74 (Leng *et al*., 2003; Shi *et al*., 2006b), in addition to, undergoing endocytosis and colocalising with the intracellular protein, Jab1, leading to Jab1/AP1 inhibition (R. Kleemann *et al*., 2000a). Studies investigating MIF/Jab1 binding using a short peptide that competed with full length MIF, have revealed that region spanning aa 50 – 65, which encompasses the CXXC motif, is critical for efficient colocalization with Jab1 (R. Kleemann *et al*., 2000a; Nguyen *et al*., 2003). Despite the results of several conflicting studies which attempt to establish if the cysteine residues are essential for binding, it is clear that most parasite derived MIF's that are also able to bind Jab1 contain some cysteine residues. Plasmodium MIF represses AP-1 activation in HEK cells to the same extent as human MIF and contains a cysteine

residue at amino acid 59 (Augustijn *et al*., 2007). Additionally, MIF derived from *Anisakis simplex* binds human Jab1 with high affinity, however, irrespective of the fact that As-MIF has a cysteine at amino acid 58, substitutions K91A and G111A disrupted the MIF/Jab1 interaction significantly (Park *et al*., 2017). With this in mind, we propose that if Ts-MIF-1 and Ts-MIF-1 P2G are unable to bind Jab1 this would lead to the observed differences in gene expression.

Future work should focus on identifying differences in p53 and Jab/1 binding between mammalian and *T. spiralis* derived MIFs and tautomerase deficient MIF proteins, encompassing the murine oxidoreductase mutants generated in this study. Identifying MIF's specific binding residues may enhance the previous knowledge surrounding MIF's role in tumorigenesis and provide additional insight into therapeutic targets. This, combined with shRNA knockout of endogenous MIF or deletion of enzymatic sites within endogenously expressed MIF using CRISPR-Cas9 editing techniques, could be utilised to characterise phenotypes in macrophages in response to additional stimuli such as LPS or other PAMPs.

Chapter 7: Assessment of MIF's role in the intestinal immune response in an *ex vivo colonic explant model*.

7.1. MIF and the intestinal immune response.

MIF is a critical modulator of innate and adaptive immune responses and is ubiquitously expressed within the small intestinal and colonic epithelium of humans and mice (Maaser *et al*., 2002c; Ohkawara *et al*., 2005; Yao *et al*., 2005). It is has been shown to positively regulate innate immune responses to bacterial antigens such as the endotoxin, LPS; peritoneal macrophages isolated from $MIF^{-/-}$ mice are hyporesponsive to LPS stimulation as a result of reduced TLR-4 cell surface expression (Roger, 2001; Roger *et al*., 2003a). Within the intestine MIF regulates antigen-sampling through specialised M-cells located within the epithelium (Man *et al*., 2008c). Additionally, the canonical MIF receptor, CD74, is highly expressed in colonic epithelial cells and promotes cell survival by inhibiting apoptosis and increasing cell viability when assessed by Annexin and Propidium Iodide (Maharshak *et al*., 2010b).

Besides this, several studies have elucidated a role for MIF in the pathogenesis of IBD and intestinal tumorigenesis. In these environments MIF is responsible for driving production of pro-inflammatory cytokines such TNF-α and IFN-γ (Ohkawara *et al.*, 2002, 2005, 2008). Antibody based blockade of MIF activity reduces the production of these cytokines. Additionally, G > C polymorphisms in the *MIF* promoter region at location -173 are strongly associated with IBD as revealed by several meta-analytic studies (Hao *et al*., 2013; Illescas *et al.*, 2018). In relation to MIF's potential tumorigenic properties, patients with colorectal cancers have increased serum MIF levels which correlates with disease severity (He *et al*., 2009)

Recently, MIF has been implicated in the modulation of cytokines essential for maintaining barrier function such as IL-6, IL-22, TNF-α and IL-17, however, even though these cytokines are involved in preserving the intricate balance of intestinal homeostasis, disorders and cancer progression, as of yet, studies which examine the interplay between MIF, IL-6, IL-22 and IL-17 are lacking.

In this study, we investigated the role of MIF on LPS-mediated intestinal inflammation by analysing both the transcriptional responses and release of the cytokine's TNF-α, IL-6, IL-22 and IL-17. The significance of these key cytokines to this study is discussed individually in detail below.

7.1.1. IL-6

IL-6 is an essential cytokine in intestinal immunology with particular importance in epithelial integrity. In humans, IL- 6 is increased in the serum of IBD patients (Aderka *et al*., 1989; Gross *et al*., 1992) and blockade of the IL-6 receptor has been shown to reduce the clinical symptoms of Crohn's disease (Danese *et al*., 2019; Ito *et al*., 2004). Studies utilising DSS - induced colitis models in mice have demonstrated that IL-6 is increased in both serum and tissue, and has a proliferative effect on colonic epithelial cells (Lee *et al*., 2012) which is not unexpected as it has been previously reported to increase the growth and survival of colitis - associated colonic tumours by upregulating survival factors such as Bcl-2 (Grivennikov *et al*., 2009). Interestingly, while mice deficient in IL-6 are protected from tumorigenesis they show increased susceptibility to DSS-induced colitis. This indicates that, like many cytokines, the context within which it is present determines the outcome.

IL-6 is a prerequisite for protection against *Citrobacter rodentium* (Dann *et al*., 2008), *Yersinia enterocolitica* (Dube *et al*., 2004) and *Giardia lamblia* (Zhou *et al*.,

2003), which are all enteric pathogens. IL-6 has been shown to be critical for the development of pathogenic and regulatory Th17+ cells by inducing expression of IL-21 (Bettelli *et al*., 2006; Voo *et al*., 2009; Zhou *et al*., 2007), and the development of Th22 cells within the GT (Basu *et al*., 2012). In addition, studies investigating crosstalk of IL-6 with other cytokines discovered that IL-6 acts synergistically with IL-17 to promote viral longevity, by diminishing apoptosis, in Theiler's Murine Encephalomyelitis (Hou *et al*., 2014).

MIF has long been recognised as a crucial cytokine for the initiation and promotion of pro-inflammatory responses. It has been shown to activate the MAPK pathway and via this pathway act in both autocrine and paracrine fashions to upregulate additional immunomodulatory cytokines such as IL-6 *(*Chuang *et al*., 2010b; Kudrin *et al*., 2006; Lang *et al*., 2018; Piddock *et al*., 2015).

7.1.2. IL-17

IL-17 is a recently discovered cytokine and has emerged as a key player in intestinal immune responses. Structurally, IL-17 lacks homology to other known cytokines, however, a study by Hymowitz *et al* (2001) found that the IL-17 protein structure contains a cysteine knot also found in morphogens, TGF-β and BMP, providing a clue as to the regulatory mechanisms of IL-17. Several cell types within the mucosal environment express IL-17 including pathogenic Th17+ cells, FOXP3+ Th17+ regulatory T cells, Innate lymphoid cells and γδ T cells. IL-23 independent IL-17 production by γδ T cells has been shown to be critical for early resolution of inflammation (Lee *et al*., 2015) while IL-17+ regulatory T cells are increased in the mucosa during colitis and within the colonic tumour niche in mice (Kryczek *et al*., 2011). Moreover, in an *Abcb1a*$^{-/-}$ multidrug resistant murine model whereby colitis spontaneously develops with age, inhibition of IL-17 and

IL-17R exacerbated the symptoms of colitis by increasing epithelial permeability causing a corresponding increase in mortality indicating that IL-17 is essential for upkeep of epithelial integrity (Maxwell *et al.*, 2015). On a transcriptional level, IL-17 has been shown to inhibit expression of chemokines associated with recruitment of Th1 cells while simultaneously upregulating expression of chemokines which are responsible for recruiting neutrophils such as CXCL1 and CXCL8 (Lee *et al*., 2008) by stabilising the mRNA of these transcripts via the cytosolic adaptor protein Act1 (Song and Qian, 2013).

A notable finding is, MIF may regulate the Th17 response; this is demonstrated in a study by Stojanovic *et al* (2012) who show that lymph node cells from $MIF^{-/-}$ mice lack the ability to produce IL-17 when compared to their WT counterparts. Additionally, MIF is directly responsible for the recruitment of tumour associated Th17 cells in nasopharyngeal cancers. However, MIF-driven IL-17 cells are associated with an increase in positive clinical outcomes (Li *et al*., 2012). Within this context of this research study, a significant finding is that the number of Th17 cells increase significantly in mice infected with the parasitic nematode *T.spiralis* and this is associated with intestinal hypermotility and an increase in worm expulsion (Fu *et al*., 2009a, 2009b; Steel *et al*., 2019). Conversely, studies utilising mice with DSS-induced colitis noted that infiltrates of Th17 and IL-17 are reduced in the presence of excretory/secretory products from *T.spiralis* (Yang *et al*., 2014). However, the exact composition of excretory/secretory products from *T.spiralis* that mediate this activity have not yet been characterised.

7.1.3. IL-22

For some time, IL-22, an IL-10 family cytokine, has been recognised as a key mediator of barrier function at mucosal sites. While IL-22 is constitutively

expressed in the small intestine to prevent pathogen colonisation, within the colon it is expressed only during inflammatory conditions such as IBD (Andoh *et al*., 2005; Cella *et al*., 2009; Sanos *et al*., 2009). Like many cytokines, IL-22 may play opposing roles depending on the context in which it is expressed. For example, it has long been known that IL-22 plays a central role in the pathogenesis of psoriasis (Ma *et al*., 2008) and collagen-induced arthritis (Geboes *et al*., 2009), however, recent advances have shown that IL-22 may play a protective role in the GT. In one such study, the genetic transfer of IL-22 into mice was shown to diminish symptoms of DSS-induced colitis (Sugimoto *et al*., 2008) and studies examining potential gene mutations in IBD have identified IL-22 as a strongly associated candidate (Silverberg *et al*., 2009). Though IL-22 is produced by numerous immune cells one of the major producers are Th17 and Th22 cells within the mucosa. Expression of IL-22 is predominantly driven by IL-6 and repressed by TGF-β (Zheng *et al*., 2006). It is essential for epithelial cell regeneration after damage and within the intestine the IL-22 receptor (IL-22R1) is exclusively expressed by epithelial cells (Wolk *et al*., 2004).

Though a number of investigations have revealed a role for MIF in IL-17 associated immune responses, one single study has revealed a link between MIF and IL-22 by utilising a MIF knockout model which failed to upregulate IL-22 in response to infection with *Toxoplasma gondii* (Cavalcanti *et al*., 2011b) demonstrating that MIF may be a requirement for IL-22 signalling in response to infection.

7.1.4. TNF-α

TNF-α is a classical pro-inflammatory cytokine first isolated in 1984 from macrophages by Aggarwal *et al* (1984) and is a potent activator of the NF-κB

signalling pathway (Hayden and Ghosh, 2014) implicated in countless immune disorders and cancers.

Within the intestine, TNF-α via NF-kB increases tight junction permeability by activation of the myosin light chain kinase gene (*MLCK3*) (Al-Sadi *et al*., 2016), it is involved in modulating the levels of mucins transcriptionally, by abrogating *MUC2* expression (McElroy *et al*., 2011), and post-translationally, increasing sulphation of secreted mucins which acts to prevent the breakdown of the mucus layer by bacterial enzymes (Arnold *et al*., 1993; Raouf *et al*., 1992). In a TNF-α knock-in mouse model, whereby TNF-α is overexpressed, mice spontaneously develop Crohn's disease (Leppkes *et al*., 2014).

Like MIF, the active, stable confirmation of TNF-α is a trimeric protein with a central channel. A relationship between MIF and TNF-α has long been established with studies showing that exogenously administered TNF-α rapidly induces MIF expression (Cao *et al*., 2006; Hirokawa *et al*., 1997) and, likewise, MIF stimulates TNF-α secretion (ref). Amaral *et al* (2007) utilised $MIF^{-/-}$ mice to reveal that MIF is responsible for the TNF-α production in response to damage to heart muscle in ischemia and reperfusion injury. However, the same study showed that there was no difference in intestinal reperfusion-associated neutrophil infiltration between WT and $MIF^{-/-}$ mice indicating that MIF may act selectively in certain types of injury. In addition to this, Coeliac patients have significantly increased levels of MIF and TNF-α when compared to their healthy matched controls (O'Keeffe *et al*., 2001). Although a number of immune cells produce TNF-α, such as pathogenic Th17 cells (Kempski *et al*., 2017; Wang *et al*., 2013), a key point to consider for the purposes of this study is that IEC's are a significant producer of TNF-α in the GT (Roulis *et al*., 2011). Furthermore,

TNF-α along with IL-6, are required for the development of Th22 cells (Trifari *et al*., 2009).

In an infection model with *T. gondii*, TNF-α levels are greatly reduced in MIF$^{-/-}$ knock out mice (Ruiz-Rosado *et al*., 2016). Conversely, MIF isolated from *B. malayi* induced the production of alternatively activated macrophages in the presence of IL-4 and TNF-α indicating that, MIF and TNF-α may in some circumstances have a role in the resolution phase of inflammation (Prieto-Lafuente *et al*., 2009b).

7.1.6. *Ex vivo* models

To date, studies examining intestinal immune responses have relied heavily on the use of transformed cell lines (Kaur and Dufour, 2012), however, although they are considered to be economical and ethical these are not without drawbacks, particularly in the case of colon adenocarcinoma cell lines such as Caco-2 and HT-29, as these cells are prone to spontaneous differentiation and mutations which cause them to behave in ways that are different from normal colonic epithelial cells (Pearce *et al*., 2018). On the other hand, *in vivo* studies are often expensive, time-consuming and unsuitable for screening purposes. A solution to the aforementioned issues are to utilise *ex-vivo* intestinal tissue as studies have indicated that tissue biopsies retain the inherent heterogenicity associated with mucosal surfaces and that cellular permeability is successfully preserved (Nunes *et al*., 2015; Pearce *et al*., 2018). In this study, the use of an intestinal explant model was employed as this has previously shown considerable success in the lab and has been successfully utilised to study drug absorption (Bareiss *et al*., 2008; Goncalves *et al*., 2014; Le May Cédric *et al*., 2013); pathogen interactions

at mucosal sites (Fábrega *et al*., 2016, 2017) and cytokine responses in disease models such as IBD (Garrido-Mesa *et al*., 2019; Powell *et al*., 2012, 2015).

7.2. Chapter aims and objectives.

To assess how MIF can modulate intestinal immune responses to an archetypical PAMP LPS, including whether MIF affects the Th17 cytokine milieu by regulating IL-6, IL-17, IL-22 and TNF-α, the following aims were suggested:

1. Assess whole tissue transcriptional changes in colonic explants via qPCR, to determine how IL-6, IL-17 and IL-22 are affected after exposure to LPS and/or MIF homologues.
2. Assess changes in IL-6, IL-22 and TNF-α, secreted into the media by explant cultures after exposure LPS and/or MIF homologues, utilising ELISA.
3. Compare and contrast the activities of murine and parasite derived MIF homologues in these assays along with mutant (P2G) MIF recombinants lacking the tautomerase activity.

7.3. MIF modulates the expression of IL-6, IL-17 and IL-22 transcripts in *ex vivo* intestinal explants.

MIF has been shown previously to be a critical modulator of immune responses and is expressed widely within the GT. To date, numerous studies have demonstrated that murine MIF knock out models have dysregulated levels of IL-6, IL-17 and Il-22 (Gomes *et al*., 2018; Stojanović *et al*., 2009), despite this, no study has investigated the role of exogenous MIF proteins on these cytokines within the context of responses to bacterial endotoxin, LPS. This investigation assessed MIF's capacity to regulate the transcriptional responses of key cytokines involved in modulating intestinal immunity such as IL-6, IL-17 and IL-

22. As discussed in chapter 4, the concentrations of LPS and MIF used in experiments utilising cell lines, primary cells and tissue are most biologically relevant between 10ng/mL – 100ng/mL. Accordingly, in intestinal explants, 100ng/mL of LPS and recombinant MIF homologues were used and from here on any reference to LPS and MIF will denote these concentrations.

To assess the differential expression patterns of IL-6, IL-17 and IL-22 in response to MIF, explants were cultured, for 20 hours, in the presence of LPS +/- recombinant MIFs. Subsequently, biopsies were removed, and RNA extraction optimised using a Trizol/Chloroform protocol (appendix figure A.5) in preparation for qPCR. Initial experiments focused on ensuring the qPCR efficiency for each primer set was between 1.9 – 2.1.

Figure 7.1 shows that IL-6 transcripts increase in intestinal tissue cultured in the presence of LPS and Mm-MIF-1 or Mm-MIF-1 P2G but not LPS alone indicating that MIF may be acting cooperatively with LPS or a component of the LPS signalling pathway to modulate IL-6 transcription. Ts-MIF-1 also increases the expression levels of IL-6 in the presence of LPS while, interestingly, Ts-MIF-1 P2G co-incubated with LPS lacks the ability to augment IL-6 transcription. It is worth considering that although these are key trends to consider, there is no statistical significance. Unexpectedly, the levels of IL-6 transcript in colonic explants failed to increase in response to LPS alone, however, studies which depict an increase in IL-6 transcription in response to LPS frequently use LPS concentrations in the range of 1µg/ml. Furthermore, due to the baso-lateral location and orientation of TLR-4 receptors within the intestine, LPS can only activate the TLR-4 pathway when barrier integrity is compromised, in this case, it is highly possible that LPS alone cannot reach the TLR-4 receptors. On the other

hand, MIF may comprise barrier function thereby allowing LPS to enter the mucosal layer and activate TLR-4.

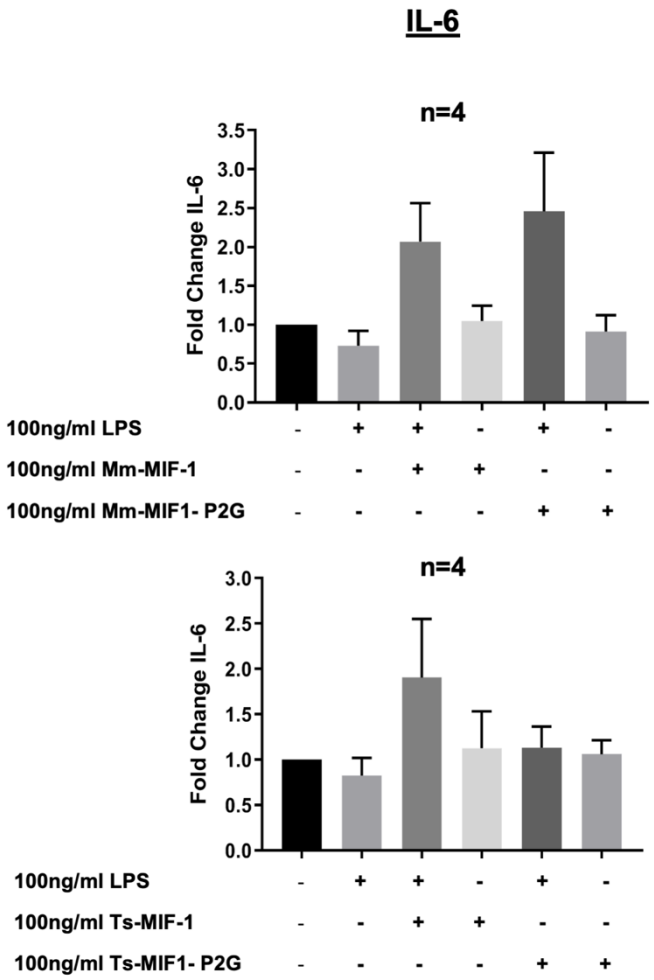


Figure 7. 1 MIF homologues modulate the transcriptional responses of IL-6 in an *ex vivo* explant model. The graphs above show the qPCR analysis of intestinal explant tissue in response to 100ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=4) of the fold change. Changes in gene expression were assessed utilising the Pfaffl equation prior to statistical testing as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

In contrast, IL-17 expression was upregulated in LPS treated samples and this was then significantly reduced ($p \leq 0.05$) in tissues cultured with LPS and Mm-MIF-1 (figure 7.2A) or Ts-MIF-1 (figure 7.2B) and, whereas Mm-MIF-1 P2G also significantly decreased IL-6 transcripts ($p \leq 0.05$), Ts-MIF-1 P2G, though reduced had no significance. Despite initially postulating that LPS and MIF may be increasing IL-6 levels and inhibiting the transcription of IL-17, there is the distinct possibility that the increase in IL-6 transcript in the tissue is responsible for impeding the transcription of IL-17 (or vice versa). Furthermore, the regulation of IL-6 and IL-17 transcripts is dependent on MIF's conserved tautomerase activities.

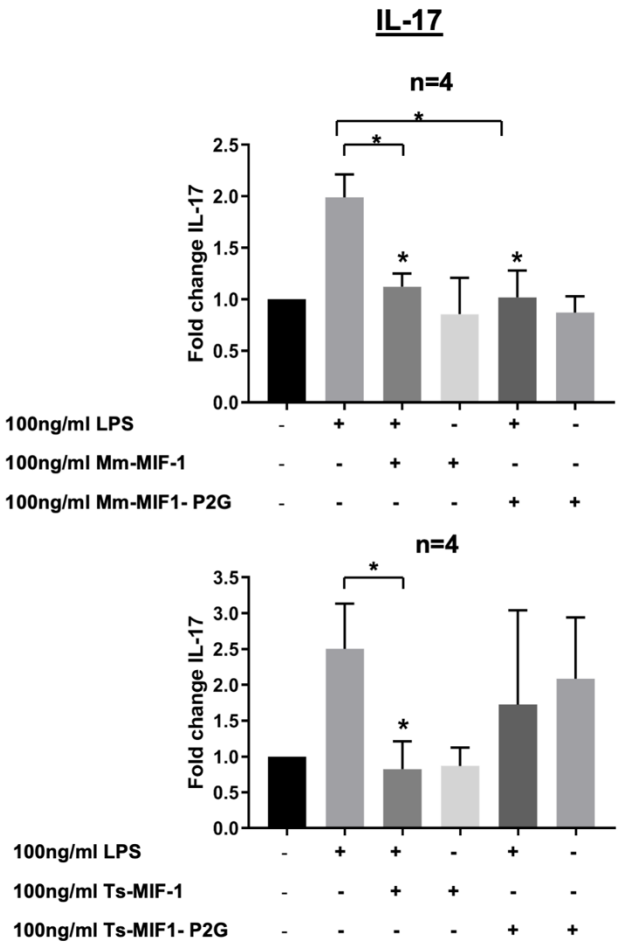


Figure 7. 2 MIF homologues supress LPS induced IL-17 transcription in an *ex vivo* explant model. The graphs above show the qPCR analysis of intestinal explant tissue in response to 100ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=4) of the fold change. Changes in gene expression were assessed utilising the Pfaffl equation prior to statistical testing. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

Bearing this is mind, it was hypothesised that MIF and LPS may be acting in concert to modify the behaviour of Th17 or potentially Th22 associated cytokines, therefore, it was decided that assessing the differential expression of IL-22 was vital. Figure 7.3 illustrates that explants cultured with LPS and Mm-MIF-1 or Ts-MIF-1 had significantly decreased transcript levels ($p \leq 0.05$) when compared to LPS alone. Remarkably, in tissues incubated solely with Mm-MIF-1 there was a significant increase in expression of IL-22 ($p \leq 0.01$) when compared to the control.

IL-22 induces pro-inflammatory IL-18 in IECs which in turn drives Th1 responses in the GT (Muñoz *et al*., 2015).

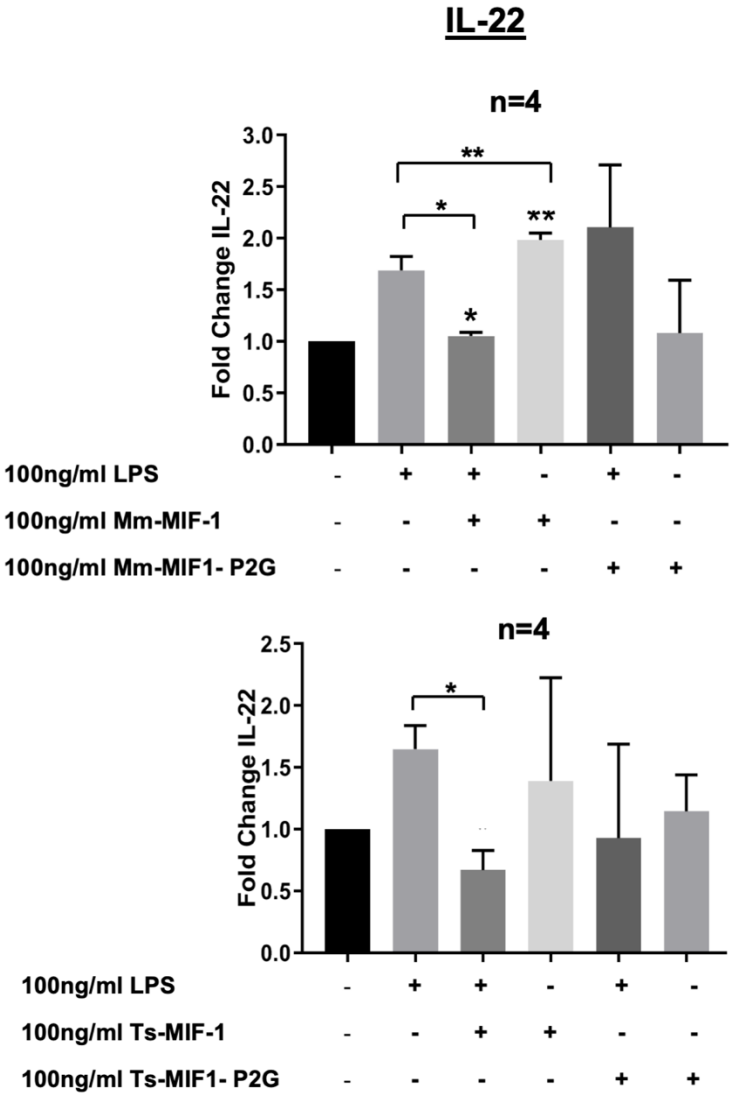


Figure 7. 3 MIFs homologues supress LPS induced IL-22 transcription in an *ex vivo* colonic explant model. The graphs above show the qPCR analysis of intestinal explant tissue in response to 100ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=4) of the fold change. All values were assessed utilising the Pfaffl equation prior to statistical testing. (*) *p*-value ≤ 0.05, (**) *p*-value ≤ 0.01 as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

7.4. MIF treatment skews Th17 cytokines in response to LPS-mediated signalling.

To confirm if the previously observed transcriptional responses were also affecting levels of secretion of these cytokines, a series of ELISA assays were employed. Early experiments focussed on assessing the secreted levels of IL-6 and IL-22 within the explant culture media. Unfortunately, the results obtained from the ELISA assays conflicted with our previous qPCR results. Figure 7.4. shows the levels of secreted IL-6 and IL-22 in response to LPS +/- MIF. In terms of IL-6, though there was a minimal decrease in samples incubated with LPS and Mm-MIF-1 or Ts-MIF-1, none were statistically significant. Notably, Ts-MIF-1 P2G alone significantly reduced ($p \leq 0.05$) the levels of IL-6 secreted protein in explant supernatants when compared to the control.

IL-22 protein quantities slightly decreased in samples with LPS alone which is contradictory to earlier results with an increase in transcriptional levels (figure 7.3 vs 7.4). In samples co-incubated with LPS and Mm-MIF-1 there was a small increase in IL-22, however, Mm-MIF-1 P2G significantly increased IL-22 in response to LPS ($p \leq 0.05$). Conversely, though LPS and Ts-MIF-1 cultures had slightly increased levels of IL-22, LPS and Ts-MIF-1 P2G decreased when compared to LPS alone, and, explants cultured in the presence of Ts-MIF-1 P2G alone did not have the ability to reduce IL-22 suggesting that the tautomerase activities of Mm-MIF-1 and Ts-MIF-1 may be modulating IL-22 responses via LPS at the protein level.

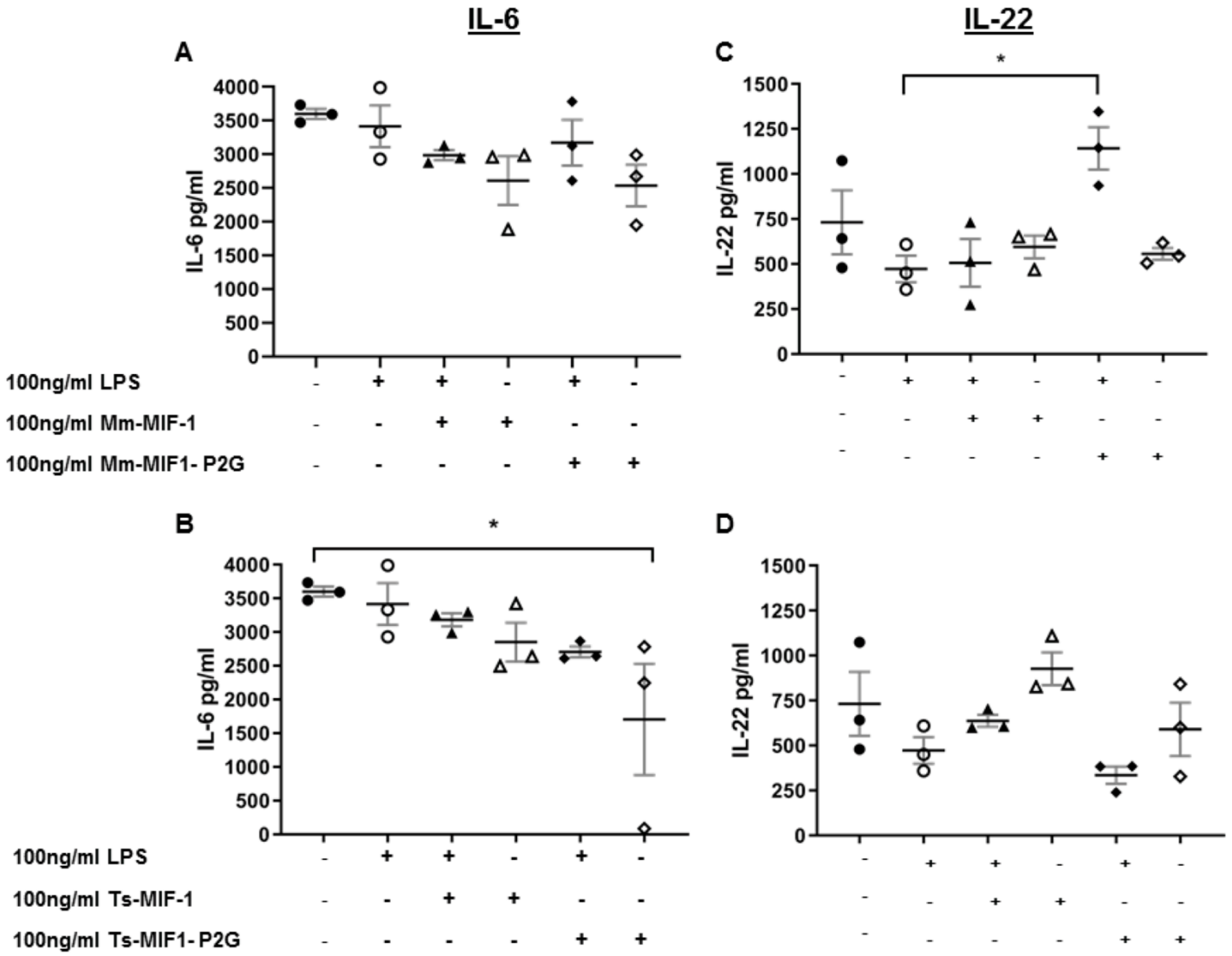

IL-6
A
IL-6 pg/ml
4000
3500
3000
2500
2000
1500
1000
500
0
100ng/ml LPS
100ng/ml Mm-MIF-1
100ng/ml Mm-MIF1- P2G
B
*
IL-6 pg/ml
100ng/ml LPS
100ng/ml Ts-MIF-1
100ng/ml Ts-MIF1- P2G
IL-22
C
*
IL-22 pg/ml
1500
1250
1000
750
500
250
0
D
IL-22 pg/ml

Figure 7. 4 MIFs modulate secretion of the cytokines IL-6 and IL-22. The graphs above show the secreted cytokine (pg/ml) levels of IL-6 and IL-22 within intestinal explant supernatants in response to 100ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=3) of the fold change. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons

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As TNF-α is a key cytokine responsible for driving the polarisation of T0 cells to Th22 cells and is chiefly produced by epithelial cells in the GT, it was vital to assess the secreted levels in response to LPS and/or MIF as earlier transcriptional results suggested that LPS and MIF may act synergistically to promote the development of a particular immune cell subset . Surprisingly, Mm-MIF-1 and Ts-MIF-1 appear to have contrasting effects when co-incubated with LPS; LPS and Mm-MIF-1 had reduced TNF-α levels when compared to LPS alone whereas secreted levels of TNF-α increased in the presence of LPS and Ts-MIF-1 suggesting that these two homologues contain a structural feature which explains the variances. In addition, despite the fact that LPS and Mm-MIF-1 P2G reduced TNF-α quantities to the same level as LPS and Mm-MIF-1, suggesting that the enzymatic activities of Mm-MIF-1 are not responsible for TNF-α modulation, the absolute amounts of TNF-α in response to LPS and Ts-MIF-1 P2G, which were decreased when compared to LPS alone, were in direct contradiction to results for LPS and Ts-MIF-1 which had greatly increased levels of TNF-α in comparison to LPS.

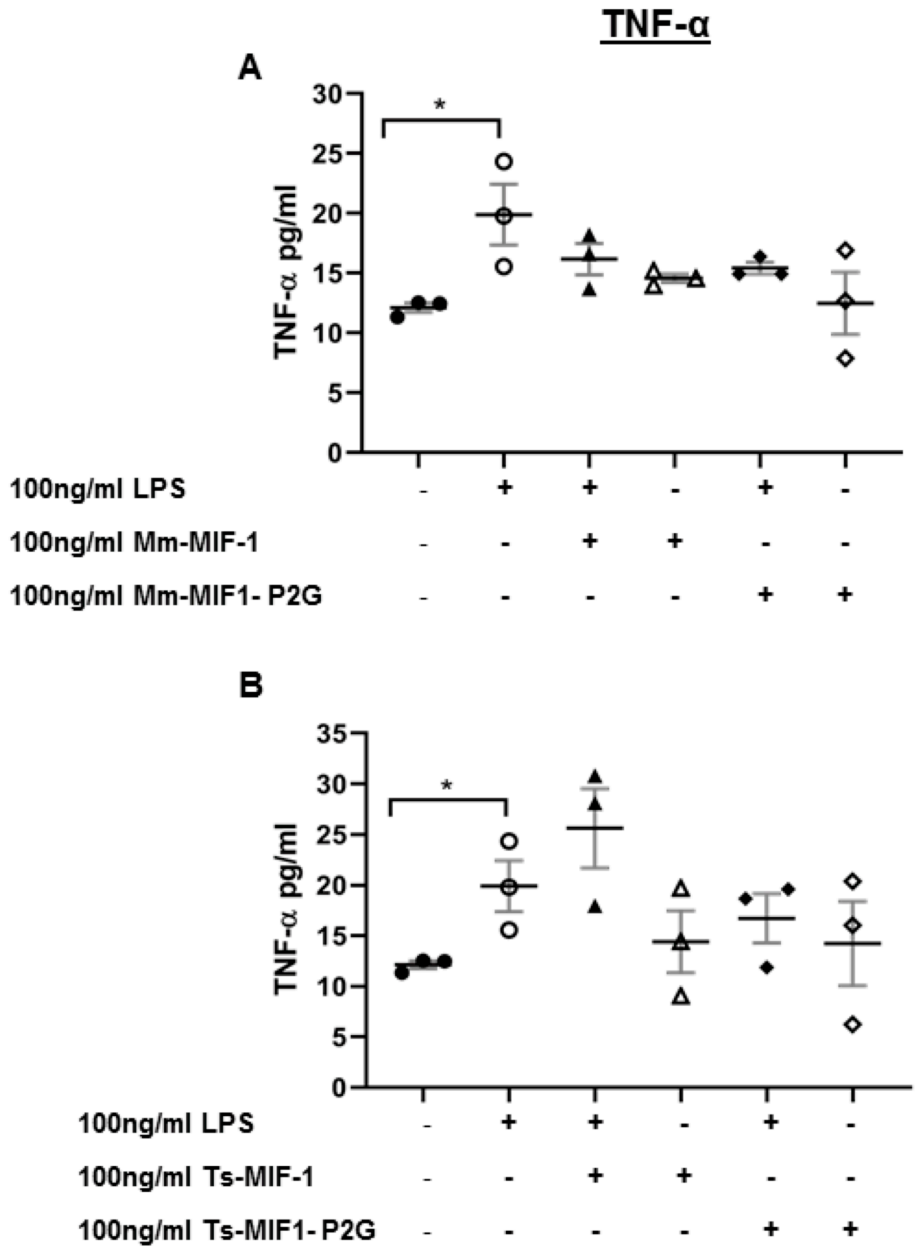

Figure 7. 5 MIFs modulate the secretion of TNF-α. The graphs above show the secreted cytokine (pg/ml) levels of TNF-α within intestinal explant supernatants in response to 100ng/ml LPS +/- 100ng/ml MIF. The data represents the mean ±SEM (n=3) of the fold change. (*) *p*-value ≤ 0.05, as determined by a one-way ANOVA with Dunnett's corrections for multiple comparisons.

7.5. Discussion of MIF's role in the intestinal immune response in an *ex vivo* colonic explant model.

The results of this study illustrate that MIF regulates cytokine production within colonic explants indicating a potential role in modulating GT immune responses. This is demonstrated at both the transcriptional level and the release of secreted protein utilising qPCR and ELISA.

Examination of the transcriptional responses to murine MIFs show that both Mm-MIF-1 and Mm-MIF-1 P2G augment IL-6 transcription in response to LPS stimulation when compared to the LPS alone, whilst the mRNA levels of IL-17 significantly decrease within the same sample set eluding to the possibility that while MIF may differentially regulate LPS-mediated IL-6 and IL-17, the increase in IL-6 may also be responsible for inhibiting IL-17 transcription. Interestingly, samples co-incubated with LPS and Mm-MIF-1 displayed decreased transcriptional levels of IL-22 in comparison to LPS alone which, surprisingly, induced IL-22 transcription substantially. Conversely, Mm-MIF-1 P2G lacked the capacity to modulate IL-22 transcription which may implicate the tautomerase enzyme activity in MIF's IL-22 modulatory mechanisms.

In respect of the secreted cytokine response to Mm-MIF-1 and Mm-MIF-1 P2G, this study revealed that murine MIF regulated the release of two key intestinal cytokines, IL-6 and IL-22 frequently associated with epithelial modulation of immune responses. We showed that Mm-MIF-1 slightly decreased IL-6 secretion in samples co-incubated with LPS in comparison to LPS alone which had no effect on IL-6 protein levels. Interestingly, the Mm-MIF-1 decrease in IL-6 was also apparent in samples that had not been stimulated with LPS and, in the context of IL-6, these results were not dependant on the presence of the Pro2

tautomerase-conferring site. On the other hand, whilst Mm-MIF-1 did not modulate IL-22 secreted levels in the presence of LPS, the tautomerase mutant, Mm-MIF-1 P2G significantly increased IL-22 secretion in the presence of LPS providing crucial insight into MIF's IL-22 regulatory role. In addition to IL-6 and IL-22, the classical pro-inflammatory cytokine secreted by IEC's, Th17 and Th22 cells (Xu *et al*., 2014), TNF-α, was assessed. As expected, LPS significantly increased TNF-α secretion in colonic explants, however, both Mm-MIF-1 and Mm-MIF-1 P2G slightly inhibited this effect within co-cultured samples. Cytokine responses to murine derived WT MIF and Pro2 mutants are summarised in figure 7.6.

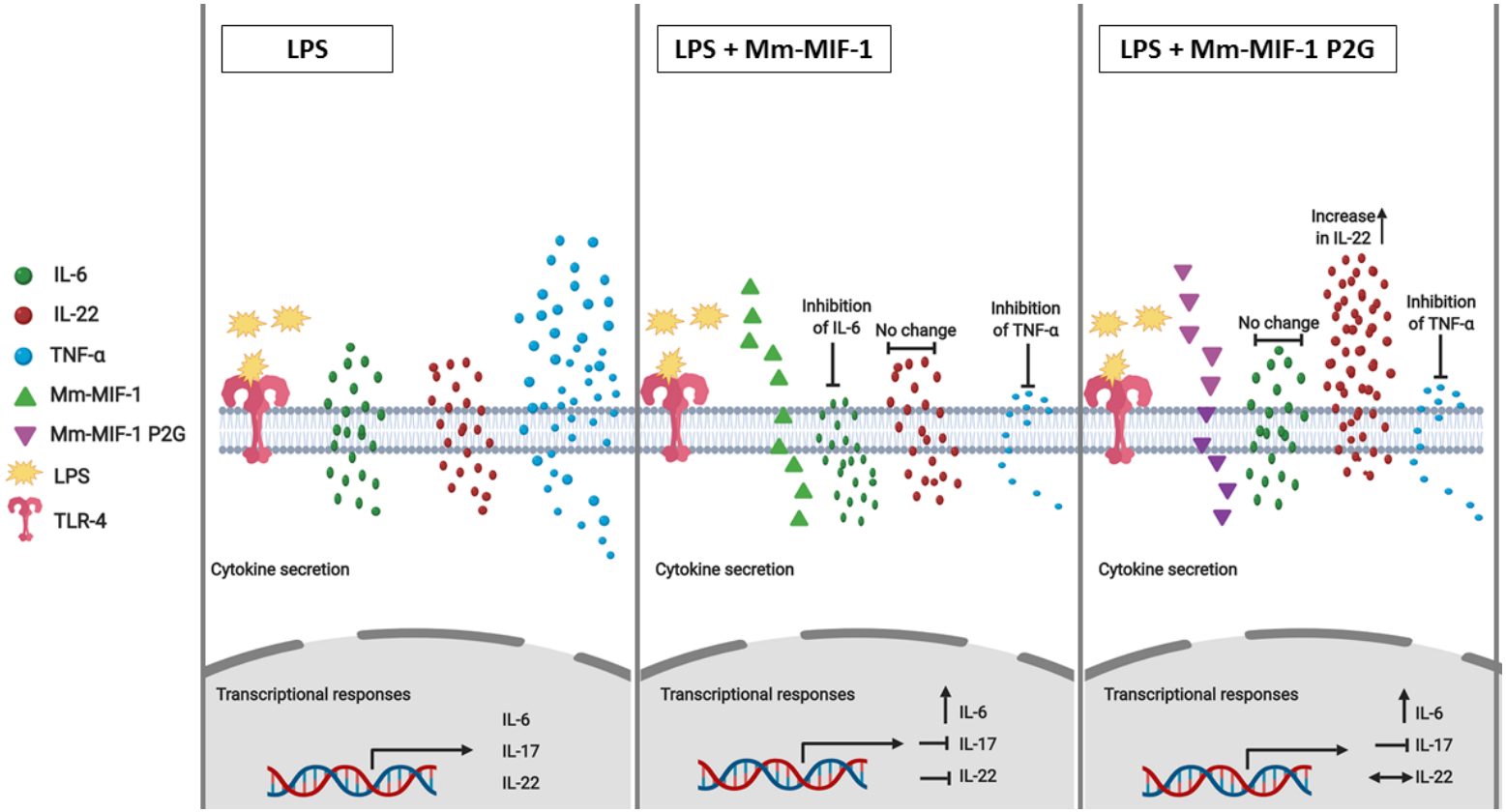
IL-6
IL-22
TNF-α
Mm-MIF-1
Mm-MIF-1 P2G
LPS
TLR-4
LPS
LPS + Mm-MIF-1
LPS + Mm-MIF-1 P2G
Inhibition of IL-6
No change
Inhibition of TNF-α
Increase in IL-22
No change
Inhibition of TNF-α
Cytokine secretion
Cytokine secretion
Cytokine secretion
Transcriptional responses
IL-6
IL-17
IL-22
Transcriptional responses
IL-6
IL-17
IL-22
Transcriptional responses
IL-6
IL-17
IL-22

Figure 7. 6 Summary of transcriptional and secreted cytokine responses to LPS + murine MIF homologues. Diagram representing murine MIF modulation of LPS mediated cytokine expression and cytokine secretion. Mm-MIF-1 potentiates the IL-6 transcriptional responses to LPS whilst inhibiting both IL-17 and IL-22 gene expression. IL-22 modulation is dependent on MIF's tautomerase activity. Mm-MIF-1 inhibits IL-6 and TNF-α secretion after LPS treatments of explants. The absence of MIF's tautomerase conferring Pro2 site leads to significant upregulation of IL-22 cytokine release.

In terms of *T.spiralis*-derived MIF's, Ts-MIF-1 in the presence of LPS, like its mammalian equivalent Mm-MIF-1, drove the transcription of IL-6 whilst inhibiting both IL-17 and IL-22 transcription. In contrast, Ts-MIF1-P2G had no effect on the transcriptional levels of IL-6 or IL-17 and only minimal inhibitory effects on IL-22 which may well be an experimental artefact. Other than the transcriptional data, experiments focussed on quantifying absolute protein concentrations of cytokines revealed that Ts-MIF homologues inhibit IL-6 secretion in response to LPS whilst samples cultured with Ts-MIF-1 P2G alone saw a significant decrease in IL-6 responses. Conversely, IL-22 secretion was increased by Ts-MIF-1 both in the presence and the absence of LPS and this effect was not maintained with substitution of the tautomerase site. In fact, conversely, IL-22 levels were decreased in samples incubated with LPS and Ts-MIF-1 P2G. A key difference between Mm-MIF-1 and Ts-MIF-1 was the observation that Ts-MIF-1 enhanced LPS mediated TNF-α secretion while Mm-MIF-1 inhibited this result somewhat. Neither Ts-MIF-1 or Ts-MIF-1 P2G played a role in the secretion of TNF-α when cultured without an additional stimulus. Cytokine responses to *T. spiralis*-derived WT MIF and Pro2 mutants are summarised in figure 7.7.

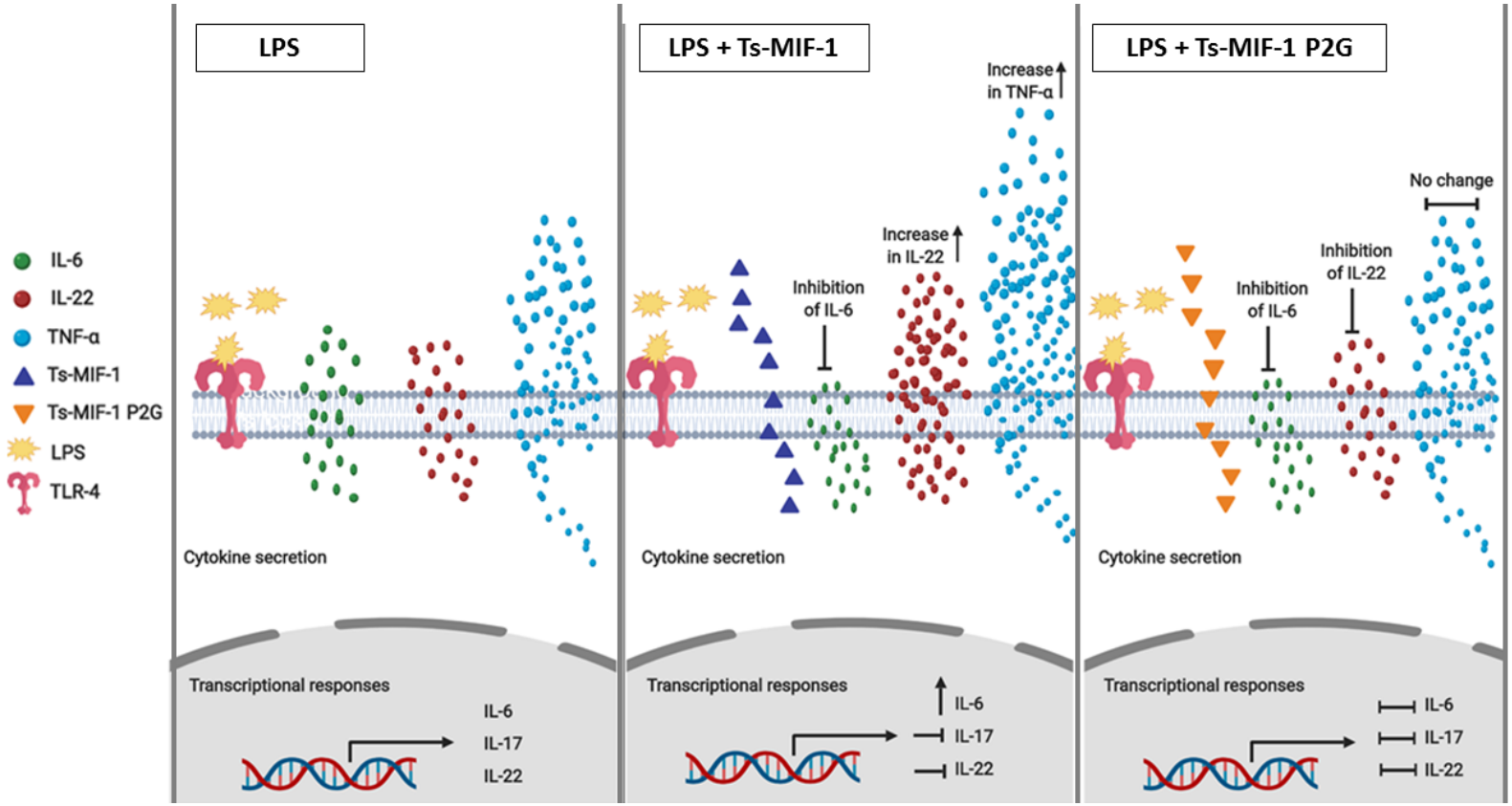
IL-6
IL-22
TNF-α
Ts-MIF-1
Ts-MIF-1 P2G
LPS
TLR-4
LPS
LPS + Ts-MIF-1
LPS + Ts-MIF-1 P2G
Increase in TNF-α
Inhibition of IL-6
Increase in IL-22
Inhibition of IL-6
Inhibition of IL-22
No change
Cytokine secretion
Cytokine secretion
Cytokine secretion
Transcriptional responses
IL-6
IL-17
IL-22
Transcriptional responses
IL-6
IL-17
IL-22
Transcriptional responses
IL-6
IL-17
IL-22

Figure 7. 7 Summary of transcriptional and secreted cytokine responses to LPS + *T. spiralis* MIF homologues. Diagram representing *T. spiralis*-derived MIF modulation of LPS mediated cytokine expression and cytokine secretion. Ts-MIF-1 potentiation of the IL-6 transcriptional responses to LPS is dependent on MIF's tautomerase activity. Ts-MIF-1 inhibits the LPS driven secretion of IL-6 whilst increasing secreted IL-22 and TNF-α both of which are highly dependent on the tautomerase activity.

Countless previous studies have attempted to characterise the immune response to MIF and, in particular, which domains confer MIF's mechanisms of actions. The assessment of cytokine responses to WT and tautomerase-null MIFs in this study is a novel investigation and demonstrates that the tautomerase activity of MIF may be responsible, in part, for the both transcriptomic and post-translation modulation of IL-6, IL-17, IL-22 and TNF-α. Here we show entirely original data evidencing that MIF's previously uncharacterised tautomerase activities are critical for murine MIF to transcriptionally regulate cytokines IL-17 and IL-22 whilst, on the contrary, the tautomerase site of Ts-MIF-1 appeared redundant in this context. Despite this, IL-6 transcriptional regulation by Ts-MIF-1 was dependent on the presence of the tautomerase-conferring Pro2. In respect of secreted cytokine levels, interestingly, the tautomerase site of Mm-MIF-1 appeared to play a substantial role in the regulation of IL-22, a critical mediator of intestinal epithelial immunity. Conversely, Ts-MIF-1-mediated modulation of IL-6, IL-22 and TNF-α all relied on the presence of Pro2 and the related enzyme activity. Several disparities between the two WT homologues and the Pro2 mutants exist including the lack of an oxidoreductase site within the protein sequence of Trichinella-derived MIF's and the fact that the tautomerase activity of Ts-MIF-1 is 6-fold greater than its mammalian counterpart in previously published works (Tan *et al*., 2001) and 2.5-fold lower than the murine MIF purified and kinetically characterised in this study.

Though the results indicate that the tautomerase activity is required for cytokine modulation, what remains unknown is precisely where the tautomerase is interacting as it may be interacting with a component of the TLR-4 signalling pathway in an undiscovered mechanism. Further work will be required to establish how the tautomerase site relates to LPS or parts of the LPS signalling

pathway. One possible explanation for this is that Mm-MIF-1 and Ts-MIF-1 target different parts of the NF-kB pathway. Several recent studies have suggested that non-classical TLR signalling, in which the YxxM PI3K binding motif is activated, leads to bypassing of the NF-kB complex and activation of CREB resulting in a response characterised by high IL-22 levels (Lutay *et al*., 2014). Archetypal TLR signalling which results in NF-kB activation promotes the transcription of inflammatory cytokines such as IL-6 and TNF-α. With this in mind, we propose that Mm-MIF-1 and Ts-MIF-1 transduce signals via different receptors, for example, while several studies have shown that mammalian MIF's can bind both the transmembrane receptor, CD74 and intracellular receptor, Jab1, to date the binding capabilities of Ts-MIF-1 is unknown.

In terms of speculating which cells may be responding to MIF, the experiments within this study do not allow for direct extrapolations as the explant tissue encompasses an incredibly heterogenous and variable population of cells. Nevertheless, the data provided here alludes to the prospect that *T. spiralis* may utilise Ts-MIF-1, along with LPS, to drive the development of an Th22 or ILC3 population, both characterised by IL-6^{-} IL-22^{+} TNF-α^{+} (Glatzer *et al*., 2013; Killig *et al*., 2014; Parks *et al*., 2016) in order to decrease intestinal expulsion by suppressing Th2-related ILC2 responses (Garrido-Mesa *et al*., 2019; Neill *et al*., 2010). In contrast, Mm-MIF-1 in this study, limits the secretion of all analysed cytokines in response to LPS-mediated TLR-4 signalling. Once again, this reiterates an earlier proposal that Ts-MIF-1 and Mm-MIF-1 have distinct mechanisms of action. Future studies, including the isolation of specific colonic cells such as epithelial cells or ILC's, will be required in order to characterise the expression of associated transcription factors such as T-bet, ROR-γt, GATA-3 and STAT3 in response to WT Ts-MIF-1 and Ts-MIF-1 P2G. Additionally, the

assessment of additional factors like the well-characterised morphogen, TGF-β, may help to determine whether this molecule polarises cells to subsets that are pathogenic or regulatory. Finally, endogenous MIF is essential for the production of key Th17, Th22 and ILC3 associated cytokines as shown by studies which report that mice lacking a functional *MIF* gene (Lang *et al*., 2018; Stojanović *et al*., 2009) or where MIF silencing by siRNA treatment (Li *et al*., 2012) have severely impaired production of IL-17, IL-22 and TNF-α. Therefore, future studies should explore the actions of exogenous MIFs, including the tautomerase-deficient mutants, on cells lacking the confounding influence of endogenous MIF.

There are numerous limitations to the results found in this study including discrepancies between transcriptional data and absolute cytokine protein concentration, however, this is not uncommon and there are several possible reasons for this as discussed in a study by Greenbaum *et al* (2003). Firstly, the cellular environment is responsible for maintaining the levels of translation by post-translationally modifying transcripts; many mRNAs will be held within the ribosome and prevented from translation. Secondly, factors affecting protein stability such as the protein half-life, rate of turnover and degradation influence the final protein concentration to varying degrees. In addition to this are issues surrounding what the methodologies represent; generally, assessing mRNA levels is correlative while quantifying absolute protein concentrations is causative. Furthermore, both qPCR and ELISA measure steady state levels and therefore do not reflect translation efficiency and protein stability. In addition to the limitations in terms of protein dynamics described above, a further potential reason for variation in protein levels of cytokines is the lack of cell surface, aside from the basal section and the epithelia, retaining the capacity to secrete cytokines into cell culture media within ex vivo intestinal explant cultures.

Importantly, there are several other caveats to this study. Namely, that studies examining the cell viability dynamics of *ex vivo* colonic explant tissue are less well established and further work using explants will need to implement a time course to assess whether there are differences in responses to MIF and LPS. However, ideally future studies should initially focus on replicating this study utilising isolated primary intestinal epithelial cells and intestinal innate immune cells. Following this, MIF homologues and the associated P2G mutants should be assessed within an *in vivo* murine model of intestinal inflammation such as DSS-induced colitis in WT and tautomerase-null MIF mice.

In summary, the work described within this study has provided novel insight into MIF's potential role in directing intestinal immune responses in the context of classical TLR-4 activation, also revealing critical differences between WT MIFs and P2G mutants

Chapter 8: General Discussion

8.1 Discussion

The rationale for this PhD study was to investigate the role of MIF proteins in driving intestinal immune responses, focussing specifically on intestinal epithelial cells and the phagocytic antigen presenting cells, macrophages. Generation of enzymatically active recombinant MIF proteins, in addition to, mutant MIF proteins that lack the critical but functionally elusive tautomerase domain formed a vital part of this body of work as initial analysis of commercially available recombinant MIF's revealed the complete absence of the conserved tautomerase activity associated with all known MIF homologues. An additional aim of this work was to further characterise the activities of *Trichinella spiralis* derived MIF homologues, Ts-MIF-1 and Ts-MIF-2, one of which (Ts-MIF-2) has not been previously described (isolated by D.B Guilliano, unpublished).

An examination of MIF's ability to modulate key immune regulatory complexes, such as NF-kB, within epithelial cell lines was undertaken to determine whether MIF might influence NF-kB signalling in this important cell type. The role of MIF's tautomerase activities play in this process and whether it was significant in epithelial immune regulation in the context of TLR4 activation was also examined.

Additionally, in order to determine the molecular profile of the intestinal environment and macrophage populations in the presence of MIF and mutant MIF homologues, several key experiments analysing either specific cytokine transcripts or whole transcriptome profiling, were performed.

In Chapter 3 the successful expression and isolation of soluble protein for all MIF homologues allowed for the enzymatic analysis of the recombinant MIF proteins and their subsequent use in cellular bioactivity assays. Previous studies using

E.coli expression systems for the production of recombinant MIF's had encountered limitations due to difficulties in removing residual endotoxin contamination (Bernhagen *et al.*, 1994; Thierry Calandra *et al.*, 1995b). Here, we establish a protocol that allows for isolation of a variety of recombinant MIF homologues and mutants from *E.coli* which, after IMAC and anion exchange chromatography based polishing, reduces endotoxin contamination to virtually undetectable levels. Additionally, protocols were developed for expression and purification active and soluble recombinants of several MIF homologues and mutants that are renowned for mis-folding, including the oxidoreductase mutant Hs-MIF-1 C57S/C60S, human and murine DDT-1/MIF-2 proteins, and the novel parasite derived MIF, Ts-MIF-2. This eliminated requirements for timely, unstandardizable and error-prone misfolding techniques to be used for production of these proteins (Kleemann *et al*., 1999b; Merk *et al*., 2011b). Furthermore, we confirmed previous studies (Jung *et al*., 2008b; Kleemann *et al*., 1999b; Robert Kleemann *et al*., 2000b; Tan *et al*., 2001), demonstrating that active and properly folded MIF proteins contain two catalytic domains conferring tautomerase activity with additional oxidoreductase activity in mammalian MIF homologues. Despite this, we noted that the tautomerase activity of the Ts-MIF-1 produced using our protocols was two-fold lower than previously published (Tan *et al*., 2001) although this may be ascribed to alternative reaction buffers and varying substrate concentrations. Notably, the work contained within these studies is the first of its kind to characterise the enzyme activities of a novel *T. spiralis* derived MIF homologue (MIF-2), that we demonstrate, resembles the mammalian MIF paralogue, DDT-1/MIF-2 in its activity and substrate preferences.

Loss of epithelial integrity within the mucosal barrier is commonly associated with intestinal disorders including IBD and infections such as diverticulitis. A distinct feature of loss of barrier integrity is the infiltration of luminal antigens or PAMPs such as LPS resulting in epithelial PRR activation. MIF is expressed and secreted from cells of the GT during tumorigenesis and intestinal infections and several previous studies have suggested that MIF may play a role in the regulation of LPS mediated TLR4 signalling modulating innate immune responses. The data presented in Chapter 4 demonstrates that, in HEK 293 and HT29 cells, while some MIF homologues appear to be capable of inhibiting or potentiating TLR4 signalling, this is largely dependent on both the cell line and the experimental system used. In the HEK-Blue™ hTLR-4 reporter assay we saw limited evidence of human and murine MIFs inhibiting TLR4 signalling. However, this was not consistent between the assay systems tested and when MIF's were tested in response to LPS in our generated HEK hTLR4-NF-kB-mCherry reporter line, though preliminary, we demonstrated that MIF caused a decrease in NF-kB activation. Interestingly, MIF recombinants could when administered on the own cause some level of NF-kB activation in HEK-hTLR4-NF-kB-mCherry reporter in indicating MIF may inhibit secretion alkaline phosphatase HEK-Blue™ hTLR-4 reporter cells independent of LPS-mediated TLR4 stimulation.

In the colonic epithelial cell line, HT29, initial results indicated that they were unresponsive to LPS even at large concentrations. In line with one study suggesting that priming of HT29 cells with IFN-γ induced LPS responsiveness by upregulating transcripts for TLR4, MD2 and CD14 (Suzuki *et al*., 2003b), we confirmed that HT-29 cells gain LPS responsiveness after 12 hours of IFN-γ priming. Interestingly, in IFN-γ primed HT29 cells, Hs-MIF-1 appears to inhibit

LPS-mediated NF-kB activation. This may be linked to observations that IFN-γ induces the canonical MIF receptor, CD74, which might enhance any effect it has on NF-kB activation.

Within the intestinal environment, the mucosal barrier is modulated by several key cytokines that exert protective effects on the epithelial cells that line the GT. Although the cytokine network within the GT is incredibly complex and intertwined, those such as IL-6, IL-17, IL-22 and TNF-a play a significant part in maintaining barrier homeostasis, both in healthy and diseased tissue states, by increasing the expression of antimicrobial peptides, mucins and, modulating epithelial tight junctions to prevent further infiltration of luminal antigens. To date, very little is known regarding the role MIF plays in regulating barrier maintenance despite the fact that it is highly expressed in epithelial cells at mucosal sites. With this in mind, transcriptional and secreted analysis of key immune-modulating cytokines levels in *ex vivo* colonic explants revealed several novel findings. Both Mm-MIF-1 and Ts-MIF-1 potentiate TLR4 driven IL-6 gene expression whilst simultaneously inhibiting IL-17 and IL-22 transcriptional responses in *ex vivo* explants. Interestingly, in this context Mm-MIF-1 alone significantly increased IL-22 expression which suggests two potential immunomodulatory mechanisms: 1) MIF, in the context of TLR4 stimulation inhibits TLR4 mediated signalling by binding to LPS or directly to TLR4; or 2) treatment of explants with LPS and MIF leads to cellular exhaustion caused by a threshold being surpassed, such as sustained activation of cell surface receptors, leading to a downregulation of IL-22. When this was translated to secreted cytokine levels, whilst IL-6 levels were slightly inhibited in the presence of Mm-MIF-1 and Ts-MIF-1 homologues, we observed distinct differences in the IL-22 response; namely, that Mm-MIF-1 does not modulate IL-22 secretion in the presence of LPS whilst Ts-MIF-1 potentiates

it. Though IL-22 is expressed and secreted by a number of cells in the colon, expression of the IL-22 receptor is unique to epithelial cells within the GT.

Aside from providing evidence that the two MIF homologues may drive opposing immune responses in the GT, we also clearly show for the first time that MIF's tautomerase activities are critical for the transcriptional and secreted cytokine response particularly in the case of secreted IL-22 and TNF-a whereby loss of the tautomerase site led to a significant increase in Mm-MIF-1 IL-22 levels in the presence of LPS and we observed a downward trend in the levels of TNF-a in Ts-MIF-1 tautomerase mutants. Importantly, the data presented here also demonstrates that the tautomerase activities are dispensable for the modulation of some cytokines. For example, MIF induced changes in IL-17 and IL-6 transcription in colonic explants after LPS stimulation did not change when tautomerase deficient MIF mutants were used although the context of explants whereby the cellular milieu is so heterogenous, there is the possibility that the effect of tautomerase mutants is compensated by the actions of additional cytokines. Consequently, we provide key evidence that MIF's tautomerase activities are essential for modulating barrier integrity in response to luminal antigens or PAMPs that gain access to basolateral spaces after epithelial damage.

Innate immune responses in the GT are a complex orchestrated process dominated by epithelial cells, professional APC's and ILC's. The importance of MIF in macrophage function has been widely researched but, aside from its role as an inhibitor of monocyte migration and counter regulator of glucocorticoid hormones, the role of MIF in innate immune regulation is still poorly understood. Interestingly, the effects of the enzymatic activities of MIF appear to be context

dependent. In this PhD thesis, we confirm, along with previous studies, that MIF does not modulate IL-6 and MCP-1 transcription in macrophages directly but synergizes with PAMPs such as LPS. This suggests MIF may be play an important role in driving immune responses during infection or inflammation where barrier function has been lost. Although IL-6 transcription was unaffected by the absence of MIF's tautomerase site in *ex vivo* explants, in the context of primary murine BMDMs this was not the case. In these experiments in macrophages, LPS and Mm-MIF-1 treatment led to a significant increase in IL-6 which was completely abrogated when the tautomerase P2G mutant was used. Surprisingly, though the Ts-MIF-1 recombinant drove a modest increase in IL-6 transcription when it was coadministered with LPS, coadministration with the tautomerase mutant of the Ts-MIF-1 yielded similar increases in IL-6 transcription. With this in mind, we propose that the mammalian oxidoreductase site, which is absent from *Trichinella*-derived MIFs, may contribute in concert with its tautomerase activities to modulate gene expression in macrophages.

Further analysis of the transcriptomes of murine bone marrow derived macrophages in response to MIF treatment led to the novel finding that mammalian and *Trichinella*-derived MIF's modulate distinct gene groups. This may provide insights into how they prime cells for responses to antigens or PAMPs such as LPS. In this work, mammalian derived Mm-MIF down regulated several targets involved in the regulation of TNF-α including a TNF-α sheddase, *Adam28*, murine p53 paralogue, *Trp63*, and regulator of exosome biogenesis, *Rab27b.* We show further evidence of the biological relevance of MIF's tautomerase activities as cells treated with tautomerase deficient MIF showed alterations in a distinct set of transcripts. Interestingly, Ts-MIF-1 upregulated a different subset of genes involved in the regulation of cell cycle processes and

cellular architecture including *BMP3*, *BMP7*, *Hhip*, *IGFbp2*, *Krt5* and *Claudin8.* Unlike transcriptome changes induced by murine MIF the expression of many of these genes remain similarly altered in cells treated with tautomerase deficient Ts-MIF-1. Furthermore, the data we show in this thesis provides additional evidence for MIF's role in the regulation of p53 and, in particular, that Ts-MIF's appear to modulate p53 activities by a mechanism distinct to that used by mammalian MIF's.

Taking together all of the data presented in this thesis we demonstrate evidently the novel prospect that Mm-MIF-1 and Ts-MIF-1 drive the development of distinct immune responses when barrier function is comprised by TLR4 activation. With this in mind, we propose two preliminary divergent models for MIF mediated epithelial-driven immune responses in the GT including WT and tautomerase inactive Mm-MIF-1 (figure 7.1) and WT and tautomerase inactive Ts-MIF-1 (figure 7.2).

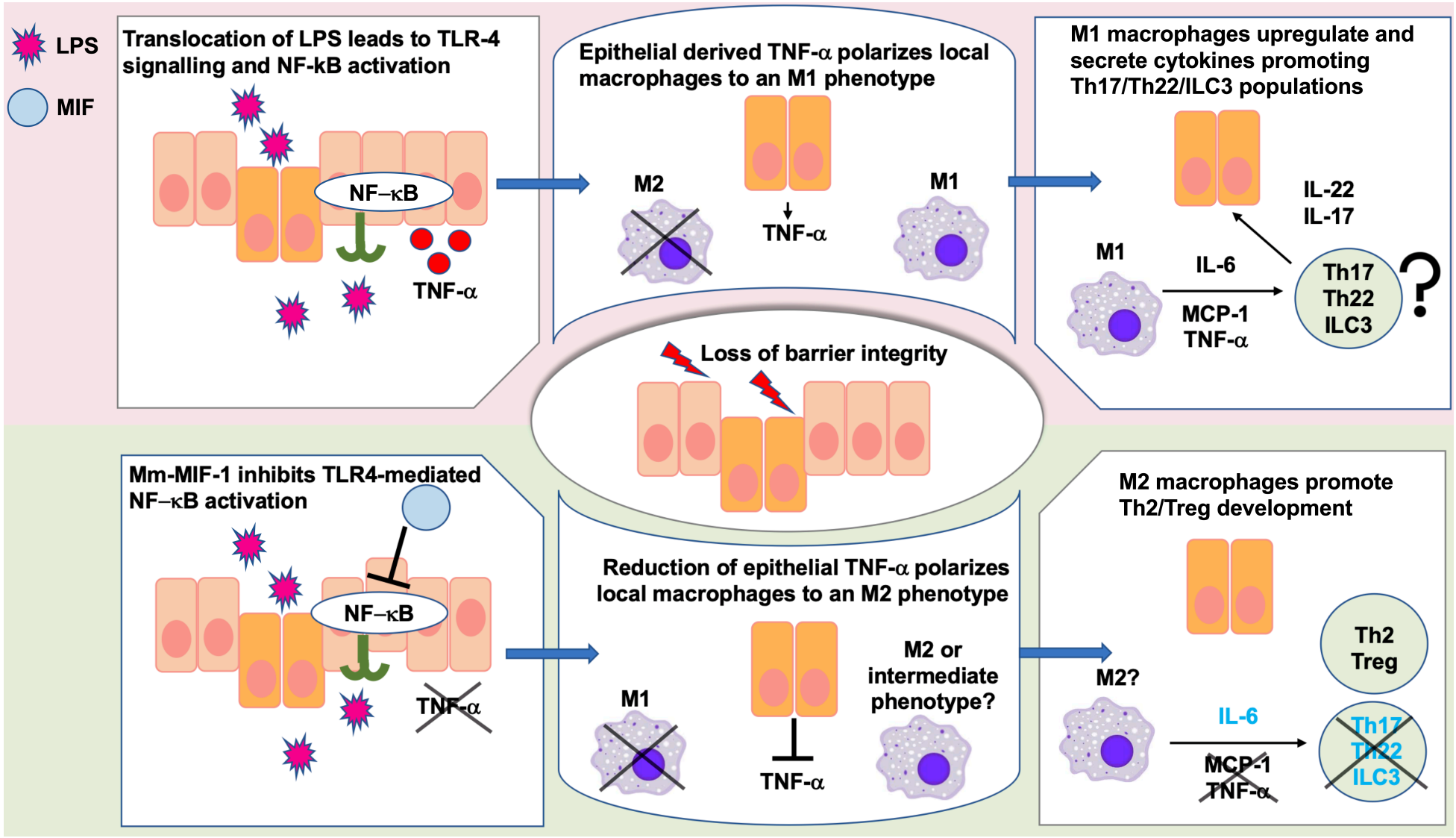
LPS
MIF
Translocation of LPS leads to TLR-4 signalling and NF-kB activation
NF–κB
TNF-α
Epithelial derived TNF-α polarizes local macrophages to an M1 phenotype
M2
TNF-α
M1
M1 macrophages upregulate and secrete cytokines promoting Th17/Th22/ILC3 populations
IL-22
IL-17
M1
IL-6
MCP-1
TNF-α
Th17
Th22
ILC3
?
Loss of barrier integrity
Mm-MIF-1 inhibits TLR4-mediated NF–κB activation
NF–κB
TNF-α
Reduction of epithelial TNF-α polarizes local macrophages to an M2 phenotype
M1
TNF-α
M2 or intermediate phenotype?
M2 macrophages promote Th2/Treg development
M2?
IL-6
MCP-1
TNF-α
Th2
Treg
Th17
Th22
ILC3

Figure 8. 1 Proposed model for Mm-MIF-1 mediated development of protective immune responses at the intestinal barrier surface after translocation of LPS. Mm-MIF-1 inhibits epithelial NF-kB driven TNF-a secretion driving the polarisation of local macrophages to an alternatively M2 phenotype. M2 macrophages inhibit typical LPS-mediated responses in the colon secreting Th2 promoting/attracting cytokine IL-6 and inhibiting pro-inflammatory cytokine MCP-1/TNF-a. Cytokines reliant on the conserved tautomerase activities are highlighted in blue.

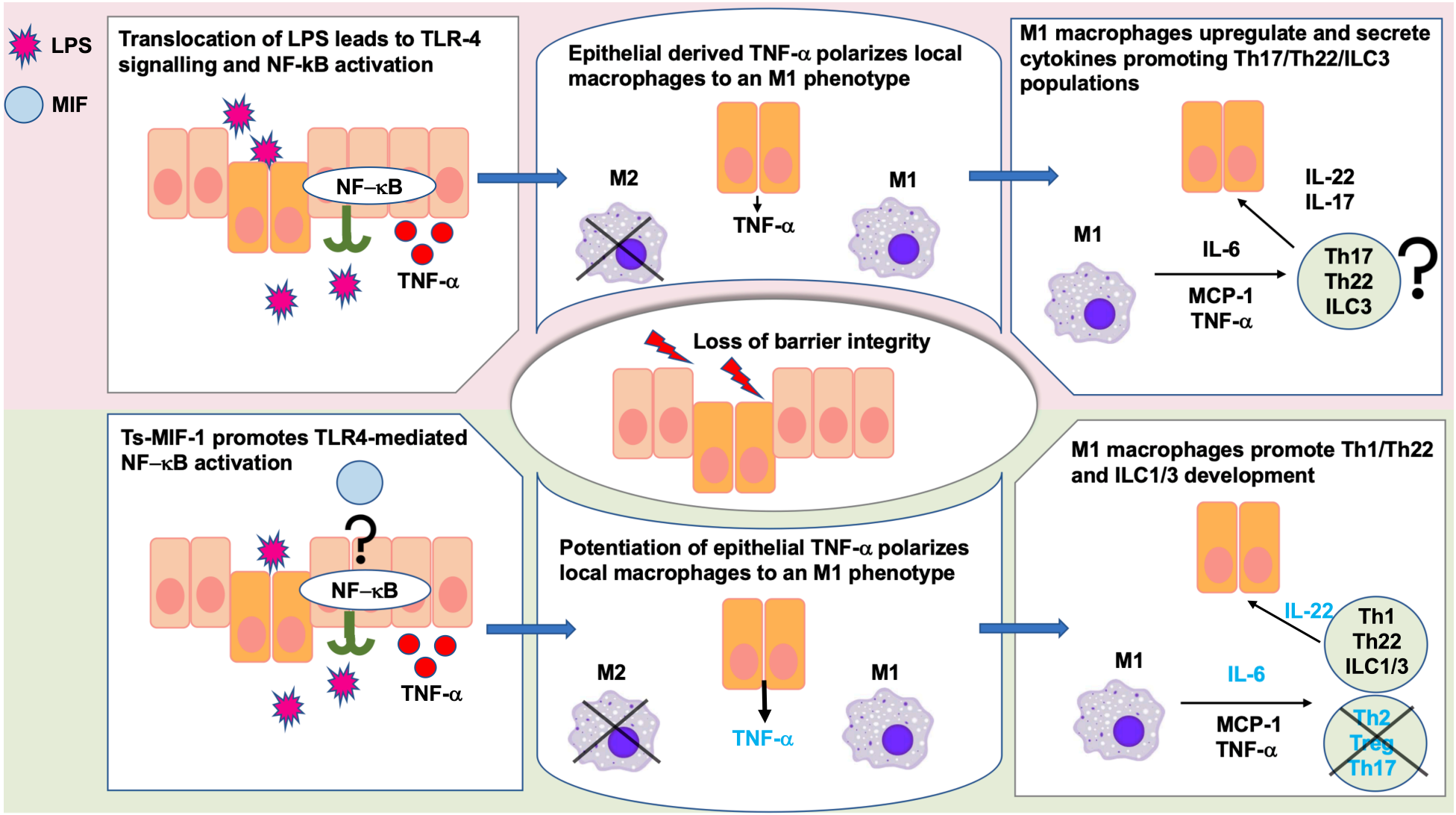
LPS
MIF
Translocation of LPS leads to TLR-4 signalling and NF-kB activation
NF–κB
TNF-α
Epithelial derived TNF-α polarizes local macrophages to an M1 phenotype
M2
TNF-α
M1
M1 macrophages upregulate and secrete cytokines promoting Th17/Th22/ILC3 populations
IL-22
IL-17
M1
IL-6
MCP-1
TNF-α
Th17
Th22
ILC3
?
Loss of barrier integrity
Ts-MIF-1 promotes TLR4-mediated NF–κB activation
?
NF–κB
TNF-α
Potentiation of epithelial TNF-α polarizes local macrophages to an M1 phenotype
M2
TNF-α
M1
M1 macrophages promote Th1/Th22 and ILC1/3 development
IL-22
Th1
Th22
ILC1/3
M1
IL-6
MCP-1
TNF-α
Th2
Treg
Th17

Figure 8. 2 Proposed model for Ts-MIF-1 mediated development of Th1, Th22 and ILC3 responses at the intestinal barrier surface after translocation of LPS. Ts-MIF-1 potentiates epithelial NF-kB driven TNF-a secretion exacerbating the polarisation of local macrophages to an inflammatory M1 phenotype. M1 macrophages inhibit protective Th2 responses in the colon secreting Th1/Th22 promoting/attracting cytokine IL-6 and TNF-a. Cytokines reliant on the conserved tautomerase activities are highlighted in blue.

8.2. Future directions.

To further address and unravel the role of MIF and MIF's evolutionarily conserved tautomerase activity in intestinal epithelial mediated immunity several future experiments are outlined below.

Whilst this PhD thesis shows key novel preliminary data to suggest MIF homologues modulate TLR4 mediated NF-kB activation, refinement of the NF-kB reporter system used earlier in our work should answer several unanswered questions such as why we see a difference in the hTLR4 mCherry NF-kB assay compared to the SEAP reporter assay when incubated with MIF alone. One possibility could be that MIF regulates secreted alkaline phosphatase post-translationally thereby skewing earlier results. To date there is no single study that has investigated MIF's role in protein regulation and synthesis. Determination of this could be achieved by, initially, analysing SEAP transcripts by qPCR after MIF treatment in order to confirm that MIF regulation of SEAP is post-translational. Additionally, co-localisation studies to determine the location of the SEAP protein within the cell could be achieved by utilising a commercially available SEAP antibody and antibodies for several subcellular locations involved in protein synthesis such as the ER (Calreticulin), the lysosome (Lamp1) and the Golgi apparatus (Golgi coiled coil protein). If SEAP is found to be inhibited post-translationally by MIF proteins additional experiments tracking general protein synthesis using newer, safer alternatives to pulse chase such as Click-iT™ (Invitrogen) technologies would establish whether MIF targets components of the secretory pathway as opposed to a specific protein.

A remaining unanswered question is, how does signalling from the multiple MIF receptor combinations confer its observed biological activities. While numerous studies have proposed that mammalian MIF has several receptors such as CD74, CD44, CXCR4 and Jab1, none have investigated how and whether *Trichinella*-derived MIF's bind to the same receptors. This is important for several reasons: identifying differences in receptor specificity and binding will offer valuable insight into which MIF domains are important for binding and signal transduction, and potential differences in the way the two homologues transduce signals will provide essential evidence for the mechanism involved in their very distinct immune responses. With this in mind, future studies should look to undertake protein-protein interaction studies to enhance the knowledge regarding MIF's tautomerase site and receptor binding. Furthermore, as discussed earlier in this thesis, it is highly likely that Ts-MIF-1 and Ts-MIF-P2G are unable to bind Jab1 due to the absence of any cysteine residues within the protein sequence. It would be important to confirm this using co-immunoprecipitation studies. An addition to this area of research should also address WT and mutant MIF interactions with p53 after our RNA-seq work revealed that Mm-MIF's and Ts-MIF's may modulate p53 using distinct mechanisms.

To build on the novel work in this PhD project evidencing that MIF homologues play a critical but discrete role in mediating intestinal immune responses particularly when barrier function is compromised, further experiments validating the observed responses should include the use of primary epithelial cells and tissue specific macrophages such as the highly specialised colonic macrophages. Additionally, this should extend to detailed immunophenotyping macrophage and T cell subsets in intestinal tissue to bridge the gap in knowledge regarding MIF's role in mediating innate responses and therefore driving adaptive

immunity. This should include but not be limited to analysis of key transcription factors involved in regulating Th responses such as T-bet (Th1), Gata-3 (Th2), FoxP3 (Treg) and critically, ROR-gT (Th17/Th22). While we show promising data here that MIF alone, and in the context of the TLR4 ligand LPS, regulates local cytokines to modulate barrier function in *ex vivo* explants, obtaining further clarification as to the identity of MIF responsive cells and the specific mechanism of action utilised by MIF.

The research encompassed in this study utilised recombinant MIF proteins for cellular assays, however, MIF-1 and MIF-1/DDT is also produced endogenously. To further extrapolate the contribution of endogenously produced MIFs, silencing techniques such as shRNA or knock out using CRISPR/CAS9 (which also allows for cleavage of specific enzymatic sites) would be useful in colonic cell lines in order to identify whether exogenous and endogenous MIFs work in concert or as a positive feedback look to regulate intestinal immunity. Treatment of MIF-silenced IEC cells, with WT and mutant mammalian and *Trichinella*-derived recombinant MIFs, versus treatment of WT IEC lines would potentially reveal whether the two MIF homologues require the presence of endogenously produced MIFs to exert their varied biological effects.

Of upmost importance is the validation of the roles of MIF-1 and MIF-2's tautomerase activities *in vivo.* The previously published MIF-1 P2G knock in murine model (Fingerle-Rowson *et al.*, 2009) would be incredibly useful when assessed in the context of loss of barrier function such as the well-documented DSS-induced colitis model. This would combine and build on our work in this study that demonstrated the importance of the tautomerase site in maintaining barrier function during damage and in the context of macrophage development.

Key experiments should include histological analysis and disease activity scoring after colitis induction to identify whether loss of the tautomerase site plays an initial role in the induction and severity of disease. Additionally, isolation and characterisation of immune cell subsets including macrophage and T cell populations would support our earlier *ex vivo studies* showing that the tautomerase site is essential for IL-22 and TNF-a production. Furthermore, to confirm that macrophages require the endogenous tautomerase site to mediate MIF's innate responses in the GT, adoptive transfer of WT macrophages into DSS induced WT and MIF-P2G knock in mice will provide crucial insight into the biological significance of MIF's tautomerase site.

8.3. Concluding remarks.

Overall, the work encompassed in this thesis provides valuable insight, through *in vitro* and *ex vivo* models, into MIF's regulatory capabilities in the intestinal environment during barrier injury. We have shown clear evidence that the tautomerase site is, at least in part, responsible for the regulation of cytokines, IL-22 and TNF-a, associated with the maintenance of barrier integrity. Additionally, we have characterised the transcriptomic profile of primary murine BMDMs after treatment with MIF which has revealed several interesting findings such as the Mm-MIF-1 specific regulation of TNF -α regulating genes and the Ts-MIF-1 driven upregulation of genes associated with cell cycle progression. Having just begun to characterise the effect of MIF and MIF tautomerase mutants on the intestinal epithelial driven immune response, the further work described previously will be required to accurately understand the role that MIF and crucially, MIF's enzymatic activities contribute in intestinal disorders such as colitis where they could be potential therapeutic target.

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Appendix

Table A. 1 List of primers used for cloning.

Primer name	Primer sequence
Hs_MIF1_Fw	CATATGCCGATGTTCATCG
Hs_MIF1_Rv	CTCGAGTTAGGCGAAGGTGG
Hs_MIF1P2G_Fw	CATATGGGCATGTTCATCGTAAACACC
Hs_MIF1C57S/C60S_Fw	AGC GCG CTC AGC AGC CTG CAC AGC ATC GGC AAG
Hs_MIF1C57S/C60S_Rv	ATG CTG TGC AGG CTG CTG AGC GCG CTC GGC
Hs_DDT_Fw	CATATGCCGTTCCTGGAGCTGG
Hs_DDT_Rv	CTCGAGGGGCTAGCTCCTTGGTGAG
Mm_MIF1_Fw	CATATGCCTATGTTCATCGTGAACACC
Mm_MIF1_Rv	CTCGAGAGCGAAGGTGGAACCGTTCCAGCC
Mm_MIF1P2G_Fw	CATATGGGCATGTTCATCGTGAACACC
Mm_DTT1_Fw	CATATGCCATTCGTTGAGTTGGAAACA
Mm_DTT_Rv	CTCGAGCAGAAATGTCATGACAGTTCCTTT
Ts_MIF1_Fw	CATATGCCTATCTTTACTCTTAATAC
Ts_MIF1_Rv	CTCGAGGAATGTAGTACCGTTCCAACCAAC
Ts_MIF1_P2G	CATATGGGCATCTTTACTCTTAATACA
Ts_MIF2_Fw	CATATGCCAATTTTCACAATAA
Ts_MIF2_Rv	CTCGAGCGCTCGCAGACAGCCACT

Table A. 2 Full sequences used for cloning.

Homologue	Accession number	Sequence
Hs-MIF-1	Z23063.1	>ATGCCGATGTTCATCGTAAACACCAACGTGCCCCGCGCCTCCGTGCCGGACGGGTTCCTCTCC GAGCTCACCCAGCAGCTGGCGCAGGCCACCGGCAAGCCCCCCCAGTACATCGCGGTGCACGT GGTCCCGGACCAGCTCATGGCCTTCGGCGGCTCCAGCGAGCCGTGCGCGCTCTGCAGCCTGCA CAGCATCGGCAAGATCGGCGGCGCGCAGAACCGCTCCTACAGCAAGCTGCTGTGCGGCCTGCT GGCCGAGCGCCTGCGCATCAGCCCGGACAGGGTCTACATCAACTATTACGACATGAACGCGGC CAATGTGGGCTGGAACAACTCCACCTTCGCCTAAGAGCCGCAG
Hs-DDT-1	NM_001084392.1	>ATGCCGTTCCTGGAGCTGGACACGAATTTGCCCGCCAACCGAGTGCCCGCGGGGCTGGAGAA ACGACTCTGCGCCGCCGCTGCCTCCATCCTGGGCAAACCTGCGGACCGCGTGAACGTGACGGT ACGGCCGGGCCTGGCCATGGCGCTGAGCGGGTCCACCGAGCCCTGCGCGCAGCTGTCCATCT CCTCCATCGGCGTAGTGGGCACCGCCGAGGACAACCGCAGCCACAGCGCCCACTTCTTTGAGT TTCTCACCAAGGAGCTAGCCCTGGGCCAGGACCGGATACTTATCCGCTTTTTCCCCTTGGAGTC CTGGCAGATTGGCAAGATAGGGACGGTCATGACTTTTTTATGA
Mm-MIF-1	NM_010798.3	>ATGCCTATGTTCATCGTGAACACCAATGTTCCCCGCGCCTCCGTGCCAGAGGGGTTTCTGTCG GAGCTCACCCAGCAGCTGGCGCAGGCCACCGGCAAGCCCGCACAGTACATCGCAGTGCACGTG GTCCCGGACCAGCTCATGACTTTTAGCGGCACGAACGATCCCTGCGCCCTCTGCAGCCTGCACA GCATCGGCAAGATCGGTGGTGCCCAGAACCGCAACTACAGTAAGCTGCTGTGTGGCCTGCTGT

		CCGATCGCCTGCACATCAGCCCGGACCGGGTCTACATCAACTATTACGACATGAACGCTGCCAA CGTGGGCTGGAACGGTTCCACCTTCGCTTGA
Mm-DDT-1	NM_010027.1	>ATGCCATTCGTTGAGTTGGAAACAAACTTGCCGGCTAGCCGCATACCCGCGGGGCTGGAGAAC CGGCTGTGTGCGGCCACAGCCACCATCCTGGACAAACCCGAAGACCGCGTGAGCGTTACGATA CGACCTGGCATGACCCTGTTGATGAACAAATCCACAGAGCCTTGTGCTCACCTTCTGGTCTCTTC CATCGGGGTTGTGGGCACCGCGGAGCAGAACCGCACTCACAGCGCCAGCTTCTTCAAGTTCCT CACCGAGGAGCTGTCCCTGGACCAGGACCGGATCGTTATCCGCTTCTTCCCCTTGGAGGCTTGG CAGATCGGAAAGAAAGGAACTGTCATGACATTTCTGTGA
Ts-MIF-1	AJ012740.1	>ATGCCATTTACNTAAACAAACATCAAAGCTACCGATGTTCCGTCGGACTTTTTGTCCAGCACAAG CGCACTTGTTGGTAATATATTATCAAAACCAGGAAGTTATGTAGCTGTGCACATCAACACAGATCA GCAGTTGTCGTTTGGCGGAAGTACAAATCCTGCTGCATTCGGTACTCTGATGTCGATTGGTGGAA TAGAACCAAGCAGAAATCGTGATCATTCGGCCAAACTGTTTGATCATCTTAACAAAAAATTGGGC ATTCCAAAAAATAGAATGTATATCCATTTCGTCAATCTGAACGGAGACGATGTTGGTTGGAACGG TACTACATTCTGA
Ts-MIF-2	Unpublished (D.Guiliano)	>ATGCCAATTTTCACAATAATTACAAATAAAAAAACTGCACCGAAAGATTTTCACCGATTGCTAACA GATCTGTTGGCGGAATTGCTGAAAAAACCGAAAGAGCTAGTGGTGGTTGATTTATTGCTTGATCA AAAAATGGAATTTGGCGGCGCTGATGATCCTTGTCTGATTGGCGTAGTTCGAGCGGTTGGAAGA ATCAGTGCAGAAGAAAATGCACAATATGCCGAAAGATTGAGTGAATTTCTACATCAGCAATTAGG CATTCTTCCACAACGAATGTACATACGGTACTTGAATATGGACGGCTTTTACGTTGGATGGAGTG GCTGTCTGCGAGCG
CD74	NM_004355.3	>ATGCACAGGAGGAGAAGCAGGAGCTGTCGGGAAGATCAGAAGCCAGTCATGGATGACCAGCG CGACCTTATCTCCAACAATGAGCAACTGCCCATGCTGGGCCGGCGCCCTGGGGCCCCGGAGAG

		CAAGTGCAGCCGCGGAGCCCTGTACACAGGCTTTTCCATCCTGGTGACTCTGCTCCTCGCTGGC CAGGCCACCACCGCCTACTTCCTGTACCAGCAGCAGGGCCGGCTGGACAAACTGACAGTCACC TCCCAGAACCTGCAGCTGGAGAACCTGCGCATGAAGCTTCCCAAGCCTCCCAAGCCTGTGAGCA AGATGCGCATGGCCACCCCGCTGCTGATGCAGGCGCTGCCCATGGGAGCCCTGCCCCAGGGG CCCATGCAGAATGCCACCAAGTATGGCAACATGACAGAGGACCATGTGATGCACCTGCTCCAGA ATGCTGACCCCCTGAAGGTGTACCCGCCACTGAAGGGGAGCTTCCCGGAGAACCTGAGACACC TTAAGAACACCATGGAGACCATAGACTGGAAGGTCTTTGAGAGCTGGATGCACCATTGGCTCCT GTTTGAAATGAGCAGGCACTCCTTGGAGCAAAAGCCCACTGACGCTCCACCGAAAGAGTCACTG GAACTGGAGGACCCGTCTTCTGGGCTGGGTGTGACCAAGCAGGATCTGGGCCCAGTCCCCATG TGA
CD44	AY101193.1	>ATGGACAAGTTTTGGTGGCACGCAGCCTGGGGACTCTGCCTCGTGCCGCTGAGCCTGGCGCA GATCGATTTGAATATAACCTGCCGCTTTGCAGGTGTATTCCACGTGGAGAAAAATGGTCGCTACA GCATCTCTCGGACGGAGGCCGCTGACCTCTGCAAGGCTTTCAATAGCACCTTGCCCACAATGGC CCAGATGGAGAAAGCTCTGAGCATCGGATTTGAGACCTGCAGGTATGGGTTCATAGAAGGGCAC GTGGTGATTCCCCGGATCCACCCCAACTCCATCTGTGCAGCAAACAACACAGGGGTGTACATCC TCACATCCAACACCTCCCAGTATGACACATATTGCTTCAATGCTTCAGCTCCACCTGAAGAAGATT GTACATCAGTCACAGACCTGCCCAATGCCTTTGATGGACCAATTACCATAACTATTGTTAACCGT GATGGCACCCGCTATGTCCAGAAAGGAGAATACAGAACGAATCCTGAAGACATCTACCCCAGCA ACCCTACTGATGATGACGTGAGCAGCGGCTCCTCCAGTGAAAGGAGCAGCACTTCAGGAGGTTA CATCTTTTACACCTTTTCTACTGTACACCCCATCCCAGACGAAGACAGTCCCTGGATCACCGACA GCACAGACAGAATCCCTGCTACCAGAGACCAAGACACATTCCACCCCAGTGGGGGGTCCCATAC CACTCATGGATCTGAATCAGATGGACACTCACATGGGAGTCAAGAAGGTGGAGCAAACACAACC TCTGGTCCTATAAGGACACCCCAAATTCCAGAATGGCTGATCATCTTGGCATCCCTCTTGGCCTT GGCTTTGATTCTTGCAGTTTGCATTGCAGTCAACAGTCGAAGAAGGTGTGGGCAGAAGAAAAAG CTAGTGATCAACAGTGGCAATGGAGCTGTGGAGGACAGAAAGCCAATTGGACTCAACGGAGAG

	GCCAGCAAGTCTCAGGAAATGGTGCATTTGGTGAACAAGGAGTCGTCAGAAACTCCAGACCAGT TTATGACAGCTGATGAGACAAGGAACCTGCAGAATGTGGACATGAAGATTGGGGTGTAA
pGEM-T Easy	>GGGCGAATTGGGCCCGACGTCGCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATnA TCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGCTCCCAACGCGTTGGAT GCATAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCC TGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAG CCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAG TCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTG CGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGC GAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGG AAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGC GTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGG CGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTC CTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCT TTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTG TGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAA CCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAG GTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACA GTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATC CGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGA AAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAA CTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTA AAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTA ATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGT CGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGA

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pET29b	>ATCCGGATATAGTTCCTCCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCAAGGGG TTATGCTAGTTATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTAGCAG CCGGATCTCAGTGGTGGTGGTGGTGGTGCTCGAGTGCGGCCGCAAGCTTGTCGACGGAGCTCG AATTCGGATCCGATATCGCCATGGAACCGCGTGGCACCAGGGTACCCAGATCTGGGCTGTCCAT GTGCTGGCGTTCGAATTTAGCAGCAGCGGTTTCTTTCATATGTATATCTCCTTCTTAAAGTTAAAC AAAATTATTTCTAGAGGGGAATTGTTATCCGCTCACAATTCCCCTATAGTGAGTCGTATTAATTTC GCGGGATCGAGATCGATCTCGATCCTCTACGCCGGACGCATCGTGGCCGGCATCACCGGCGCC ACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGGCTCGCCAC TTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGACTG TTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTAC TACTGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGAGATCCCGGACACCATC GAATGGCGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGAGAGTCAATTCAGGGTG GTGAATGTGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGACCGT TTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGGGAAAAAGTGGAAGCGGC GATGGCGGAGCTGAATTACATTCCCAACCGCGTGGCACAACAACTGGCGGGCAAACAGTCGTTG CTGATTGGCGTTGCCACCTCCAGTCTGGCCCTGCACGCGCCGTCGCAAATTGTCGCGGCGATTA AATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTCGATGGTAGAACGAAGCGGCGTCG AAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAACGCGTCAGTGGGCTGATCATTAACTA TCCGCTGGATGACCAGGATGCCATTGCTGTGGAAGCTGCCTGCACTAATGTTCCGGCGTTATTT CTTGATGTCTCTGACCAGACACCCATCAACAGTATTATTTTCTCCCATGAAGACGGTACGCGACT GGGCGTGGAGCATCTGGTCGCATTGGGTCACCAGCAAATCGCGCTGTTAGCGGGCCCATTAAG TTCTGTCTCGGCGCGTCTGCGTCTGGCTGGCTGGCATAAATATCTCACTCGCAATCAAATTCAGC CGATAGCGGAACGGGAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAACCATGCAAATGCT GAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAACGATCAGATGGCGCTGGGCGCAAT GCGCGCCATTACCGAGTCCGGGCTGCGCGTTGGTGCGGACATCTCGGTAGTGGGATACGACGA TACCGAAGACAGCTCATGTTATATCCCGCCGTTAACCACCATCAAACAGGATTTTCGCCTGCTGG

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CGTGTTTCGTAAAGTCTGGAAACGCGGAAGTCAGCGCCCTGCACCATTATGTTCCGGATCTGCA
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CTGGACGCGGATGAACAGGCAGACATCTGTGAATCGCTTCACGACCACGCTGATGAGCTTTACC
GCAGCTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGA
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Table A. 3 qPCR primers used in this study.

Primer name	Primer sequence
Hs_TBP_Fw	ACCCAGCAGCATCACTGTTTC
Hs_TBP_Rv	CAAGCCCTGAGCGTAAGGTG
Mm_GAPDH_Fw	CTCCCACTCTTCCACCTTCG
Mm_GAPDH_Rv	GCCTCTCTTGCTCAGTGTCC
Mm_IL-6_Fw	CCCCAATTTCCAATGCTCTCC
Mm_IL-6_Rv	AGGTTTGCCGAGTAGATCTCAA
Mm_IL-22_Fw	TTGACACTTGTGCGATCTCTGA
Mm_IL-22_Rv	AGGTGCGGTTGACGATGTAT
Mm_IL-17_Fw	TCTCCACCGCAATGAAGACC
Mm_IL-17_Rv	CACACCCACCAGCATCTTCT
Mm_MCP-1_Fw	GCTCAGCCAGATGCAGTTAA
Mm_MCP-1_Rv	TCTTGAGCTTGGTGACAAAAACT
Mm_IL-10_Fw	ATAACTGCACCCACTTCCCA
Mm_IL-10_Rv	GGGCATCACTTCTACCAGGT

Table A. 4 Differentially expressed genes in MIF treated BMDMs

Sample ID	Gene ID	Accession Number	Gene Name	Log2FC	Adjusted p value
Mm-MIF-1					
	Snx31	NM_025712	sorting nexin 31	22.805	0.0084773099
	Foxa1	NM_008259	forkhead box A1	15.93	0.0026540933
	Hhip	NM_020259	Hedgehog-interacting protein	13.146	0.0019448917
	Krt7	NM_033073	keratin 7	12.893	0.0031562406
	Krt15	NM_008469	keratin 15	2.242	0.0002851333
	Wfdc2	NM_026323	WAP four-disulfide core domain 2	-14.026	0.0001562889
	Upk2	NM_009476	uroplakin 2	-14.277	0.0002851333
	Fam25c	NM_183278	family with sequence similarity 25, member C	-15.053	0.0001831548
	Adam28	NM_010082	a disintegrin and metallopeptidase domain 28	-16.523	0.0002241468
	Rab27b	NM_030554	RAB27B, member RAS oncogene family	-16.628	0.0040745852
	Upk1b	NM_178924	uroplakin 1B	-17.593	0.0002217725
	Sprr1a	NM_009264	small proline-rich protein 1A	-23.883	0.0000397287
	Trp63	NM_001127259	transformation related protein 63	-28.424	0.0000397287
Mm-MIF-1 P2G					

Snx31	NM_025712	sorting nexin 31	29.907	0.0000023700
Foxa1	NM_008259	forkhead box A1	25.244	0.0002818600
Krt7	NM_033073	keratin 7	24.462	0.0000155000
Bmp3	NM_173404	bone morphogenetic protein 3	20.858	0.0087996100
Nfe2l3	NM_010903	nuclear factor, erythroid derived 2, like 3	20.779	0.0087996100
Krt19	NM_001313963	keratin 19	20.674	0.0090747000
Fhl1	NM_001077362	four and a half LIM domains 1	20.393	0.0102459000
Hhip	NM_020259	Hedgehog-interacting protein	20.118	0.0102459000
Tmprss2	NM_015775	transmembrane protease, serine 2	20.088	0.0102459000
Krt15	NM_008469	keratin 15	20.056	0.0102459000
Krt5	NM_027011	keratin 5	19.952	0.0102459000
Igfbp2	NM_008342	insulin-like growth factor binding protein 2	19.914	0.0102459000
Psca	NM_028216	prostate stem cell antigen	19.838	0.0102459000
Krt8	NM_031170	keratin 8	19.749	0.0105545800
Krt4	NM_008475	keratin 4	19.074	0.0157563500
Fam84a	NM_029007	family with sequence similarity 84, member A	18.974	0.0158858600
Bmp7	NM_007557	bone morphogenetic protein 7	18.969	0.0158858600
Klhdc7a	NM_173427	kelch domain containing 7A	18.944	0.0158858600
Sprr2a3	NM_001309382	small proline-rich protein 2A3	18.929	0.0158858600
Cldn8	NM_018778	claudin 8	18.682	0.0177027400

	Upk1b	NM_178924	uroplakin 1B	14.525	0.0016875100
	Upk2	NM_009476	uroplakin 2	10.227	0.0017721000
	Wfdc2	NM_026323	WAP four-disulfide core domain 2	9.692	0.0018324200
	Rab27b	NM_030554	RAB27B, member RAS oncogene family	8.657	0.0018796900
	Fam25c	NM_183278	family with sequence similarity 25, member C	8.252	0.0018833900
	Adam28	NM_010082	a disintegrin and metallopeptidase domain 28	6.566	0.0025662800
	Sprr1a	NM_009264	small proline-rich protein 1A	4.13	0.0026212200
Ts-MIF-1					
	Snx31	NM_025712	sorting nexin 31	21.513	0.0000068970
	Hhip	NM_020259	Hedgehog-interacting protein	20.356	0.0000199348
	Krt15	NM_008469	keratin 15	19.253	0.0000535239
	Tmprss2	NM_015775	transmembrane protease, serine 2	18.814	0.0000800932
	Krt5	NM_027011	keratin 5	18.782	0.0000824047
	Klhdc7a	NM_173427	kelch domain containing 7A	18.368	0.0001195510
	Krt7	NM_033073	keratin 7	17.916	0.0000163666
	Psca	NM_028216	prostate stem cell antigen	17.741	0.0001954905
	Nfe2l3	NM_010903	nuclear factor, erythroid derived 2, like 3	17.728	0.0002257294
	Igfbp2	NM_008342	insulin-like growth factor binding protein 2	15.924	0.0008349219
	Upk2	NM_009476	uroplakin 2	15.414	0.0011005480

	Bmp7	NM_007557	bone morphogenetic protein 7	15.327	0.0013297415
	Krt8	NM_031170	keratin 8	14.837	0.0018887475
	Fam84a	NM_029007	family with sequence similarity 84, member A	14.797	0.0019448134
	Bmp3	NM_173404	bone morphogenetic protein 3	14.785	0.0021270654
	Krt4	NM_008475	keratin 4	13.982	0.0036324032
	Cldn8	NM_018778	claudin 8	13.941	0.0037382788
	Sprr2a3	NM_001309382	small proline-rich protein 2A3	13.905	0.0036606252
	Trp63	NM_001127259	transformation related protein 63	-28.39	0.0000000024
Ts-MIF-1 P2G					
	Hhip	NM_020259	Hedgehog-interacting protein	18.953	0.0000763000
	Bmp3	NM_173404	bone morphogenetic protein 3	14.928	0.0019237500
	Foxa1	NM_008259	forkhead box A1	12.725	0.0087344700
	Sprr1a	NM_009264	small proline-rich protein 1A	-6.967	0.0140244100
	Wfdc2	NM_026323	WAP four-disulfide core domain 2	-13.602	0.0048860700
	Upk2	NM_009476	uroplakin 2	-14.265	0.0031559400
	Fam25c	NM_183278	family with sequence similarity 25, member C	-14.566	0.0025819400
	Trp63	NM_001127259	transformation related protein 63	-27.989	0.0000000040

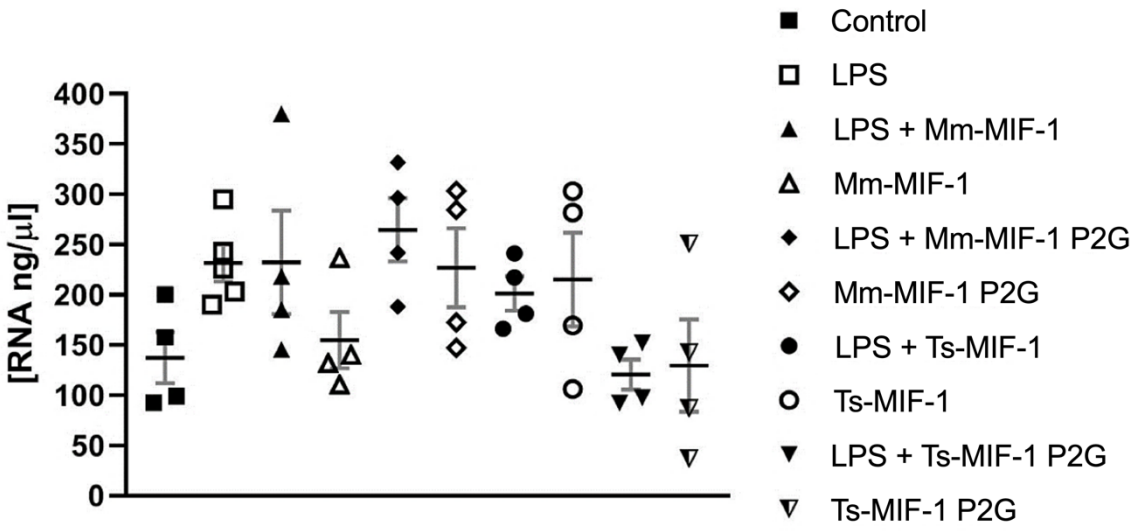

Figure A. 1 Explant RNA concentration. Colonic explants were cultured for 20 hours in the presence or absence of 100ng/ml LPS +/- 100ng/ml MIF. Explant tissue was weighed and lysed in an appropriate volume of Trizol using beads to homogenise. Chloroform was added to Trizol and, after incubation, the aqueous phase was precipitated with isopropanol and put through a direct-zol column. RNA concentration and integrity were analysed using a Nanodrop 2000. Figure represents RNA concentration obtained from experiments represented in this study. The data represents the mean ±SEM (n=4).