PERSEVERANCE OF MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF) IN PULMONARY LEPTOSPIROSIS

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Bharathidasan University in the partial fulfillment
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IN
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Submitted By

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CERTIFICATE

This is to certify that the thesis entitled **"Perseverance of macrophage migration inhibitory factor (MIF) in pulmonary leptospirosis"** submitted to Bharathidasan University, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** is a record of original and independent research work done by **Ms. K. Sumaiya (Register No. 27668)** in the Department of Microbiology, Centre for Excellence in Life Sciences, Bharathidasan University, Tiruchirappalli - 620 024, under my supervision and guidance and the thesis has not formed the basis for the award of any Degree/Diploma/ Associateship/Fellowship or other similar title to any University.

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DECLARATION

I, **K. SUMAIYA**, hereby declare that the dissertation, entitled **"Perseverance of Macrophage migration inhibitory factor (MIF) in pulmonary leptospirosis"**, submitted to the Bharathidasan University, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Microbiology** is a record of original and independent research work done by me under the supervision and guidance of **Dr. K. Natarajaseenivasan**, **Professor, Department of Microbiology,** Centre for Excellence in Life Sciences, Bharathidasan University and it has not formed the basis for the award of any Degree/ Diploma/ Associateship/ Fellowship or other similar titles to any candidate in any University.

Signature of the candidate

K. SUMAIYA

DEDICATED

TO MY TEACHER

TO MY FAMILY

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"A favour done, not as return for another, is more valuable than
Heaven and earth put together."

- Thirukkural (101)

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Abstract

Abstract

The casting about for promising early diagnostic biomarkers and severity predictors for leptospirosis and the quest for an insight picture of leptospirosis pathophysiology is still ongoing. The goal of the present study was to investigate the diagnostic and severity predictive value of macrophage migration inhibitory factor (MIF) and the role of MIF in the pathogenesis of leptospirosis. MIF is a multifaceted pro-inflammatory cytokine that regulates the host's immune responses to infection and contributes to several biological functions including pathological and physiological processes. This study was initiated with seroprevalence of leptospirosis, for which confirmed leptospirosis cases (N=143), other febrile cases (N=101) and healthy controls (N=57) were included. The seroprevalence of leptospirosis was 47.3%, and the highly prevalent serovars were found to be Autumnalis, followed by Australis and Canicola with the gold standard reference microscopic agglutination test (MAT) with a titer range between 1:80 and 1: 2560. MIF-Enzyme linked immunosorbent assay (MIF-ELISA) showed that serum MIF levels were significantly ($P < 0.001$) higher in patients with different clinical manifestations of leptospirosis including, febrile illness, pulmonary hemorrhages, Weil's syndrome, and renal failure than in healthy controls. Serum MIF has considered a valuable diagnostic marker of leptospirosis with 100% sensitivity and >90% specificity and an area under the curve (AUC) value of >0.9 ($P < 0.0001$). The optimum concentration of leptospiral lipopolysaccharide (LPS) to generate *in vitro* (THP-1 cells) and *in vivo* (BALB/c mice) models were 1µg/mL and 20mg/kg, respectively. Leptospiral LPS stimulated the initiation of high MIF profile at 30 minutes post-treatment, which reached a maximum level of expression at 24h *in vitro*, whereas the elevated and differential MIF expression in lymphoid and other vital organs of *in vivo* model at the early stage of infection. Thus, MIF is considered an early stage secreted cytokine which serves as a promising

diagnostic marker of leptospirosis. In the present study, we found that leptospiral LPS triggers the intracellular calcium influx $[Ca^{2+}]_i$ in a time-dependent manner. LPS mediated induction of intracellular $[Ca^{2+}]_i$ signaling was achieved through enhanced expression of store operated calcium entry (SOCE) proteins (Inositol 1,4,5- trisphosphate receptor [IP3R], ORAI1, Stromal interaction molecule 1 [STIM 1]). The knocking down of ORAI1 has downregulated the MIF expression in LPS stimulated conditions, evidencing intracellular Ca^{2+} drives MIF expression. In addition, MIF expression is significantly regulated by the activation of the transcription factor, cAMP-response element binding protein (CREB). The CREB activation was accomplished by the phosphorylation of Calcium/calmodulin-dependent protein kinase II (CAMKII), which may regulate MIF expression. MIF knockdown preserves the THP-1 cells from cytotoxicity, reactive oxygen species (ROS) production, mitochondrial membrane potential ($\Delta\Psi_m$), and cell death by reducing the cytochrome c release in MIF siRNA transfected cells rather than scrambled siRNA (Scr siRNA) transfected cells. (S,R)-3-(4 hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) suppressed the leptospiral infection mediated histopathological alterations of the lung including immune cell infiltration, hemorrhage, and alveolar congestion. Leptospiral infection and MIF induction significantly increased TNF-α, IL-1β, and IL-4 mRNA levels whereas the administration of ISO-1 increased the IL-10 levels *in vivo*. Kaplan Meier survival plots illustrated that the blockade of MIF prevented the mice from LPS-induced lethality by increasing their survivability. Given the importance of MIF in infectious diseases, these reports proposed the blockade of MIF as an efficient therapeutic strategy for the MIF mediated leptospiral pathogenesis. As MIF is associated with the severity of leptospirosis, gene polymorphism (MIF 173G/C) single nucleotide polymorphism (SNP) was analyzed by PCR based Restriction fragment length polymorphism (PCR-RFLP). The C allele related genotype

GC (OR: 28.4; 95% CI: 10.9–73.6; $p < 0.001$) and CC (OR: 40; 95% CI: 2.3–686.5; $p < 0.001$) was associated with susceptibility and severity of leptospirosis respectively. In severe leptospirosis cases, a significantly higher number of cases were CC genotype carriers, whereas in mild leptospirosis cases, a higher number of cases were found to be the GC genotype carriers. High MIF expression genotypes GC and CC cause the upregulation of MIF mRNA and protein expression. Pearson correlation test showed a significant positive correlation between elevated serum MIF and -173*C allele ($r = 0.99$, $p < 0.001$) and suggested that MIF -173G/C SNP is correlated with susceptibility and severity of leptospirosis and also MIF genetic polymorphism could be a promising severity predictor of leptospirosis. To develop the MIF directed therapeutic approach for the treatment of leptospirosis, we identified potential MIF inhibitors by screening 10 essential tautomerase inhibition classes of chemical compounds and 7 existing anti-inflammatory and anti-microbial drugs. Among 17 chemical compounds, ibudilast showed the MIF tautomerase IC_{50} value at very lower concentration (9.5 ± 5.6 µM) which is similar to the IC_{50} of standard MIF antagonist, ISO-1 (6.2 ± 3.8 µM) with high biocompatibility. The *in vitro* and *in vivo* analysis of the therapeutic potential of MIF inhibitor revealed that ibudilast significantly reduced the leptospiral LPS mediated inflammatory responses in THP-1 cells and downregulates the inflammatory responses and histopathological changes and protects the leptospiral BALB/c model from lethality by increasing the survival rate from 25% to 66%. The biocompatibility assays revealed that ≤50 concentration of ibudilast showed no significant cytotoxicity, Red blood cell (RBC) lysis, and cell death.

Introduction

1. Introduction

Leptospirosis is a globally important emerging infectious disease caused by pathogenic species of the genus *Leptospira*. Leptospires are spirochetes with length of about 6-20 μm and 0.1 μm in diameter. The genus *Leptospira* comprises 20 species and >300 serovars under 20 serogroups. Leptospirosis has transmitted by direct or indirect exposure to infected host animals. Humans are only the occasional hosts in the wild and domestic animals are involved transmission cycle (Faine and World Health Organization, 1982; Haake & Levett, 2015; Bharti *et al*., 2003). Every year 1.03 million leptospirosis cases and 58,900 deaths are estimated worldwide. In resource poor countries, leptospirosis is underrated due to the lack of proper diagnostic features leading to high morbidity and mortality (Costa *et al*., 2015). It has been considered an emerging worldwide public health problem. Nowadays, it constitutes a major challenge in the healthcare system. Leptospirosis has two major limitations such as scarcity of knowledge on leptospirosis prevalence and the importance of disease consideration, and the lack of promising diagnostic markers of leptospirosis lead to poor disease documentation and misdiagnosis of leptospirosis. Such limitations still exist in developing countries and cause several complications in treatments (Picardeau, 2015). Every year, leptospirosis has been reported worldwide, especially in tropical and subtropical areas. Though it has been reported for several decades, it is not well profiled in developing countries. The developing countries have no effective control programs for leptospirosis, so natural disasters, especially flooding increase the risk of disease onset and transmission (Karpagam & Ganesh, 2020; Kobayashi, 2005; Soo *et al*., 2020).

Leptospirosis encompasses a broad range of clinical and subclinical illnesses in hosts. The majority of infections are subclinical, and the symptoms are non-specific. Only 10% of patients have developed the severe stage of leptospirosis by accelerating host inflammatory responses in

response to leptospiral infection. The non-specific clinical symptoms overlapping with other febrile illnesses and lack of proper early diagnostic assay and improper disease monitoring system cause leptospirosis still be underdiagnosed (Chaudhry *et al*., 2002; Cagliero *et al*., 2018). The available diagnostic tests for leptospirosis are MAT, polymerase chain reaction (PCR), ELISA, complement fixation test, macroscopic slide agglutination test, microcapsule agglutination test, dipstick assay, indirect hemagglutination assay (IHA) and other techniques. The diagnosis of leptospirosis is largely dependent on serological tests, so these assays are not suitable for early diagnosis. MAT is the most widely used serovar specific gold standard technique to diagnose leptospirosis with the major limitations of time consuming, needs the proper maintenance of live leptospiral antigens, and requires experts to perform the test and analyze the results. Although PCR is another confirmatory diagnostic test, it is time consuming and not performed in small healthcare settings (Musso and La Scola, 2013; Pinto *et al*., 2022). Thus, the frequent estimation of leptospirosis prevalence and early diagnostic tools are imperative for the early detection and management of leptospirosis.

MIF is a pluripotent cytokine that plays essential role in inflammation and immune responses through its chemokine like function and regulation of systemic stress responses. It is a pro-inflammatory cytokine, secreted by immune cells and released into the bloodstream during the early stage of inflammatory diseases. It is constitutively expressed in several tissues of all vital organs and organs of the immune system. Because of its variety of biological functions, MIF exerts multifaceted activities in pathophysiological states. MIF acts as a potential initiator to promote the expression and secretion of inflammatory cytokines such as tumor necrosis factor- α (TNF-α), IL-6, and IL-1 while it inhibited the actions of glucocorticoids mediated anti-inflammatory cytokines (Calandra *et al*., 1994; Calandra and Roger, 2003). Previous studies have

shown that MIF is overexpressed in inflammatory cells and involved in the pathogenesis of several diseases (Stosic-Grujicic *et al*., 2009). Several studies reported the potential diagnostic value of MIF in inflammatory diseases, autoimmune diseases, and cancer (Hertelendy *et al*., 2018; Gunther *et al*., 2019). Comparative evaluation of serum, plasma, urinary, and tissue localized MIF expression in diseased/induced and physiological conditions was mostly studied for the development of diagnostic and therapeutic strategies (Zhao *et al*., 2011; Fouda *et al*., 2022; Baugh and Bucala, 2002). In this study, we validated the serum MIF as an early diagnostic marker of leptospirosis with a large number of sera samples obtained from different febrile cases and healthy control subjects.

LPS is the dominant outer membrane antigen of *Leptospira*. Leptospiral LPS was antigenically active and serogroup specific, which directed the innate immune responses during infection (Bulach *et al*., 2000). The presence of LPS in the leptospiral outer membrane can distinguish it from other invasive Spirochaetes. It is the basis for the antigenic diversity of leptospires. LPS consists of an endotoxic component lipid A, a core oligosaccharide, and O antigen, a polysaccharide (Isogai *et al*., 1986). The unique structure of lipid A promotes the LPS-mediated inflammatory signaling through Toll-like receptor 2 (TLR2) rather than TLR4 in human hosts. Commonly, Gram negative bacterial LPS can stimulate macrophage activation by using CD14 and TLR family. Although the substantial body of knowledge confirmed that TLR4 is the prominent receptor activating LPS mediated signals, the biochemical and genetic analysis provided the evidence that leptospiral LPS activates the inflammatory cells by TLR2 dependent signaling mechanisms (Werts *et al*., 2001; Murray *et al*., 2010). In this study, we used the LPS to develop the experimental model of leptospirosis.

Hamsters, guinea pigs and gerbils are the ideal experimental models to study the pathophysiology of acute leptospirosis while rats are used for chronic leptospirosis. Even though mice has resistant to leptospiral infection, recently the inbred and transgenic mice have been reported to be the perfect study model to understand the immune responses and inflammation mediated pathogenesis in sublethal infection, which facilitates to conducting the experiments for the development of protective vaccines (Gomes-Solecki *et al*., 2017). Immunologically mature mice and rats are resistant to leptospirosis, so young mice were selected and injected with mouse adapted or lethal challenge strain (MACS) of *Leptospira* (Adler and Faine, 1977). The biological activities of LPS in experimental models are measured by investigating the endotoxemia and endotoxemic inflammation, which contributes to the pathogenesis of several inflammation mediated diseases (Pussinen *et al*., 2022).

Uncontrolled stimulation of inflammatory responses during leptospiral infection causes cytokine storm, which leads to tissue damage, septic shock, and severe multi-organ failure especially, the kidneys, lungs, and liver. The cytokine storm process initiates and progresses several inflammatory cascades leading to persistent inflammation which drives the leptospiral infection to severe leptospirosis (Cagliero *et al*., 2018). In this study, we have investigated the role of one of the captivating pro-inflammatory cytokines, MIF. Recent studies evidenced that LPS enhanced the upregulation of MIF and its receptors and receptor complexes leading to increased secretion of MIF by inflammatory cell types (Klasen *et al*., 2018). MIF regulates the LPS-activated inflammatory responses of immune cells, endotoxemia, apoptosis, and cell damage. In response to *E. coli* LPS induction, MIF was secreted by anterior pituitary cells and elevated the serum MIF levels, and contributes to endotoxemia and septic shock (Bernhagen *et al*., 1993).

MIF has been believed to be an early pro-inflammatory cytokine that stimulates inflammatory injury. After the cellular MIF expression upon infection, MIF releases at the early stage of diseases. The secreted MIF enhanced the activation of other proinflammatory cytokine expressions, hence it acts as an early upstream facilitator of inflammatory tissue damage. Several studies investigated an early MIF expression profile; mRNA and protein levels of MIF were increased as early as 0.5 h after external stimulation (Liu *et al*., 2018). MIF expression and its potential contribution in the early stage of inflammatory diseases, autoimmune disorders and cancers have been analyzed for a couple of decades (Llamas-Covarrubias *et al*., 2013; Rahman *et al*., 2007; Lee *et al*., 2008; Arcuri *et al*., 2001). In this investigation, we deal with the development of efficient leptospirosis experimental models and analyze the molecular MIF profiling in leptospirosis.

Cellular immediate early genes (IEGs) can be transcribed rapidly within several minutes of cellular exposure to external stimuli. Most of the IEGs are translated to be transcription factors, which regulate the cellular responses of cells against stimuli. Greenberg *et al*., (1992) demonstrated that calcium influx or membrane depolarization regulates the IEGs activation (West *et al*., 2001). As MIF profiling clearly indicated that MIF expression was initiated at 30 minutes post leptospiral LPS stimulation, we attempted to investigate whether the early MIF gene expression was regulated by intracellular calcium influx $[Ca^{2+}]_i$. Different types of external stimuli induce distinct levels of early response gene expression (Bartel *et al*., 1989).

The microbial product, LPS, and other virulent proteins required calcium signals to promote inflammatory pathways (Chiang *et al.*, 2012). LPS increased the intracellular free calcium $[Ca^{2+}]_i$ when compared with baseline expression of untreated control. LPS primed cells showed the peak levels of resting $[Ca^{2+}]_i$ which further enhances the stimulus-induced cellular activity including,

activation of inflammatory cytokine mediated pathway, iNOS release, and cell death (Forehand *et al*., 1989; Azenabor *et al*., 2009; Sherwin and Fern, 2005). As a second messenger, calcium and its signaling play a crucial role in many fundamental processes such as cell growth and proliferation, and regulation of metabolism and gene expression. The $[Ca^{2+}]_I$ initiate the calcium mediated signal transduction cascades by activating Ca^{2+} /calmodulin dependent protein kinase II (CAMKII), which promotes the phosphorylation of cAMP response element-binding protein (CREB), a calcium regulated transcription factor of MIF (Wu *et al*., 2002; Chiang *et al*, 2012).

$[Ca^{2+}]_i$ acts as a second messenger in many cell types and serves notable diverse physiological processes (Vig and Kinet, 2009). Store operated calcium (SOC) channel is the major mechanism of $[Ca^{2+}]_i$ entry in a wide variety of cell types including both excitable and predominantly in non-excitable cells. STIM1 (stromal interaction molecule 1), IP3R (Inositol 1,4,5-trisphosphate receptor), and ORAI1 is the functional players of SOC dependent calcium influx, in which IP3R releases calcium from the endoplasmic reticulum (ER) under stimulation, STIM1 serves as a calcium sensor that activates ORAI1 which is a store operated channel that allows the entry of calcium (Martin-Romero *et al*., 2002; Giachini *et al*., 2009; Parekh and Putney, 2005). $[Ca^{2+}]_i$ undergo regulation of transcription and enzymatic activity by activation and nuclear import of the calcium dependent transcription factors and mediating phosphorylation respectively. Among the transcription factors of the MIF gene, specificity protein (Sp)1 and cAMP response element-binding protein (CREB) is an effective positive regulators of human MIF gene expression (Roger *et al*., 2007). This study attempts to explain the poorly described molecular mechanisms of MIF expression.

MIF is associated with several physiological processes including leukocyte recruitment, cell proliferation, host immune responses, and inflammatory responses, and counter-regulation of

anti-inflammatory glucocorticoids. Among the multifaceted activity of MIF, the role of MIF in the pathogenesis of several inflammatory diseases was extensively studied. Therefore, MIF has emerged as a novel and captivating therapeutic target for inflammatory and autoimmune diseases including sepsis and septic shock (Bozza *et al*., 1999), dengue (Chuang *et al*., 2015), rheumatoid arthritis (Kim *et al*., 2011), acute respiratory distress syndrome (Donnelly *et al*., 1997), diabetes (Sanchez-Zamora *et al*., 2010), multiple sclerosis (Benedek *et al*., 2017), hepatic inflammatory disease (Marin *et al*., 2017), psoriasis (Bezdek *et al*., 2018), systemic lupus erythematosus (Tu *et al*., 2019), and solid tumors/ cancer (Nobre *et al*., 2017).

The physiological and pathophysiological role of MIF was achieved by contributing several signaling pathways. The notable signaling cascades are Src kinase/PI3K/Akt/NuclearFoxO3a/ GILZ/MKP-1 and Src kinase/ERK1/2, PLA2/Arachidonic acid/COX-2/PGE2 to generate inflammatory responses of the host against pathogens; MIF/TXNIP/NF-κB/inflammatory cytokine to promote chemotaxis and various immune responses; Src kinase/PI3K/Akt/pBAX-pBAD to carryout tissue regeneration as physiological aspect and tumor formation on pathological events; MIF/JAB1/JNK/AP-1/CyclinD1 to promote immune cell proliferation and tumor cell invasion, MIF/p53/Mdm2/↓P21/↓BAX to inhibits apoptosis and tumorigenesis (Sumaiya *et al*., 2021).

MIF is a critical regulator of inflammatory pathways by stimulating the secretion of inflammatory mediators such as tumor necrosis factor-α, IFN-γ, IL-1β, IL-6, IL-8, IL-12, macrophage inflammatory protein-2, cyclo-oxygenase-2, nitric oxide, mitogen-activated protein kinases, matrix metalloproteinases, ICAM-1, VCAM-1, PGE_2, COX-2, arachidonic acid, iNOS and counteracts the glucocorticoids activity (Kasama *et al*., 2010; Calandra and Roger, 2003). The balanced regulation of the inflammatory response to an infection is essential for the healthy

maintenance of the host system; the exacerbated inflammatory response worsens the pathological conditions. Blockage of MIF by knockdown of MIF gene (Choi *et al*., 2013), administration of anti-MIF antibody (Burger-Kentischer *et al*., 2006) and MIF antagonist (Fu *et al*., 2019) significantly reduced the secretion of inflammatory cytokines and chemokines. In this study, we were particularly focused on analyzing the role of MIF in the pathogenesis of leptospirosis.

An unresolved mystery of leptospirosis is the heterogeneity of disease outcomes. Only about 10% of leptospirosis patients are complicated with multiorgan failure including hepatic dysfunction, acute renal failure, pulmonary involvement, circulating system dysfunction, acute pancreatitis and rhabdomyolysis (Bourquin *et al*., 2011). Leptospiral infection in some cases results in severe leptospirosis with multi-organ involvement and high mortality while others have mild subclinical manifestations. The factors driving the disease progression towards severe leptospirosis with fatal outcomes remain unclear. Therefore, studies on human genetics associated with susceptibility and severity of infectious diseases are essential for advancing our knowledge of disease pathogenesis, stratification of risk, and the development of specific drug targets (Kwok *et al*., 2021).

Several studies revealed that MIF gene promoter polymorphisms exert a multiple roles in the susceptibility and severity of inflammatory and autoimmune diseases. The chromosome location of the human MIF gene is 22q11.23 which has 0.8kb long. The gene contains 2 introns and 3 exons. The MIF protein consists of 115 amino acids with a molecular weight of 12.5kDa. MIF is a pluripotent cytokine that specifically has the proinflammatory and immunoregulatory activities responsible for correlation with many diseases (Liu *et al*., 2018). Recent studies indicated that genetic polymorphism of genes directs the contribution of an appropriate protein in physiological and pathological processes. Studies reported that the human MIF gene has six major

polymorphisms, such as single nucleotide polymorphisms (SNPs) including rs755622, rs2096525, rs2070766, and rs1007888, and a microsatellite -794 $CATT_{5-8}$ tandem repeats (Du *et al*., 2020). Out of these gene polymorphisms, functionally important MIF gene promoter polymorphisms -173G/C SNP (rs755622) and -794 $CATT_{5-8}$ microsatellites tightly regulate the MIF gene expression and play a key role in disease progression and severity of several diseases such as cancer (Zhang *et al*., 2015), rheumatoid arthritis (Baugh *et al*., 2002), tuberculosis (Liu *et al*., 2018), coronary artery disease, pneumococcal meningitis (Savva *et al*., 2016), and many autoimmune diseases (Du *et al*., 2020). MIF gene promoter polymorphisms upregulate the transcriptional activation of MIF gene expression and follow mRNA levels and secreted MIF levels in circulation. Several investigations remarked the elevated levels of MIF in serum, plasma, and tissues in inflammatory and autoimmune diseases (Gregersen and Bucala, 2003). The longer CATT repeats (6-8 repeats of CATT variants) and -173*C are considered as high expression MIF genotypes whereas shorter CATT repeats (5-CATT variants) and -173*G is the low expression MIF genotypes (Das *et al*., 2016). Based on this knowledge, we decided to examine the association between MIF gene promoter polymorphism and leptospirosis susceptibility and severity in the study subjects. We report herein the correlation of high MIF expression genotypes (GC and CC) with susceptibility and severity of leptospirosis and low MIF expression genotype (GG) has no effect on disease onset and progression.

As MIF is a potent mediator of the host's inflammatory responses and counter regulator of anti-inflammatory effects of glucocorticoids during pathological conditions, MIF is regarded as an attractive therapeutic target for inflammatory and autoimmune diseases. Host inflammatory responses primarily rely on the neuroendocrine regulatory system; certainly MIF is remarkably secreted by anterior pituitary cells in response to external stimuli. Therefore, researchers focused

on the MIF targeting therapeutic strategies for multiple inflammatory diseases (Bernhagen *et al*., 1996). The major MIF targeting therapeutic approaches including small molecule inhibitors of MIF, anti-MIF neutralizing antibodies and indirect stabilization are studied over the decades. Antibodies against MIF (Imalumab, BaxG03, BaxB01 and BaxM159) and their receptors, CD74 (Milatuzumab), CXCR4 (MEDI3185, Ulocuplumab) and CXCR2 were developed and evaluated in several pathologies including bacterial infections, sepsis, tumorigenesis, atherosclerosis, arthritis, and ischemia (Hussain *et al*., 2013, Cheng *et al*., 2009, Kuhne *et al*., 2013, Wente *et al*., 2006, Liu *et al*., 2010, Simons *et al*., 2011).

Bioengineered anti-MIF nanobodies are also developed and demonstrated the effective inhibitory effect on MIF mediated inflammatory responses during multiple pathological states (Sparkes *et al*., 2018). Several preclinical studies and clinical trials have been conducted to evaluate the safety and biological activity of anti-MIF antibodies and MIF antagonists (Kerschbaumer *et al*., 2012; Mahalingam *et al*., 2020). MIF antagonists have been reported to be efficient inhibitors of MIF's tautomerase activity. But the translation of developed MIF inhibitor into clinical success has not yet been shown. The extensively studied small molecule antagonists of MIF are (S,R)-3-(4 hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), AV411 (Ibudilast), N-acetyl-p-benzoquinone imine (NAPQI), 4 - iodo-6-phenylpyrimidine (4-IPP), P425, and CPSI-1306 (Al-Abed and Van Patten, 2011, Cho *et al*., 2010, Senter *et al*., 2002, Winner *et al*., 2008, Bai *et al*., 2012, and Choudhary *et al*., 2013). The major classes of MIF inhibitory chemical compounds are the derivatives of dopachrome, acetaminophen, isoxazolines, acetylenic compounds, phenyl pyrimidine, isothiocyanate, benzoxazinone, benzoxazol-2-one, chromene, isoxazoline, pyrimidazole, isocoumarin, phenyl pyruvic acid, Schiff bases, cinnamates, plant derived compounds, oxygen heterocycles, and curcumin (Kok *et al*., 2018). Due to reduced cost

and low risk of failure in biocompatibility analysis, repurposing existing drugs has emerged as an effective approach to developing novel MIF antagonists (Yang *et al*., 2022). Therefore, we selected the important categories of suggested MIF inhibitory small molecule derivatives and available drugs for screening of anti-MIF activity.

Objectives

2. Objectives

To address the issues in leptospirosis diagnosis and disease management such as i) leptospirosis is not yet well documented in developing countries and considered a neglected tropical disease, ii) Due to the non-specific symptoms, complex standard diagnostic techniques and lack of early diagnostic marker, leptospirosis are underdiagnosed, and iii) lacunae in the understanding of the mechanism of pathogenesis of leptospirosis, we framed our study objectives as follows,

1. Appraise the early diagnostic value of serum MIF in leptospirosis patients
2. Determinate the *in vitro* and *in vivo* MIF expression profile in the leptospiral LPS induced model
3. Understand the mechanism of $[Ca^{2+}]_i$ dependent, transcriptionally activated MIF expression by *in vitro* system of ORAI1 knockdown
4. Demonstrate the role of MIF in the pathogenesis of leptospirosis by developing *in vitro* MIF knockdown and *in vivo* MIF blockade strategy
5. Understand the role of MIF gene polymorphism (-173G/C SNP) in the severity of leptospirosis
6. Screening and evaluation of chemical compound inhibitors for the anti-MIF activity as the drug for leptospirosis

Review of Literature

3. Review of Literature

3.1. Leptospirosis and its clinical features

Leptospirosis is a spirochaetal zoonotic disease caused by pathogenic leptospires infecting both humans and animals. The route of transmission is either direct or indirect contact with infected animals or excretion of an infected animal and contaminated water or soil respectively (Haake and Levett, 2015). Leptospirosis has a spectrum of clinical presentations ranging from mild, nonspecific flu like illness to severe fatal conditions include kidney injury, inflammation in the liver, pulmonary hemorrhage, myocarditis, and meningitis. For past decades, pulmonary manifestations are increasingly recognized as the most severe form of leptospirosis (Tan *et al*., 2017). Mortality and morbidity remain significant, and every year 1.03 million people are affected worldwide (Costa *et al*., 2015). The signs and symptoms of leptospirosis are simulated to that of well-known disease like typhoid, dengue, malaria, and acute hepatitis (Safa *et al*., 2017) and lead to under-diagnosis and inadequate treatment on time (Izurieta *et al*., 2008). Approximately 10% of leptospirosis cases develop a severe form of the disease with organ involvement such as pulmonary hemorrhage, acute kidney injury, and Weil's syndrome associated with Jaundice. Among these, pulmonary hemorrhage has been increasingly considered a major, frequently distributed manifestation with a high mortality rate (Bharti *et al*., 2003).

3.2. Pulmonary involvement in leptospirosis

Over the years, pulmonary leptospirosis has been identified to be the frequently increasing complication of leptospirosis and highly lethal and affects about 70% of leptospirosis patients and emerging as a life threatening condition, however, it is still poorly documented in endemic regions. It is present in both anicteric and icteric forms of leptospirosis (Luks *et al*., 2003, Dolhnikoff *et al*., 2007). In patients, pulmonary leptospirosis was characterized by chest pain,

cough, pneumonia, acute respiratory distress syndrome, pleural effusion, patchy pulmonary infiltrate, hemoptysis, and dyspnea. Patients with dyspnea and alveolar infiltration are an important predictors of severe leptospirosis (Budiono *et al*., 2009, Dupont *et al*., 1997, Dolhnikoff *et al*., 2007). The severe pulmonary form of leptospirosis (SPFL) is the most widespread manifestation of leptospirosis in Brazil (Silva *et al*., 2002). Smoking increases the risk of pulmonary hemorrhage in patients by increased permeability and damage of lung capillaries, injury in the alveolar basement membrane, and induced local inflammatory responses (Donaghy and Rees, 1983). The observed histological findings are pulmonary congestion, hemorrhage, monocyte and neutrophil infiltration in alveolar spaces, fibrin deposition, pulmonary edema, formation of hyaline membrane, and proliferative fibroblastic conditions (Arean, 1962, Dolhnikoff *et al*., 2007, Leptospirosis associated severe pulmonary hemorrhagic syndrome has emerging in many countries which significantly reduced the survival rate of patients, but the knowledge of insight mechanism of pathophysiology needs the extensive studies (Herath *et al*., 2019). Recently, leptospirosis associated acute respiratory distress syndrome (ARDS) patients were successfully treated with penicillin and venous-venous extracorporeal membrane oxygenation (v-vECMO) in China (Wang *et al*., 2020).

3.3. Inflammatory responses during leptospirosis

The major reason for the differential disease outcome of leptospirosis is uncontrolled inflammatory responses as described in **Figure 3.1**. The stimulation of immune responses such as cytokine secretion and other inflammatory mediator activation upon infection plays a key role to eradicate the pathogens from the host. But the non-specific, elevated uncontrolled production of inflammatory cytokines leads to the event of a cytokine storm (Cagliero *et al*., 2018). Consequently, it drives the mild disease into severe form with organ dysfunction.

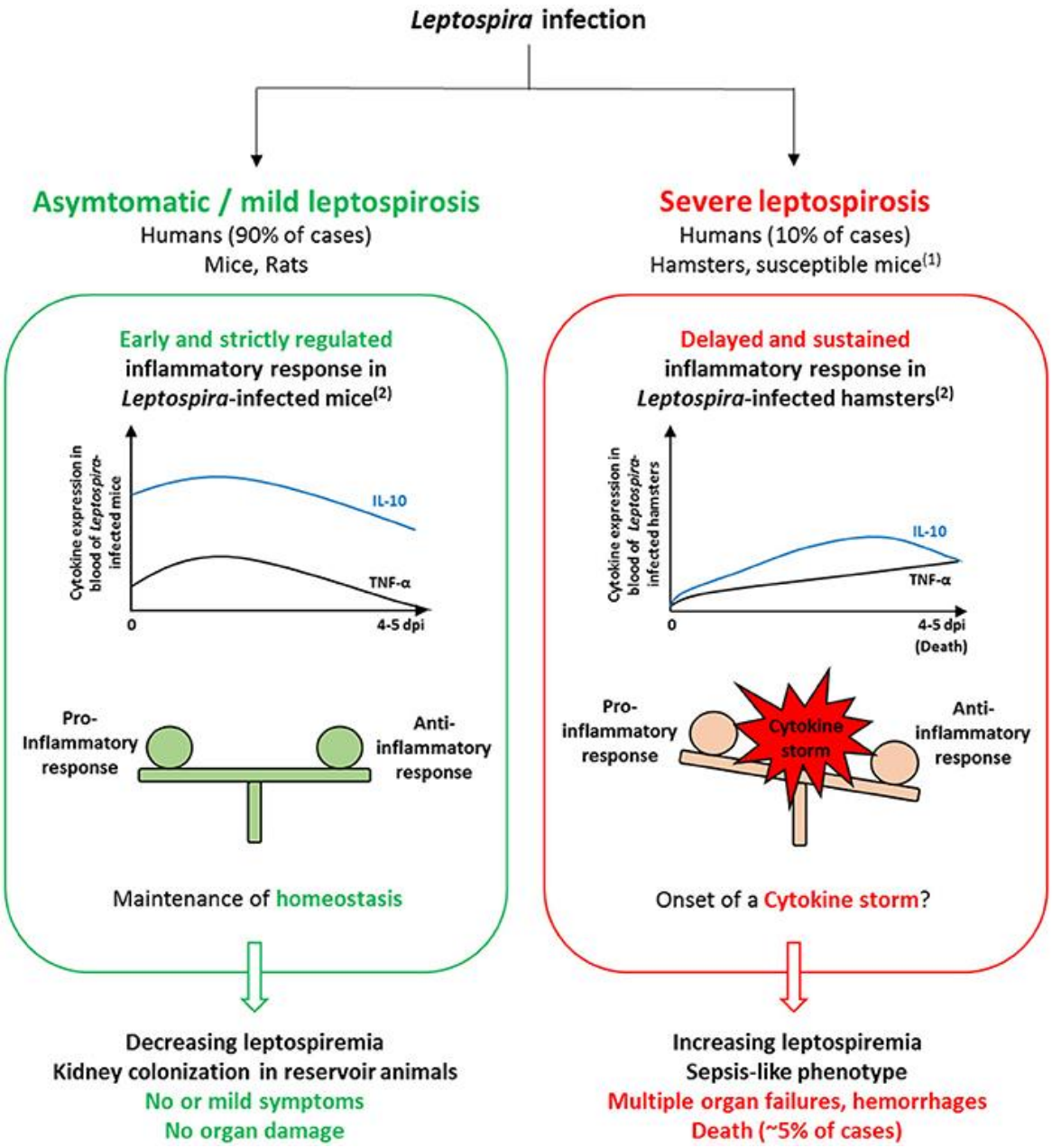

Figure 3.1. Importance of inflammatory responses in influencing the disease outcome

The innate immune response was activated by the host interaction with leptospires via pattern recognition receptor (PRRs) expression especially TLR and Nod like receptors (NLRs). It enhances the inflammatory cascade reaction by activating inflammatory pathways, Nuclear factor kappa B (NF-κB), Janus kinase (JAK)-signal transducer and activator of transcription (JAK-STAT) (Schroder and Tschopp, 2010), and production of prostaglandins (PGs), nitric

oxide (NO), cytokines and chemokines (Turner *et al*., 2014, Tisoncik *et al*., 2012, Zhang *et al*., 2020). Leptospires regulate the host immune response by TLRs through their surface adhesion proteins (Lsa21). Lsa21 significantly activates the TLR2 and 4 of macrophages and enhances the inflammatory cascades events, which are mainly relied on mitogen activated protein kinases (MAPKs) which activate several inflammatory pathways (Faisal *et al*., 2016). In human cells, the principal receptor to leptospiral LPS are TLR2/TLR1, whereas in murine cells TLR2 and TLR4 play a key role in the recognition of LPS during infection (Nahori *et al*., 2005). Leptospiral outer membrane protein, LipL32 (prominent lipoprotein) activates the effective inflammatory responses in renal proximal tubule cells by NF-κB pathway (Yang *et al*., 2006).

3.4. Macrophage migration inhibitory factor (MIF)

Macrophage migration inhibitory factor (MIF) is a homo-trimeric protein that acts as a pleiotropic pro-inflammatory cytokine. It is involved in various functions, including leukocyte recruitment, inflammation, immune responses, cell proliferation, tumorigenesis, and counter-regulation of glucocorticoids (GC). The *MIF* gene is located on chromosome 22 (22q11.23) of the human genome and contains 3 exons (107, 172, and 66 bp) and two introns (188 and 94 bp). Almost six decades ago, migration inhibition activity was identified during a delayed-type hypersensitivity study (Bloom and Bennett, 1966), and later researchers first cloned human MIF that facilitated the characterization of the biological activities of MIF (Weiser *et al*., 1991). In 1993, MIF was discovered to be a secreted pro-inflammatory protein (Bernhagen *et al*., 1993), and subsequently, the physiological and pathological roles of MIF and MIF receptors were elucidated (Leng *et al*., 2003). The multifaceted role of MIF in cellular signaling pathways are shown in **Figure 3.2**

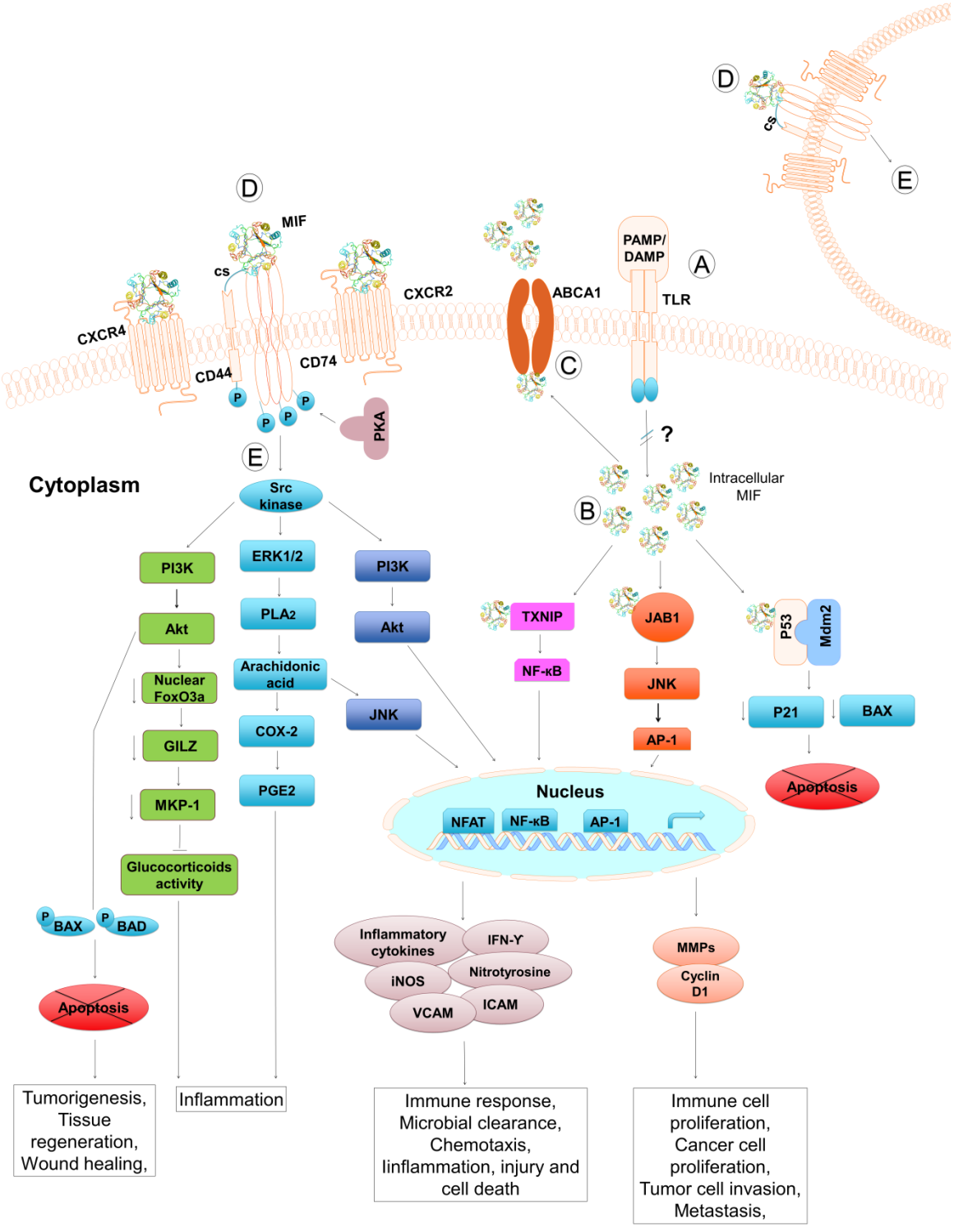

Figure 3.2. The multifunctional activity of Macrophage migration inhibitory factor

3.5. Mechanism of the role of MIF in diseases

LPS-induced MIF plays a critical role in lethal endotoxemia by stimulating TNF-α, secretion. MIF counteracts the anti-inflammatory and immunosuppressive activity of GC. GC stimulates the MKP-1 (MAP kinase phosphatase-1) leading to the inhibition of inflammatory mediators, thereby achieving anti-inflammatory properties. The functional activity of MIF drives the inflammation targeted cascade ↑Akt / ↓Nuclear FoxO3a (Forkhead transcription factors of the O3 class) / ↓GILZ (Glucocorticoid-induced leucine zipper protein)/ ↓MKP-1 to override the action of GC (Fan *et al*., 2014). Due to its broad regulatory properties, MIF is a potential mediator of many inflammatory and immune diseases, including sepsis (Bozza *et al*., 2004), rheumatoid arthritis (Kim *et al*., 2011), diabetes (Sanchez-Zamora *et al*., 2010), solid tumors/cancer (Nobre *et al*., 2017), acute respiratory distress syndrome (Donnelly *et al*., 1997), hepatic inflammatory diseases (Marin *et al*., 2017), multiple sclerosis (Benedek *et al*., 2017), systemic lupus erythematosus (Tu *et al*., 2019), psoriasis (Bezdek *et al*., 2018), and dengue (Chuang et al., 2015).

Several studies proposed the mechanism of MIF in the pathogenesis of diseases in part by enhanced production of inflammatory molecules (TNF-α, nitric oxide, IL-1, IL-6, IL-8, and cyclo-oxygenase (COX). MIF regulates the LPS-mediated host responses in an autocrine manner by upregulating the TLR4-MD2 (Toll-like receptor 4 / myeloid differentiation factor 2) expression (Roger *et al*., 2003). During infection, MIF has secreted in response to endotoxin/exotoxin and increased glucocorticoids (GC) levels. MIF counter-regulates the immunosuppressive effect of GC on inflammatory cytokine production. The immunosuppressive effects of GC are inhibition of TNF-α mRNA translation via the suppressed activity of *c-jun* N-terminal kinase/ stress-activated protein kinase and inhibition of LPS-induced arachidonic acid

release. After secretion, MIF activates the p44/42 ERK (extracellular signal-regulated kinase) MAP kinase and cytosolic phospholipases A2 ($cPLA_2$), an important proinflammatory cascade factor. MIF-induced $cPLA_2$ activation produces inflammatory components, including prostaglandins, arachidonic acid, and leukotrienes. The release of arachidonic acid activates *c-jun* N-terminal kinase / stress-activated protein kinase, which is responsible for the translation of TNF-α mRNA. Thus, MIF plays a key role in the inflammatory cascade and acts as an upstream regulator of inflammatory cytokine production (Mitchell *et al*., 1999; Sampey *et al*, 2001). MIF triggers inflammatory responses by MIF signaling pathways during infections. Thus, MIF has emerged as a promising drug target for many diseases.

3.6. Biological strategies influence the MIF activity

3.6.1. Genetic polymorphism of MIF

About 200 genetic polymorphisms are identified in the human MIF gene, of which 2 functionally important polymorphisms are described in the promoter region of the MIF gene (Garcia-Orozco *et al*., 2020). Two functional polymorphisms of the MIF gene locus are CATT tetranucleotide repeats (CATT5-8) and G/C SNP. G/C SNP is the polymorphism of G to C transition located at -173 (rs755622) in the untranslated 5' flanking region of the MIF gene, whereas CATT5-8 is a microsatellite repeat located at −794 (rs5844572) in the upstream region of G/C SNP (Barton *et al*., 2003). The existence of MIF -794 $(CATT)_6$, $(CATT)_7$, $(CATT)_8$ nucleotide repeats, and -173*C MIF genetic variants are identified as the higher MIF expression gene variants, and the $(CATT)_5$ repeat and -173*G allele is the low expression gene variants. These polymorphisms are associated with host susceptibility and severity of infectious diseases and immune disorders. Indeed, MIF gene polymorphisms have regulated the level of MIF mRNA and serum MIF

protein (Matia-García *et al*., 2015). In most cases, high expression MIF genotype contributes to disease worsening but recent studies showed that high expression MIF gene variants are

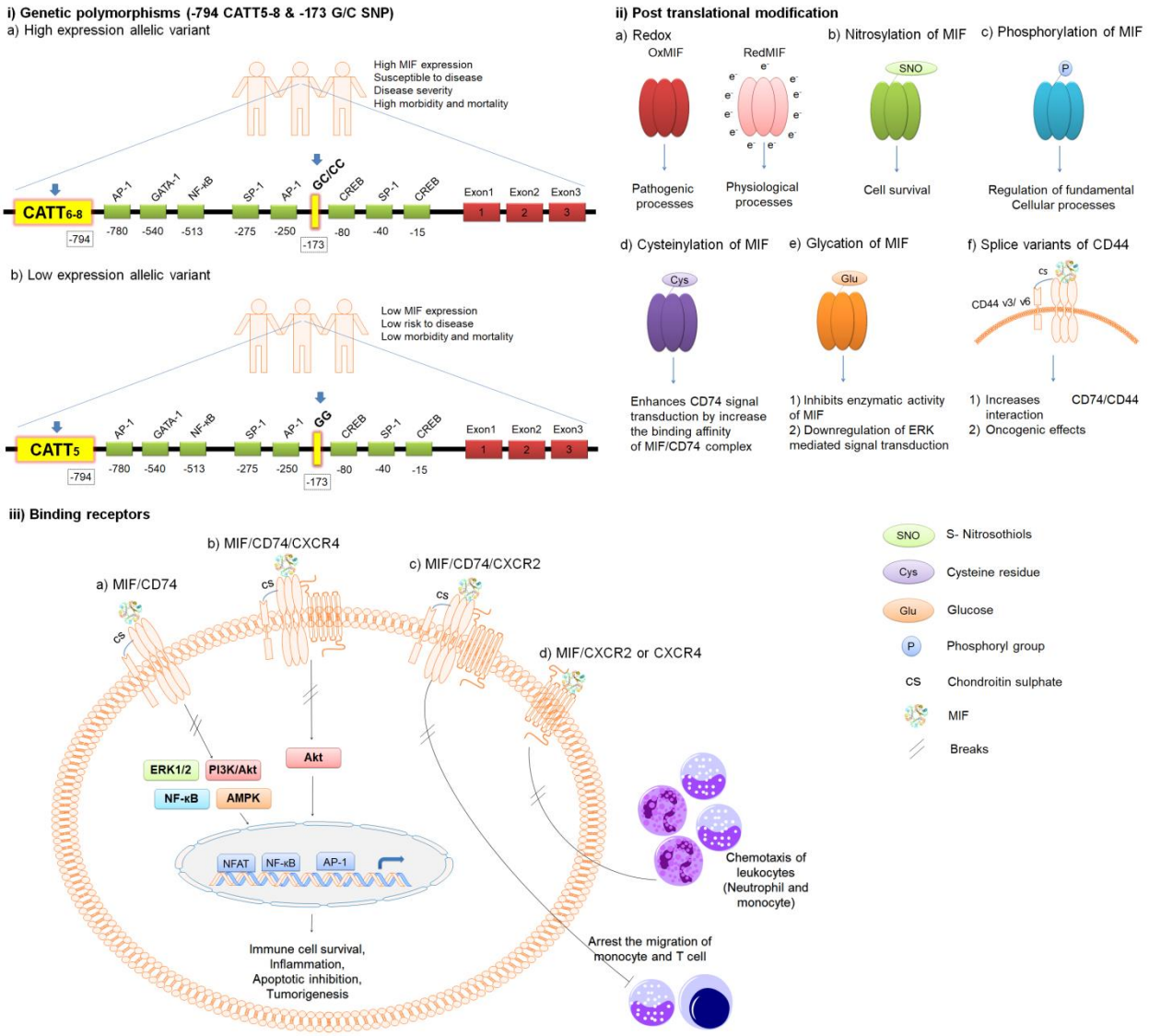

Figure 3.3. Biological strategies influence the activity of MIF

markedly reduced the susceptibility of a few diseases, thereby the correlation between the MIF polymorphism and disease must have relied on the appropriate role of MIF in such diseases (Bucala, 2012). In addition, MIF polymorphism determines the morbidity and mortality rate of the host against several diseases (Baugh *et al*., 2002). MIF gene polymorphism and elevated

serum MIF may be critical biomarkers and severity predictors of complex diseases (Okazaki *et al*., 2018). Thus, MIF genotype analysis may serve as an early severity predictor and helps to classify the high-risk population.

3.6.2. Other biological strategies

Researchers revealed that genetic polymorphism, epigenetic mechanisms, post-translational modifications (PTMs), and differential binding receptors and receptor complexes of MIF may determine the activity of MIF in physiology and pathology by being involved inappropriate signaling events (Sumaiya *et al*., 2021). The strategies which regulate the versatile functions of MIF were summarized in **Figure 3.3.**

Chapter I

4. Appraise the early diagnostic value of serum MIF in leptospirosis patients

4.1. Materials and Methods

4.1.1. Study site, duration, and study design

A case control was carried out by active hospital-based surveillance at the Annal Mahatma Gandhi Memorial General Hospital, Tiruchirappalli, Tamil Nadu, India from June 2017 to February 2018. This study was performed to determine the following: i) seroprevalence of leptospirosis in Tiruchirappalli and ii) serum MIF levels in patients with leptospirosis and other common febrile illnesses and healthy control subjects to appraise the early diagnostic value of serum MIF for leptospirosis.

4.1.2. Study population and case definition

In this study, a total of 300 study subjects were recruited to participate. Among which, 243 sera samples were collected to diagnose leptospirosis from clinically suspected leptospirosis cases with a range of clinical presentations including fever, body ache, myalgia, icterus, rigors, arthritis, abdominal pain, breathlessness, jaundice, conjunctival suffusion, and subconjunctival hemorrhages. The sera samples were collected before any treatment was given to the patients. A total of 57 seronegative healthy individuals from the neighborhood of the leptospirosis suspected cases matching for age (±5 years) and sex were recruited as control subjects. A number of healthy control subjects who had a fever in the past 15 days were excluded from this study. The collected sera samples were stored at -80°C until use.

4.1.3. Leptospiral antigens

Live antigens were cultured and maintained by regular subculturing in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium supplemented with bovine serum albumin and Tween-80 at the Medical Microbiology Laboratory, Bharathidasan University, Tiruchirappalli.

Based on our earlier observation, below mentioned panel of serovars was used as live antigens in MAT assay and IgM ELISA, Australis (serovar Australis, strain Ballico), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Pyrogenes (serovar Pyrogenes, strain Salinem), and Sejroe (serovar Hardjo, strain Hardjoprajitno).

4.1.4. MAT assay

The gold standard technique of leptospirosis, MAT was carried out to diagnose leptospirosis by using the panel of 12 live leptospiral serovars against the collected sera samples from patients. Seven days old well gown live leptospiral cultures at the concentration of 1-2x10^8 leptospires/ml were used as antigens in MAT. Each serum sample was assayed in double dilutions with Phosphate-Buffered Saline (PBS) ranging from 1:20 to 1:2560. The diluted serum was added with live leptospiral antigens, mixes thoroughly in a micro shaker, and incubated at 30ºC for 3 hours for agglutination. MAT titers were stated as the appropriate dilution which agglutinating ≥50% of live antigens. A titer of ≥1:80 was considered positive for MAT.

4.1.5. Preparation of heat extracted antigens

Seven days old leptospiral cultures at the concentration of 1-2x10^8 leptospires/ml were used to prepare the heat extracted antigens. The pelleted cells were washed twice with PBS and treated with formalin with a final concentration of 0.5%. Then incubated at 100ºC for 30 minutes and bacterial lysates were centrifuged at 10,000 rpm for 30 minutes. The supernatant was collected as heat extracted antigen and the protein concentration was quantified by the bicinchoninic acid

(BCA) method (Sigma-Aldrich, St. Louis, MO, USA). The heat extracted antigens were stored at -80ºC as separated aliquots until use.

4.1.6. IgM Enzyme-Linked Immunosorbent Assay

IgM ELISA was performed for the further confirmation of leptospirosis in patients by evaluating the primary immune response to leptospirosis. The samples were prepared by adding 0.2 µg of heat extracted antigen with carbonate coating buffer (pH 9.6) at the final volume of 100 µL and coated on an appropriate well of microtiter plates. After incubating the plates at 4ºC overnight, each well was washed thrice with 200 µl of PBST (1X PBS and 0.1% Tween-20) for 10 minutes each. 200 µl of 3% skim milk powder blocking solution was added to wells and the plates were incubated at 37ºC for 1-2 h. After washing the wells properly as mentioned previously, 100 µl of test sera (1:100 dilution) were added to appropriate wells and incubated at 37ºC for 1h. The unbound antibodies were washed thrice with PBST. To detect the bound IgM antibodies, 100 µl of alkaline phosphatase (ALP) conjugated anti-human IgM antibody (1:1000 dilution) was added to appropriate wells and incubated at 37ºC for 1 h. Followed by developing with 100 µL of *o*-phenylenediamine dihydrochloride (OPD) solution by incubating at dark and room temperature for 10-15 minutes. To stop the developing reaction, 50 µL of 1N H_2SO_4 solution was added and immediately read the optical densities (OD) at 490 nm by using a microplate reader (BioRad, Hercules, CA, USA).

4.1.7. MIF ELISA

The determination of serum MIF levels of leptospirosis cases, other febrile cases, and healthy control subjects were performed by using a Human MIF ELISA kit (Sigma-Aldrich, St.Louis, Mo, USA) according to the manufacturer's instructions. The samples and reagents were brought to room temperature prior to use. All samples and MIF standards were run in triplicates. The

standards and samples were added to the appropriate wells and incubated on the ELISA plates for 2.5 h at room temperature with gentle shaking. After washing the wells with 1X wash solution four times, 1X Biotinylated Detection Antibody was added to each well and incubated for 1 hour under the previously mentioned conditions. After washing, HRP-Streptavidin solution was added to each well and incubated for 45 minutes. Washed the unbound particles, added the ELISA colorimetric TMB Reagent to each well, and incubated for 30 minutes under dark conditions. After adding the stop solution, the absorbance was detected immediately at 450 nm by using a microplate reader. The mean absorbance of each set of standards, samples, and controls was calculated and subtracted from the average OD of zero standards. The standard curve was plotted using SigmaPlot 11.0 software.

4.1.8. Statistical significance

Data from biological triplicates were calculated and showed as Mean ± Standard Deviation (SD) (n=3). The results of serum MIF ELISA comprising outlier points were shown as Median (Interquartile Range, IQR). Statistical data were computed either with GraphPad Prism version 9.2.0 or SigmaPlot 11.0 software. To determine the sensitivity and specificity of serum MIF as a leptospirosis diagnostic marker, Receiver operating characteristic (ROC) analysis was performed. To demonstrate the association of serum MIF levels with the duration of leptospirosis and the patient's age, Pearson's correlation coefficient test was carried out. A p-value ≤ 0.05 was considered significant.

4.1.9. Ethical Compliance

This study comprising human subjects (for blood sample collection) was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (DM/2014/101/51) and the

informed written consent was obtained from both cases and healthy control subjects before blood sampling; in case of minor study subjects, the consent form was provided by their surrogates.

4.2. Results

4.2.1. Seroprevalence of human leptospirosis

As per the observations of MAT assay and IgM ELISA, the seroprevalence of leptospirosis in Tiruchirappalli was determined using the panel of 12 most circulating leptospiral serovars. Among the 300 cases included in this study, a total of 142 cases were tested positive for antibodies against prevalent pathogenic leptospires at ≥1:80 titer of agglutination, which revealed the alarmingly high seroprevalence of 47.3% of human leptospirosis. The MAT assay revealed that out of the 12 leptospiral antigens tested, 6 showed seropositive among the sera samples of leptospirosis cases.

Table 4.1. Demographic characteristics of study subjects

S.No.	Variables	Leptospirosis confirmed cases	Other febrile cases	Healthy control subjects
01.	Age Range (in years) Mean (years) ± S.D	 6 - 75 34.2 ± 17.7	 5 - 75 28 ± 16.8	 8 - 74 35.8 ± 18.0
02.	Sex Female (%) Male (%)	 48 52	 43.5 56.5	 54.4 45.6
03.	Duration of disease Range (days) Mean (days) ± S.D	 7 - 31 19.8 ± 7	 2 - 41 11.9 ± 7.5	 - -
04.	Serology MAT titres (Range) IgM ELISA titre	 1:80 - 1:2560 1:100	 - -	 - -

Table 4.1 showed the demographic characteristics of the study participants. No significant differences were observed in seropositivity with respect to age and gender. The predominantly infecting serovars were Autumnalis, Australis, and Canicola with the seroprevalence of 50.7%,

21.2%, and 16.2% respectively, whereas the other infecting serovars are Icterohaemorrhagiae, Grippotyphosa, and Ballum with 5.6%, 3.5% and 2.9% of seroprevalence, respectively.

The range of MAT titers observed in sera of leptospirosis cases was between 1:80 and 1:2560. The frequency of infecting serovars, median MAT titers, and all the positive MAT titers in respect to prevalent serovars were shown in **Table 4.2**. The results of IgM ELISA were significantly similar to MAT positivity except for 6 cases, which showed the seronegative for IgM ELISA.

Table 4.2. MAT titres and serovar distribution in study subjects

Serovar	**Frequency (n) (%)**	**Median MAT titres**	**1:80 (%)**	**1:160 (%)**	**1:320 (%)**	**1:640 (%)**	**1:1280 (%)**	**1:2560 (%)**
Autumnalis	72 (50.7)	1:640	12.5	13.8	16.6	33.3	16.6	6.9
Australis	30 (21.1)	1:640	16.6	16.6	13.3	26.6	23.3	3.3
Canicola	23 (16.2)	1:320	17.3	17.3	26	26	8.6	4.3
Icterohaemorrhagiae	8 (15.6)	1:640	12.5	12.5	0	50	12.5	12.5
Grippotyphosa	5 (3.5)	1:320	20	20	40	20	20	0
Ballum	4 (2.9)	1:160	25	25	50	0	0	0

4.2.2. Increased serum levels of MIF in leptospirosis cases

After defining the seroprevalence of leptospirosis among the study population, we performed the profiling of circulating MIF in serum samples obtained from laboratory confirmed leptospirosis cases, other febrile illness cases, and seronegative healthy control subjects. To analyze the level of serum MIF in study subjects, we carried out MIF ELISA with sera samples of both cases and controls. Human MIF ELISA considered the MIF level at the concentration of 0.008ng/mL as the cutoff point for assay positivity. The overall result of serum MIF profiling was represented in **Figure 4.1**.

The Mean +2 SD (Standard Deviation) calculated from seronegative healthy individuals was defined as the cut-off concentration. The cut-off value was determined as 2.5ng/ml. The data

obtained from MIF ELISA showed the outlier points; so it presented as (Median (IQR)) ng/mL. Serum MIF levels of all study subjects were displayed in **Table 4.3.** The sera of leptospirosis cases with different clinical conditions showed elevated levels of serum MIF when compared with other common febrile cases and healthy control subjects.

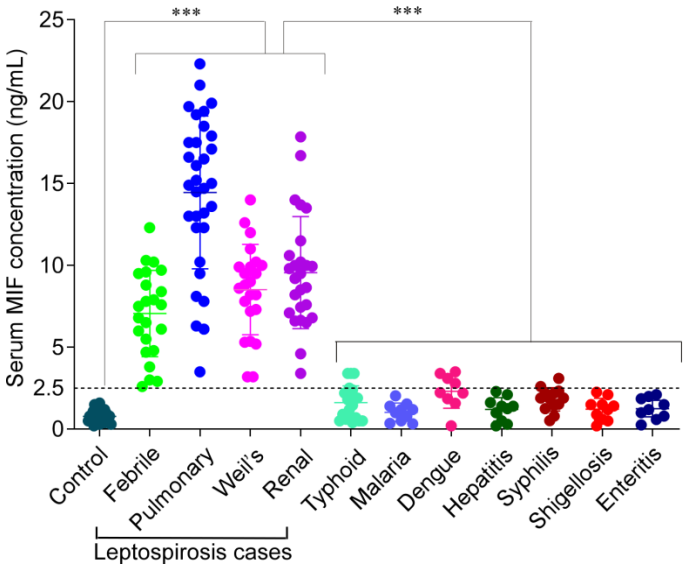

Figure 4.1. Serum MIF profiling by MIF ELISA. Profiling of MIF in serum of patients with leptospirosis with different clinical manifestations, other febrile illnesses, and healthy control. Study groups are indicated on the *x*-axis and MIF concentration on the *y*-axis. The dotted line represents the cut-off with the absolute values on the left. n = 3 experiments. ***P<0.001.

In detail, serum MIF levels of febrile illness cases, pulmonary hemorrhage, Weil's syndrome and renal failure were (7.5(5.32-8.97)), (13.2(11.77-16.72)), (8.8(7.25-9.95)) and (8.65(7.18-10.5)) respectively; whereas serum MIF levels of other febrile cases including typhoid, malaria, dengue, hepatitis, syphilis, shigellosis and enteritis were (1.32(0.57-1.9)), (1.1(0.57-1.35)), (2.2(1.58-2.8)), (1.32(0.62-1.56)), (1.9(1.46-2.2)), (1.31(0.67-1.5)) and (1.2(0.8-1.85)) respectively and those in healthy controls was (0.65(0.5-1.1)). The serum MIF levels are significantly elevated in leptospirosis cases than in other febrile cases and healthy control

subjects, indeed, among leptospirosis cases, pulmonary hemorrhage showed remarkably higher serum MIF levels than other clinical conditions of leptospirosis.

Table 4.3. Serum MIF level in patients and healthy controls

Study subjects (n)	**Serum MIF level (ng/mL)**		
	Range	**Median (Interquartile Range)**	**P value**
Laboratory confirmed leptospirosis cases (142)			
Febrile illness (41)	2.6 - 12.3	7.5 (5.32-8.97)	<0.001
Pulmonary hemorrhages (37)	3.5 - 22.3	13.2 (11.77-16.72)	<0.001
Weil's syndrome (35)	3.2 - 14	8.8 (7.25-9.95)	<0.001
Renal syndrome (29)	3.4 - 17.85	8.65 (7.18-10.5)	<0.001
Other febrile cases (101)			
Typhoid (40)	0.4 - 3.4	1.62 (0.57-1.9)	1
Malaria (10)	0.32 - 2.03	1.1 (0.57-1.35)	1
Dengue (9)	0.2 - 3.5	2.23 (1.58-2.8)	1
Hepatitis (10)	0.2 - 2.3	1.32 (0.62-1.56)	1
Syphilis (13)	0.52 - 3.1	1.9 (1.46-2.2)	1
Shigellosis (10)	0.2 - 2.25	1.31 (0.67-1.5)	1
Enteritis (9)	0.25 - 2.1	1.2 (0.8-1.85)	1
Healthy controls (57)	0.2 - 1.6	0.65 (0.5-1.1)	-

4.2.3. Diagnostic value of serum MIF as a biomarker of leptospirosis

To predict the diagnostic efficacy of serum MIF in leptospirosis, ROC analysis was performed and found that serum MIF potentially discriminated against the leptospirosis cases from other febrile cases. The area under the curve (AUC) value of various clinical conditions of leptospirosis including febrile illness, pulmonary hemorrhage, Weil's syndrome, and renal failure was 0.9910, 0.9999, 0.9960, and 0.9989 respectively (p<0.0001) **(Figure 4.2A-D)**. The optimal cutoff value of MIF was determined as 2.5 ng/mL. The specificity of MIF profiling for leptospirosis cases with febrile illness, pulmonary hemorrhage, Weil's syndrome, and renal failure were 91.7%, 99%, 95.4%, and 95.4% respectively, while sensitivity was reported as

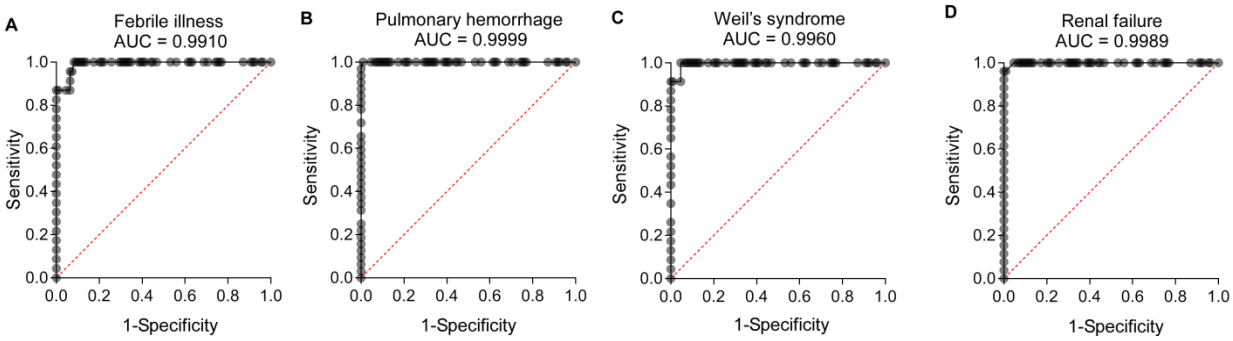

Figure 4.2. Analysis of the diagnostic value of MIF for early diagnosis of leptospirosis. (A-D) ROC curve of MIF for different clinical manifestations of leptospirosis such as (A) febrile illness (AUC=0.9910), (B) pulmonary hemorrhage (AUC=0.9999), (C) Weil's syndrome (AUC=0.9960), (D) Renal failure (AUC=0.9989). AUC values represent the diagnostic value of MIF, >0.9 was considered the outstanding ability of the biomarker to diagnose the disease.

100%. The positive predictive value (PPV) and negative predictive value (NPV) for different forms of leptospirosis were >91% and 100% respectively. The detailed parameters of ROC analysis are shown in **Table 4.4**. The overall results of ROC analyses showed that serum MIF has a strong diagnostic potential for leptospirosis cases; hence serum MIF will be a good candidate as an early diagnostic marker of human leptospirosis which will strongly discriminate the leptospirosis cases.

Table 4.4. ROC analysis of MIF as an early diagnostic marker for leptospirosis

Clinical Parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC±SE (*p* value)
Febrile illness	100	91.7	91.54	100	0.9910 ± 0.006 (<0.0001)
Pulmonary hemorrhage	100	99	98.9	100	0.9999 ± 0.0003 (<0.0001)
Weil's syndrome	100	95.4	95.12	100	0.9960 ± 0.003 (<0.0001)
Renal syndrome	100	95.4	95.12	100	0.9989 ± 0.001 (<0.0001)

4.2.4. Correlation between serum MIF and leptospirosis disease progression

As per the results of MIF profiling, this study revealed that non-hospitalized cases of leptospirosis with the mild febrile illness have remarkably higher serum MIF levels than hospitalized cases with severe organ and/ or multi-organ dysfunction. To investigate the

correlation between serum MIF levels and leptospirosis disease progression, we performed Pearson's correlation co-efficient analysis and found a significantly strong positive correlation (r = 0.75, p<0.0001) **(Figure 4.3)**.

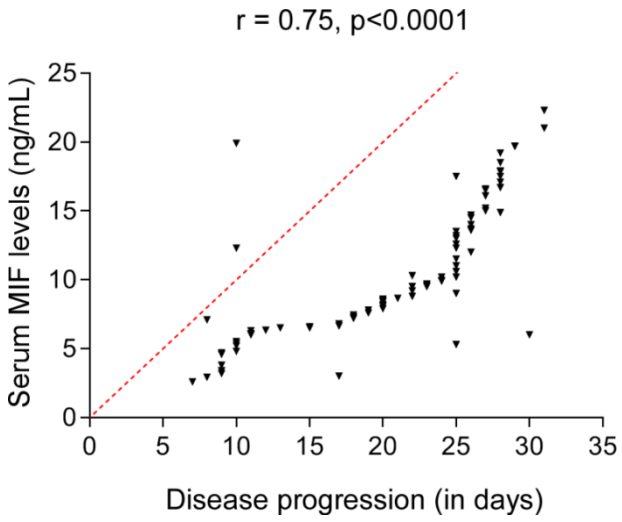

Figure 4.3. Pearson correlation co-efficient of serum MIF level and disease progression. This analysis showed that serum MIF was positively correlated with disease progression which indicates the contribution of serum MIF to disease severity (r = 0.75, P<0.0001) and acts as a severity predictor.

The mean difference in serum MIF levels between the groups was significantly high (2.5, p<0.001). No significant correlation between serum MIF levels and the patient's age was found (r = 0.006). Therefore, this study proposed that serum MIF may be a promising severity predictor of human leptospirosis.

4.3. Discussion

Leptospirosis remains under-diagnosed in most urban and rural regions of India due to the inappropriate awareness of leptospirosis prevalence among people and physicians, lack of attention on leptospirosis specific diagnosis, the varied clinical spectrum of and unavailability of the early diagnostic tool leads to delayed antibiotic treatment, multi-organ dysfunction and increased mortality rate (Le Turnier and Epelboin, 2018). In this study at Tiruchirappalli of

Tamil Nadu State, India, the seroprevalence of leptospirosis is 47.3%, which is near to the disease incidence in Kerala and Andaman and Nicobar Islands where the frequent outbreaks happen every year during post-natural calamities (Rajendran *et al*., 2021, Vijayachari *et al*., 2008). Studies demonstrated the serovar prevalence in the reservoir, carrier hosts, and human and risk factor analysis to enlighten the knowledge of the mode of transmission in specific geographical regions. This study found the highly circulating leptospiral serovars in the Tiruchirappalli district such as Autumnalis, Australis, Canicola, Icterohaemorrhagiae, Grippotyphosa, and Ballum, which are similar to our previous investigation (Vanithamani *et al*., 2015). In Southern peninsular India, the predominant serovars are Icterohaemorrhagiae, Javanica, Hurstbridge, Australis, and Pyrogenes (Balamurugan *et al*., 2021); in Chennai city, serovars are Canicola, Australis and Autumnalis (Jacob *et al*., 2018); in Kerala, the major serovars are Autumnalis, Australis, Louisiana, and Grippotyphosa (Kuriakose *et al*., 2008); in Andhra Pradesh, the prevalent serovars are Pomona, Autumnalis, and Hardjo (Alamuri *et al*., 2019), in Karnataka, Australis is the prominent serovar; in Andaman and Nicobar Island, Icterohaemorrhagiae, Grippotyphosa, and Valbuzzi (Vijayachari *et al*., 2008, Vimal *et al*., 2018). Leptospirosis infection gets worsened by enhanced inflammatory responses through the secretion of cytokines. The cytokine storm-mediated immunoparalysis causes septic shock and multi-organ failure (Cagliero *et al*., 2018). Growing shreds of evidence suggested that inflammatory cytokines were shown to be the best candidate of biomarkers for early diagnosis and severity predictor of leptospirosis. Because the cytokine levels in healthy controls and leptospirosis patients varied significantly, such as IL-6, IL-8 and Il-10 were predominantly higher in leptospirosis cases (Chirathaworn *et al*., 2016). Growing pieces of evidence revealed the role of MIF in disease development and showed that MIF may serve as a better molecular marker for

diagnosis and also severity predictor of several diseases. Since MIF was found to be highly secreted by several inflammatory cells during infection followed by the activating the major inflammatory pathway and plays a pro-oncogenic role, MIF serves as an early indicator of systemic infections, inflammatory diseases, autoimmune diseases, metabolic disorders, and cancer. MIF has chemokine-like functions to promote chemotaxis and recruitment of inflammatory leukocytes during infection (Grieb *et al*., 2010, Grieb *et al*., 2014). MIF acts as a disease modifier due to its immunoregulatory activities such as the counter-regulation of glucocorticoid activities and followed by the overexpression and secretion of inflammatory cytokines. Serum and/or plasma MIF are correlated with high mortality of dengue hemorrhagic fever patients by enhancing the level of serum IL-6, TNF-α, IFN-γ, and IL-10 and also progress to dengue shock syndrome (Chen *et al*., 2006).

In this study, we found the serum MIF as an early diagnostic marker of leptospirosis which potentially discriminates the leptospirosis cases from healthy controls as well as the levels of serum MIF assist to predict the leptospirosis severity. The range of serum MIF in mild leptospirosis with febrile illness was 2.6-12.3 and that of severe leptospirosis with organ involvement was 3.2-22.3. Therefore, MIF will be a valuable candidate biomarker and severity predictor of leptospirosis.

4.4. Conclusion

This study revealed the alarmingly high seroprevalence in Tiruchirappalli district, one of the important agro-climatic zones of Tamil Nadu, India. The serovar distribution in the studied area was identical to the previous investigations. For the first time, our study provided evidence of serum MIF level variations among leptospirosis confirmed cases, other common febrile cases,

and healthy control subjects. We suggested that the assessment of serum MIF will be a promising early diagnostic marker of leptospirosis.

Chapter II

5. Determinate the *in vitro* and *in vivo* MIF expression profile in the leptospiral LPS induced model

5.1. Materials and Methods

5.1.1. Animals and experimental conditions

An inbred strain of BALB/c mice (weighing about 20 ± 2 gm) was used throughout the study. To accustomed, the mice were housed under ambient room temperature (25 ± 2°C), 12-hour light/dark cycle, and given standard feed and water ad libitum over a period of 10 days before the experiments began. All experimental protocols were approved by the Institutional animal ethical committee (IAEC), Bharathidasan University, Tiruchirappalli (BDU/IAEC/P30/2018).

5.1.2. Mouse adapted challenge strain (MACS)

Leptospira interrogans serovar Autumnalis strain N2 - MACS were developed by repeated challenging with an appropriate reference laboratory strain and re-isolating of leptospires from circulation and/ or kidney tissues. Initially, the mice were immunocompromised with cyclophosphamide monohydrate by intraperitoneal administration at the concentration of 300mg/kg body weight of mice. In BALB/c mice, the successful MACS were obtained after more than 15 times of passages. The developed MACS strain which has passaged in synthetic media less than 3 times was used for experiments (Adler and Faine, 1976; Adler and Faine, 1977; Kanagavel *et al*., 2017).

5.1.3. LPS extraction

For *in vitro* and *in vivo* experiments, leptospiral LPS was extracted by the standard hot phenol-water method. The developed MACS strain was grown in EMJH broth at 30ºC to reach the cell number of ~ $5x10^8$ leptospires/mL. Nucleic acid and protein contaminants were lysed by RNase H, DNase, and proteinase K respectively. LPS containing phenol phase was dialyzed extensively against water and the insoluble material was removed by centrifugation. The extracted LPS were

quantified by the phenol / sulfuric acid method using sucrose solution as a standard (Westphal, 1965).

5.1.4. MTT assay

For *in vitro* optimization of LPS dose, THP-1 cells were treated with different concentrations of LPS (0.1, 0.5, 1, 2, 3, 4, 5, 10, and 20 µg), and the percentage of cell proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The THP-1 cells (NCCS, Pune) were seeded in 96-well tissue culture plates at a cell number of 2 x 10^3 cells/ well and incubated for 12h. Then, the cells were treated with various concentrations of LPS for 12 h. PBS and Triton X-100 were used as vector and positive control respectively. After the treatment, 20 µL of MTT solution (5mg/mL) was added and incubated at 37ºC for 3 h. Then, the medium was removed and 100 µl of Dimethyl sulfoxide (DMSO) was added to each well to completely dissolve the formazan precipitates. The absorbance was read at 490 nm by using a microplate reader (Bio-Rad, Hercules, CA, USA). The median inhibitory concentration (IC_{50}) value was calculated by the GraphPad prism.

5.1.5. Determination of median lethal dose (LD_{50})

For *in vivo* optimization of LPS dose as LD_{50}, LPS mediated survivability was determined by Kaplan Meier analysis. Young, 4-6 weeks old, immunocompromised mice were segregated into 5 groups (n=5). After 24 hrs of immunosuppression, mice were challenged intraperitoneally with different concentrations of leptospiral LPS (5, 10, 20, and 30 mg/kg body weight of mice). Group 1 mice injected with PBS alone were maintained as untreated control. The mice of Group II, III, and IV were injected with different doses of LPS respectively as mentioned above. The mice were monitored frequently for a week for clinical outcome and survival. The dead mice

were dissected and moribund animals were euthanized to harvest the tissues. The LD_{50} value was calculated by using a GraphPad prism.

5.1.6. Dot blot assay

To investigate the successful development of leptospirosis experimental models, mice blood was tested for endotoxemia. Dot blot assay was performed by plasma samples of both untreated control and LPS injected mice. 2 µL of plasma was loaded on a 0.2 µm nitrocellulose membrane and incubated with a 3% blotting grade blocking solution (Bio-Rad, Hercules, CA, USA). After blocking, the membrane was incubated with leptospirosis patient's sera in the dilution of 1:100 for 1 hour at room temperature. The membrane was washed with Tris buffer saline with 0.1% Tween 20 (TBST) 3 times for 10 minutes each. After washing, the membrane was incubated with ALP conjugated anti-human IgM antibody (1:2000) for 1 h. After washing, Super Signal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was added to develop the bands and documented by Fusion Solo™ Personal Blot and Gel Imaging System (Vilber Lourmat, Paris, France). The densitometric analysis of blot was performed by ImageJ software and presented in arbitrary units.

5.1.7. Cytokine Profiling

Quantitative real-time PCR (qRT-PCR) was performed to confirm the endotoxemic inflammation by *in vitro* and *in vivo* experimental models by profiling the inflammatory cytokine expression. RNA was extracted from LPS treated cell lysates and monolayer cells of blood plasma from infected mice by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and quantified by using the Biophotometer Plus system (Eppendorf, Hamburg, Germany). Complementary DNA (cDNA) was synthesized by the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA) and qRT-PCR was performed using a CFX96 Touch™ Real-Time PCR

Detection System (Bio-Rad, Hercules, CA, USA) to demonstrate the expression profiling of TNF-α, IL-1β, IL-4, and IL-10. The reaction mix (10 µL) was prepared by adding 2X SYBR Green PCR Master Mix, 20 µM of each primer, and 20 ng cDNA). The primer sequences used in this experiment are provided in **Table 5.1** The housekeeping gene, GAPDH was used as an internal control.

Table 5.1. List of primer sequences used for gene expression analysis

Target Gene	**Sequence**
Human MIF: FP	5'-CGC AGA ACC GCT CCT ACA G-3'
Human MIF: RP	5'-GGA GTT GTT CCA GCC CAC AT-3'
Mouse MIIF: FP	5'-GGC CTT GCG TCT TGT CC-3'
Mouse MIF: RP	5'-GCA AGC TGC CCG CGA TA-3'
Mouse TNF-α: FP	5'-GGA CTA GCC AGG AGG GAG AA-3'
Mouse TNF-α: RP	5'-CGC GGA TCA TGC TTT CTG TG-3'
Mouse IL-1β: FP	5'-AAA CAG ATG AAG TGC TCC TTC CAG G-3'
Mouse IL-1β: RP	5'-TGG AGA ACA CCA CTT GTT GCT CCA-3'
Mouse IL-4: FP	5'-CAA ACG TCC TCA CAG CAA CG-3'
Mouse IL-4: RP	5'-AAG CCC GAA AGA GTC TCT GC-3'
Mouse IL-10: FP	5'-GCC CTT TGC TAT GGT GTC CT-3'
Mouse IL-10: RP	5'-TTT TCA GGG ATG AAG CGG CT-3'
Human GAPDH: FP	5'-AAC GAC CCC TTC ATT GAC-3'
Human GAPDH: FP	5'-TCC ACG ACA TAC TCA GCA C-3'
Mouse GAPDH: FP	5'-TGG TGA GTG GAG TGA TCC CTG AGT-3'
Mouse GAPDH: FP	5'-AGA TTT CTT AGG GGC ATG GTC GGC-3'

5.1.8. In vitro and *in vivo* LPS induction

For *in vitro* induction, THP-1 cells were seeded and grown in a 6-well plate at the cell number of 5 x 10^5 cells per well. Cells were treated with 1 µg of leptospiral LPS for different time intervals

at 0, 5, 10, 20 minutes and 1, 2, 3, 6, 12, 24, 48, and 60 h. All the treatments were performed in triplicate.

For *in vivo* induction, the immunocompromised mice were injected intraperitoneally with a 20 mg/kg dosage of leptospiral LPS. LPS treated mice were frequently observed for clinical symptoms and survivability for up to 8 days. Dead and moribund mice were dissected. The vital organs such as lungs, liver, kidney, heart, and spleen and lymphoid organs such as bone marrow, thymus, lymph node, and spleen were harvested and stored in liquid nitrogen until use.

5.1.9. Western blotting

For MIF protein expression profiling by western blotting, total protein was extracted from LPS treated THP-1 cells and various tissues of infected mice by using Radioimmunoprecipitation assay (RIPA) lysis buffer. The cells were washed twice with PBS and the cell lysates were prepared by using 1X ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, and 1% NP-40) (Thermo Fisher Scientific, Waltham, MA, USA) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The tissues were homogenized completely and lysed with a 1:4 ratio of RIPA buffer. The supernatant containing solubilized proteins was collected, quantified by the BCA method, and stored at -80ºC until use. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with 25 µg protein in 12% gel by using Mini- PROTEAN Tetra System (Bio-Rad, Hercules, CA, USA). Blotting was performed in a 0.2µm pore-sized nitrocellulose membrane by using a V20 semi-dry blotter (Scie-Plas, Cambridge, UK). Then, the membrane was incubated with a 5% blocking solution for 1h at room temperature. After washing thrice with TBST, the membrane was incubated with an anti-MIF antibody (Invitrogen, Carlsbad, CA, USA) in the dilution of 1:1000 for 12h at 4ºC. The housekeeping protein, GAPDH was used as an internal control. After

washing, the bound antibodies were captured and detected by 1:5000 dilution of HRP-conjugated anti-rabbit antibody (Sigma-Aldrich, St. Louis, MO, USA). Bands were developed and documented and the densitometric analysis of blot was performed to normalize the target with internal control.

5.1.10. MIF ELISA

To measure the secreted MIF levels in the culture medium of THP-1 cells, supernatants were collected from LPS treated THP-cells and performed the MIF ELISA as mentioned previously in Materials and Methods 4.1.7. The concentration (ng/mL) of secreted MIF was calculated by using the MIF standards.

5.1.11. Quantitative Real-Time PCR

For MIF gene expression profiling by qRT-PCR, cDNA was synthesized from extracted RNA by (Bio-Rad, Hercules, CA, USA) and qRT-PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) to demonstrate the gene expression profiling of MIF gene. The primers used in this experiment are listed in **Table 5.1.**

5.1.12. Statistical analysis

Data from biological triplicates were represented as Mean ± SD (n=3). The significant differences of variation between study groups were determined by a two-tailed paired Student's t-test or the Mann- Whitney U test. Data were computed either with GraphPad Prism version 9.2.0 or SigmaPlot 11.0 software. Kaplan Meier plot analysis was performed to quantify the survival rate of LPS injected mice by using GraphPad Prism. Differences were considered significant at p values ≤ 0.05.

5.1.13. Ethical compliance

The experimental protocols were reviewed and approved by the Institutional Animal Ethical Committee (BDU/IAEC/P30/2018), Bharathidasan University. The procedures of the administration of biological materials and dissection were strictly adopted with Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

5.2. Results

5.2.1. Optimization of IC_{50} dose of LPS

Mouse adapted challenge strain was developed and leptospiral MACS LPS was extracted and purified. **Figure 5.1** showed the polyacrylamide gel image of purified LPS.

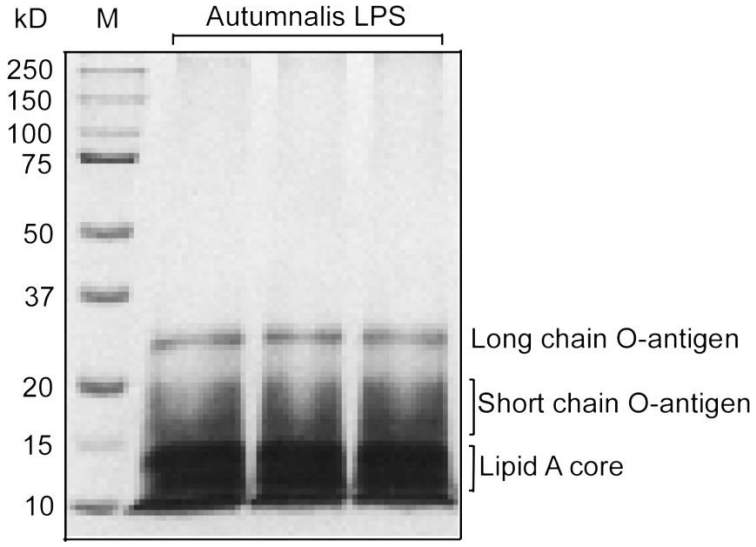

Figure 5.1. Extraction of Leptospiral lipopolysaccharide by hot phenol water method. Representative image of extracted and purified LPS from *L. interrogans* serovar Autumnalis strain N2 showed the three major components of LPS, O antigen, core oligosaccharide, and lipid A.

In vitro IC_{50} dose of leptospiral LPS was optimized by MTT assay, a technique that measure cellular respiration by mitochondrial dependent reduction of MTT to formazan. THP-1 cells treated with 1 µg of LPS showed about 50% of cell viability. LPS concentration, 0.1, 0.5, 1, 2, 3, 4, 5, 10 and 20 µg showed the cell viability of 100%, 92%, 87%, 58%, 32%, 18%, 9%, 5%, 0%,

and 0% respectively **(Figure 5.2A)**. Therefore, 1 µg of LPS was determined as the optimized dose of leptospiral LPS to carry out the *in vitro* experiments.

5.2.2. Determination of LD_{50} value of LPS

In vivo LD_{50} dose of leptospiral LPS was determined by survivability analysis. Kaplan Meier survival plot **(Figure 5.2B)** was prepared by recording frequent observations of experimental mice injected with various concentrations of LPS, which revealed that 20 mg/kg LPS showed the survival of about 50% of mice at the end of experiments. Furthermore, the mice treated with 5, 10, and 30 mg/kg LPS showed 80%, 80%, and 20% of survivability respectively. Therefore, we considered 20 mg/kg LPS as the optimized dose used for further profiling experiments.

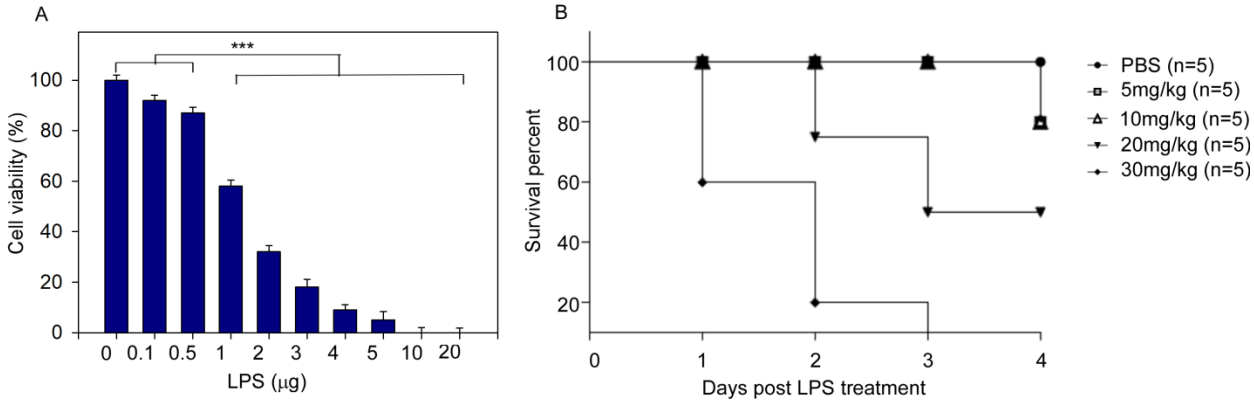

Figure 5.2. Determination of *in vitro* and *in vivo* infection dose of leptospiral LPS for THP-1 cells and BALB/c mice. A) Representative graph of LPS IC50 determination showed the cell viability in different doses (0.1, 0.5, 1, 2, 3, 4, 5, 10, 20 µg) of LPS treated THP-1 cells, B) Representative Kaplan-Meier plot of LD_{50} determinations showed the survivability of different doses (5mg/kg, 10mg/kg, 20mg/kg and 30mg/kg) of leptospiral LPS injected mice. n = 3 experiments. ***P<0.001.

5.2.3. Determination of endotoxemia and endotoxemic inflammation

To confirm the development of an effective experimental mice model of leptospirosis, LPS mediated endotoxemia and inflammation were investigated. **Figure 5.3A** showed the presence of LPS in the circulation of the LPS injected mice model. The dot intensities of blot indicated that a

significant concentration of LPS occurred in the blood of infected mice at 4 days post infection when compared with PBS alone injected control mice **(Figure 5.3B).**

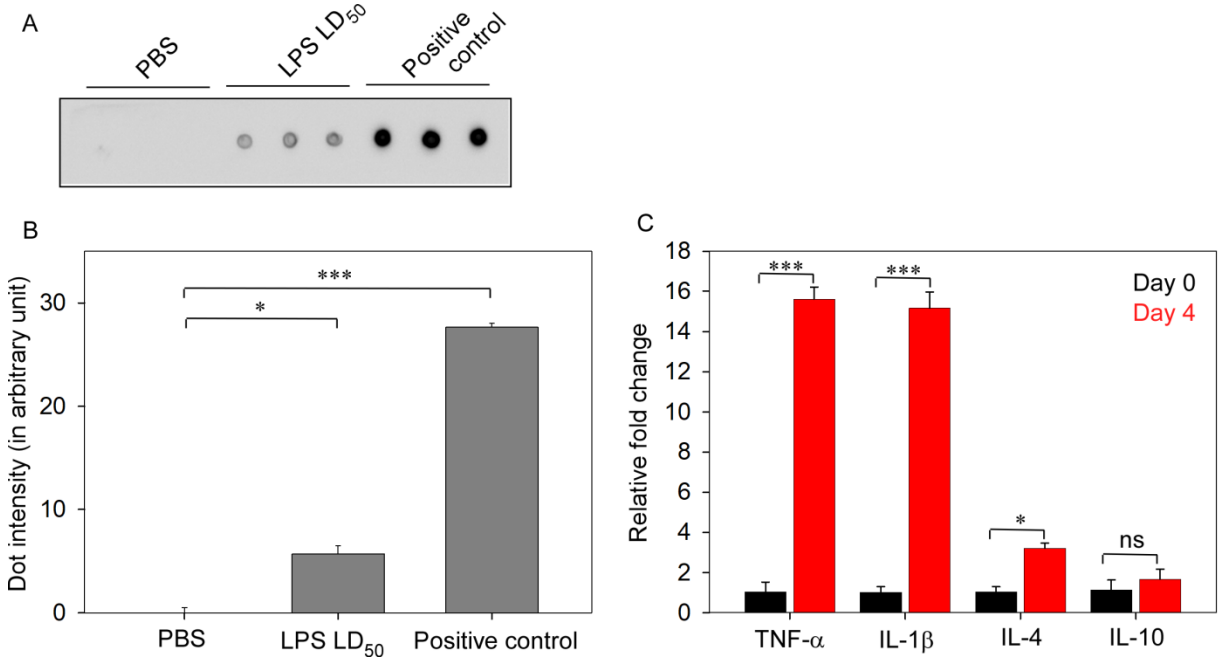

Figure 5.3. Leptospiral LPS LD_{50} induced endotoxemia and endotoxemic inflammation. A) Dot blot assay showed the LD_{50} (10mg/kg) of leptospiral LPS caused endotoxemia in infected mice. The plasma samples of PBS and LPS LD_{50} injected mice were assayed. The purified LPS was loaded as a positive control. B) Representative graph showed the dot intensity of samples. C) Analysis of inflammatory cytokine expression in leptospiral LPS injected mice model by qRT-PCR. n = 3 experiments. *P<0.05, ***P<0.001

Followed by the endotoxemic inflammatory response in infected mice was characterized by cytokine profiling. **Figure 5.3C** demonstrated that the administration of the LD_{50} dose of LPS significantly enhanced the expression of inflammatory cytokines, TNF-α (15-fold), IL-1 (15-fold), and IL-4 (3-fold). The upregulation of inflammatory cytokines and non-significant expression profile of anti-inflammatory clearly remarked the endotoxemic inflammation in LPS LD_{50} injected BALB/c mice models.

5.2.4. *In vitro* profiling of MIF

THP-1 cell lysates were investigated for MIF gene and protein expression profiling at different time intervals. The qRT-PCR analysis further revealed the LPS induced MIF gene overexpression in a time-dependent manner at an early stage of infection. Equivalent to Western blotting results, MIF gene expression was significantly increased at 30 minutes of LPS treatment and reached the maximum level of expression at 12-48 h, followed by a decline to baseline expression **(Figure 5.4A)**. The relative fold change of MIF expression at 0min, 10min, 30min, 1h, 2h, 3h, 6h, 12h, 24h, and 48h were 1, 1, 1, 7, 9, 12, 19, 26, 40, 43, and 12-fold respectively which confirmed that MIF is an early expressed gene during leptospiral infection.

The Western blotting determined that administration of 1 µg of leptospiral LPS significantly ($p < 0.001$) upregulates the MIF protein expression gradually from 30 minutes post-treatment in a time-dependent manner when compared with untreated cells. The gradually increased expression of MIF reached the peak level of expression at 24 hrs and depleted at 60 h post-treatment **(Figure 5.4B and C)**.

The examination of LPS treated THP-1 cell supernatants for secreted MIF analysis by MIF ELISA revealed that IC_{50} dose of LPS induction significantly ($p < 0.001$) upregulates the MIF protein secretion gradually from 1-hour post-treatment in a time-dependent manner when compared with untreated cells which showed the baseline MIF expression. MIF secretion was elevated gradually and reached the sustained maximum level of expression after 12 h **(Figure 5.4D)**.

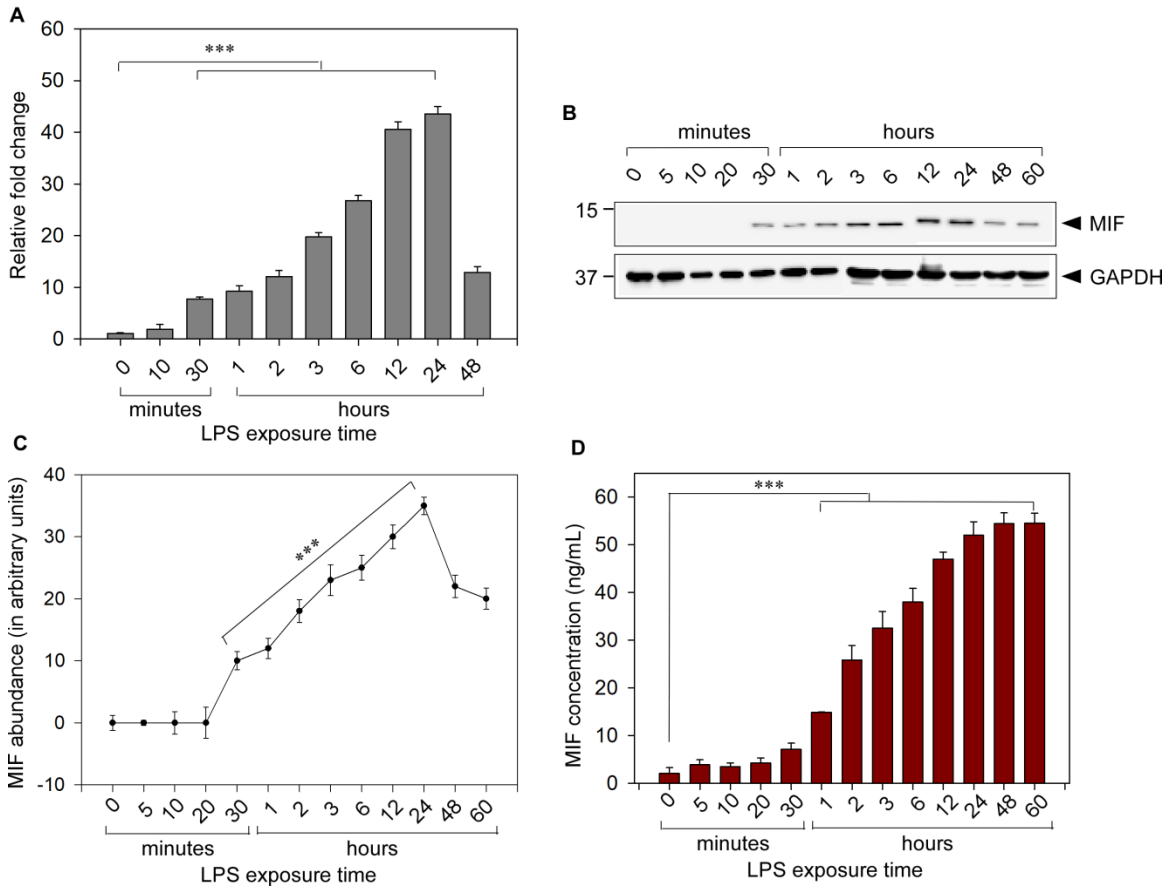

Figure 5.4. Analysis of MIF profiling in leptospiral LPS induced in vitro experimental model, THP-1 cells. A) Analysis of Leptospiral LPS stimulated MIF mRNA expression in THP-1 cells by qRT-PCR. B) Representative western blot analysis showed increased expression of MIF in LPS treated THP-1 cells in a time-dependent manner. C) Quantification of MIF abundance by densitometric measurement from B. D) Measurement of secreted MIF levels in the culture medium of THP-1 cells by MIF ELISA. n = 3 experiments, *P<0.05, **P<0.01, ***P<0.001.

5.2.5. *In vivo* profiling of MIF

For *in vivo* profiling of MIF expression, vital organs such as heart, lung, liver, spleen, kidney, and lymphoid organs including bone marrow, thymus, lymphnode, and spleen were collected and lysates were subjected to Western blotting and qRT-PCR. Among the vital organs of infected mice, LD_{50} dose of LPS significantly ($p < 0.01$) stimulated the expression of MIF in the lung followed by the spleen and heart when compared with the kidney and liver **(Figure 5.5A and B)**.

Tissue specific differential MIF expression was observed in vital organs of untreated control mice.

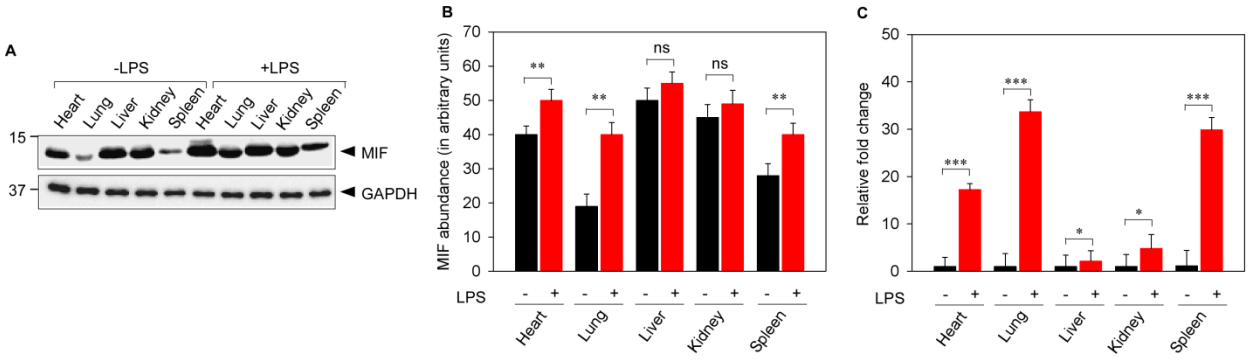

Figure 5.5. MIF profiling in vital organs of leptospiral LPS induced *in vivo* experimental model, BALB/c mice. A) Representative western blot analysis showed the significantly upregulated MIF expression in lungs and spleen among the vital organs of LPS induced BALB/c mice. B) Quantification of MIF abundance by densitometric measurement from A. C) Analysis of Leptospiral LPS stimulated MIF mRNA expression in vital organs n = 3 experiments, *P<0.05, **P<0.01, ***P<0.001.

Similar to Western blotting observations, qRT-PCR results confirmed that LPS injected mice showed a significant increase in MIF gene expression. Leptospiral LPS stimulated the MIF gene expression significantly in the lung, followed by the spleen and heart. The relative fold change of MIF gene expression in the heart, lung, liver, kidney, and spleen was 17, 33, 2, 4, and 29-fold respectively **(Figure 5.5C)**.

Among lymphoid organs, LPS stimulated overexpression of MIF protein was observed in all organs gradually at 1, 2, 3, and 4 days post-infection. No significant differential expression was observed among primary and secondary lymphoid organs **(Figure 5.6A and B)**. Consistent with Western blotting observations, MIF gene expression was gradually increased till 3 days post-infection, and on the 4th day, a drastic increase of MIF expression was observed among lymphoid organs. On the 4th day, the drastic relative fold change of MIF gene expression in thymus, bone marrow, spleen, and lymph node were 32, 29, 28, and 37-fold respectively **(Figure 5.6C)**.

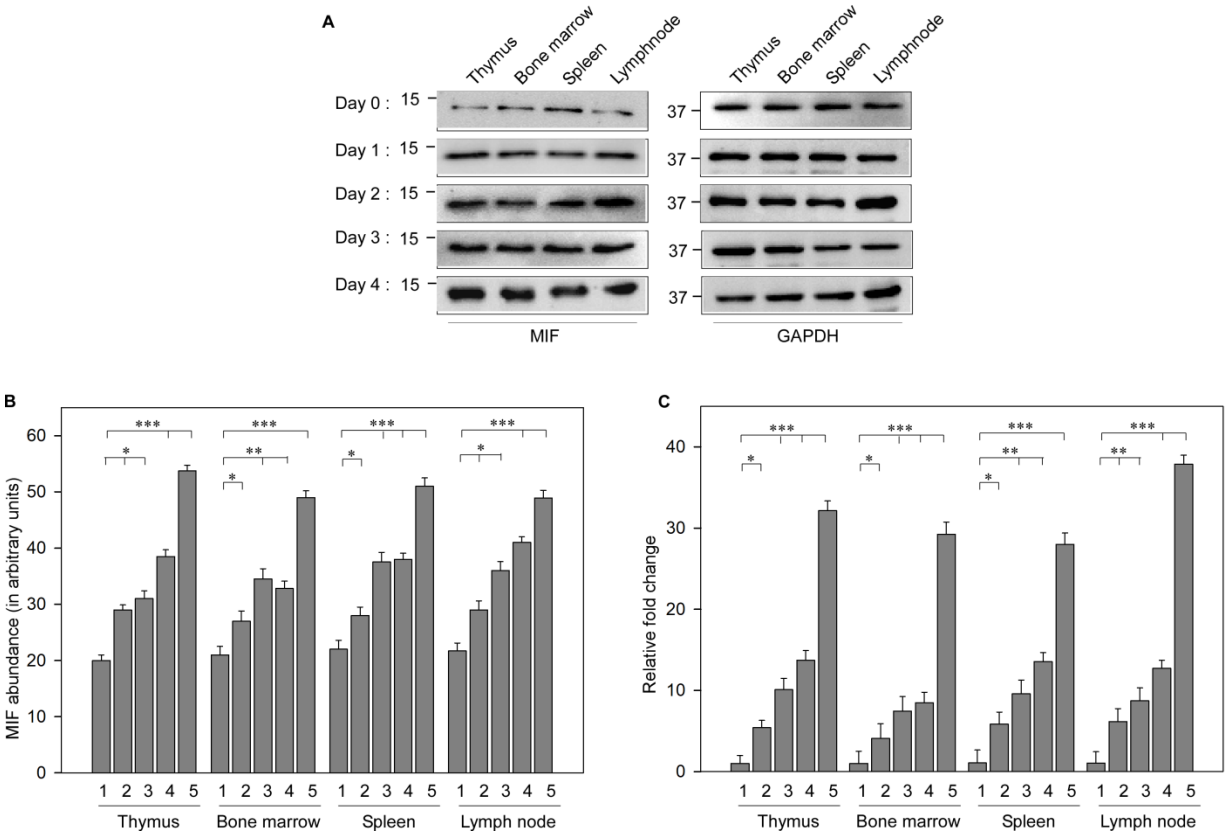

Figure 5.6. MIF profiling in vital organs of leptospiral LPS induced *in vivo* experimental model, BALB/c mice. Representative western blot analysis showed the progressive MIF upregulation in primary and secondary lymphoid organs in different time intervals. B) Quantification of MIF abundance by densitometric measurement from A. C) Analysis of Leptospiral LPS stimulated MIF mRNA expression in lymphoid organs n = 3 experiments, *P<0.05, **P<0.01, ***P<0.001.

Therefore, MIF is an early expressed and secreted cytokine, which may be an effective candidate for early diagnostic tools of leptospirosis and may play a crucial role in the pathogenesis of leptospirosis.

5.3. Discussion

The major findings of this part of the study are the determination of LPS dose in *in vitro* and *in vivo*, the development of efficient leptospirosis experimental models, and molecular MIF profiling. Differential expression of MIF and their family members, D-dopachrome tautomerase activity (DDT), DDT- like (DDTL), and their receptor, CD74 were verified in previous studies

(Verjans *et al*., 2009, Florez-Sampedro *et al*., 2020). Our study reported for the first time leptospiral LPS mediated *in vitro* and *in vivo* expression profile of macrophage migration inhibitory factor was determined. MIF, a constitutively expressed cytokine was differentially expressed in mice tissues of treated and infected groups. Naturally, mice are not susceptible to leptospiral infection, so a single dose of cyclophosphamide (300mg/kg body weight of mice) injection rendered the mice susceptible to sublethal leptospiral infection. Cyclophosphamide is well known to specifically inhibit the functional activities of B cell generated immunity; therefore, the studies on cell-mediated immunity and inflammatory cytokines have no significant impacts (Adler and Faine, 1976).

In this study, leptospiral LPS induced THP-1 cell (monocytic leukemia cell line) and young BALB/c mice were used as *in vitro* experimental models. Isogai *et al*. (1990) studied the leptospiral LPS mediated macrophage activity. As a potent activator of macrophages, MIF stimulates the expression of interleukin-1 and interferons, inflammatory responses, necrosis of the liver, deposition of LPS in the liver, spleen, and lymph nodes, and efficient bactericidal activity of non-immune macrophages. The lethal dose of LPS varied depending on the strain type and maturity of mice; the immunologically matured mice and rats are resistant to leptospiral LPS challenge while the young 4-6 weeks old mice were the suitable responder for endotoxemic stimulation. Mouse lethal leptospiral challenge strain has been reported to render the mice model susceptible to challenge (Haake, 2006).

Since MIF is the primary mediator which has been identified to be secreted from inflammatory immune cells during endotoxemic conditions. In this study, MIF expression was found at 30 minutes post-infection with LD50 of LPS and enhance the production of proinflammatory cytokines. Previous studies confirmed that Interferons (IFN-γ), pro-inflammatory cytokines

including TNF-α, IL-1β, IL-2, IL-4, IL-8, and IL-6, and other pro-inflammatory molecules such as prostaglandins, nitric oxide were upregulated in leptospirosis. Indeed, the transcription factors involved in inflammatory pathways such as AP-1 and NF-κB were significantly increased during leptospirosis (Cagliero *et al*., 2018). As a pro-inflammatory cytokine, MIF is expressed in a variety of immune and non-immune cell types namely, lymphocytes, monocytes, macrophages, eosinophils, mast cells, neutrophils, pituitary cells, endothelial cells, epithelial cells, smooth muscle cells, mesenchymal cells and synovial fibroblasts (Sumaiya *et al*., 2021).

Several reports indicated that MIF expression was increased in several diseased conditions. In response to LPS induction, human umbilical vein endothelial cells (HUVECs) secrete higher levels of MIF at 12 h post-treatment (Nishihira *et al*., 1998). In pancreatic ductal adenocarcinoma (PDAC), MIF was overexpressed in exosomes and developed PDAC liver metastasis (Costa-Silva *et al*., 2015). In THP-1 cells, the ethanol treatment has not overexpressed the MIF mRNA and protein but the LPS stimuli enhanced the expression of MIF mRNA and protein secretion (Marin *et al*., 2017). Therefore, cell types and the differential response to stimuli determine the MIF expression and secretion. Besides, MIF expression was tightly regulated in a time and dose-dependent manner. TNF-α enhances the MIF gene overexpression by 67% with 10ng/mL and by 95% at 24 hrs. IL-1β facilitates the upregulation of 15% with 100ng/mL and at 48h (Lai *et al*., 2003). This study revealed that leptospiral LPS had a potent up-regulatory effect on MIF expression at 30 minutes and reached a peak at 24h post-treatment in THP-1 cells.

5.4. Conclusion

For the first time, we analyzed the *in vitro* and *in vivo* profiling of MIF in the leptospirosis experimental model and revealed that MIF is an early phase secreted cytokine. The mRNA and

protein expressions of MIF were significantly increased as the duration of infection increased, with MIF possibly driving the pathogenesis of leptospirosis. In vitro and in vivo MIF profiling determined the timeline of early MIF gene expression and its differential expression in different organs during leptospiral infection. Thus, the present findings provide the framework for further studies for understanding the role of MIF in leptospirosis.

Chapter III

6. Understand the mechanism of calcium [Ca^{2+}] dependent, transcriptionally activated MIF expression by *in vitro* system of ORAI1 knockdown

6.1. Materials and Materials

6.1.1. Intracellular calcium [$Ca^{2+}]_i$ dynamics

Intracellular Ca^{2+} dynamics of LPS treated cells were optically imaged by Fluo-4 staining using Confocal Laser Scanning Microscopy (CLSM) (De Jonge *et al*., 2004, Natarajaseenivasan *et al*., 2018). THP-1 cells with 70% confluence were grown and adherent on Cell-Tak (BD Biosciences, Bedford, MA) coated 25 mm diameter glass coverslips. Then, the cells were loaded with ratiometric fluorescent Ca^{2+} indicator dye, Fluo-4 AM (Molecular Probes, Eugene) with the concentration of 1-5 µM diluted in Hank's Balanced salt solution (HBSS), and incubated at 37ºC for 30 minutes in the dark state for maximal binding to $[Ca^{2+}]_i$. The cells were pelleted and washed three times with calcium free HBSS and stimulated with leptospiral LPS (1µg). Fluo-4 AM loaded untreated THP-1 cells in calcium free HBSS was considered a negative control while dye loaded cells with 1µM ionomycin were used as a positive control of $[Ca^{2+}]_i$ influx. The coverslips were mounted on an open chamber and images were acquired using confocal microscopy (Zeiss LSM710, Germany) at 488 nm using a 63× oil objective. The Fluo-4 AM fluorescence was quantified using ImageJ.

6.1.2. LPS induction *in vitro*

THP-1 cells were seeded and grown in a 6-well plate at the cell number of 5×10^5 cells per well. Cells were treated with 1 µg for 12h. All the treatments were performed in triplicate. LPS treated cell lysates were used to carry out the Western blotting, ChIP assay, and luciferase assay.

6.1.3. Western blotting

To analyze the LPS stimulated store operated calcium entry (SOCE) protein expression, protein kinase signaling and CREB activation, Western blotting was performed. THP-1 cells were treated with leptospiral LPS (1µg) and protein was extracted from cell lysates by RIPA lysis buffer. 25 µg of protein was separated by SDS-PAGE and the proteins were blotted with a 0.2µm nitrocellulose membrane. The target proteins were probed with antibodies specific for IP3R (1:1000, Millipore), STIM1 (1:1000, Cell Signaling Technology), ORAI1 (1:1000, Santa Cruz Biotechnology), CAMKII (1:1000, Cell Signaling Technology), pCAMKII (1:1000, eBioscience), pCREB (1:1000, Cell Signaling Technology), CREB (1:1000, Cell Signaling Technology), p38 MAPK (1:1000, Cell Signaling Technology), p-p38 MAPK (1:1000, Cell Signaling Technology), p44/42 (1:1000, Cell Signaling Technology), p-p44/42 (1:1000, Cell Signaling Technology), and GAPDH (1:1000, Cell Signaling Technology). The HRP-conjugated anti-mouse or anti-rabbit antibodies are used to detect the unbound antibodies. Finally, the bands were developed, documented, and normalized with internal control.

6.1.4. Chromatin immunoprecipitation (ChIP) analysis

ChIP assay was performed to identify the binding interactions between the transcription factor pCREB and the promoter region of the MIF gene. Pierce Magnetic ChIP kit (Thermo Scientific, USA) was used and all the procedures were followed as per the manufacturer's instructions. Initially, THP-1 cells were cultured and treated with leptospiral LPS, and untreated control was also maintained. Crosslinking of proteins to the DNA was enhanced by adding 1% formaldehyde and harvesting the cells. Added 10µL of diluted micrococcal nuclease per $4x10^6$ cells and incubated at 37ºC for 15 minutes. Cells were sonicated and the supernatant containing digested chromatin was collected for immunoprecipitation. Antibodies used for immunoprecipitation were

anti-pCREB antibody (target-specific), anti-NF-κB antibody (non-target transcription factor specific), and anti-RNA polymerase II antibody (positive control), normal rabbit IgG (negative control). After the incubation for overnight at 4ºC, 20 µL of magnetic beads were added and incubate for 2 hours at 4°C with mixing. Magnetic beads were collected and the protein-chromatin complex was eluted with 150µL of elution buffer. Finally, 750µL of DNA binding buffer was added to recover the DNA. Extracted DNA was then amplified by real-time PCR using oligonucleotides surrounding the CRE^{P} region of the MIF promoter.

6.1.5. Luciferase assay

HeLa cells were cultured in 6 well plates and transfected with MIF promoter plasmid (LightSwitch Promoter Reporter GoClone plasmid with *MIF* promoter sequence, SwitchGear Genomics; S721866) and corresponding control plasmids when cells reached 95% confluency. After 48h post-transfection, cells were treated with leptospiral LPS (1µg) overnight and untreated control was maintained. Then, luciferase activity was measured using the Dual-Luciferase® Reporter Assay System (Promega, USA) following the manufacturer's protocol. In brief, cells were lysed by using 500µL of 1X PLB lysis buffer. 20 µL lysate was added to each well of 96 well plates and 100µL of LAR II (Luciferase assay substrate reagent) was dispensed. Then, the firefly luciferase activity was measured. The reaction is quenched, and the *Renilla* luciferase reaction is initiated simultaneously by adding Stop & Glo® Reagent to the same sample and the activity was monitored.

6.1.6. Generation of ORAI1 transient knockdown (KD) model

Transient knockdown of Orai1 was performed using the ON-TARGETplus SMARTpool siRNA (Dharmacon, USA). THP-1 cells grown in 6-well plates were transfected with ORAI1 siRNA using Lipofectamine RNAiMAX transfection reagent (Thermo Scientific, USA) according to the

manufacturer's instructions. In brief, THP-1 cells were seeded in 6-well plate to be 70% confluent at the time of transfection. Lipofectamine RNAiMAX transfection reagent and siRNA were diluted with Opti-MEM medium as per the manufacturer's instructions. The reagent and siRNA were added in the ratio of 1:1 and mixed well. After incubation at room temperature for 5 minutes, the reagent/siRNA mixture was added to the cells at the final concentration of 3 pmol / well of 6-well plates and stir the plates gently, and incubated at 37ºC for 1-3 days. Seventy-two hours post-transfection cells were used for the experiments. Non-targeting siRNA, scrambled siRNA (SCR-siRNA) duplex (Dharmacon, USA) was used as control. The transfected experimental model was subjected to Western blotting as described earlier.

6.1.7. LPS induction and protein expression analysis

To verify the $[Ca^{2+}]_i$ influx induced CREB activation and MIF expression, the cells were treated with LPS and performed the Western blotting as described earlier. The transfected cells were treated with leptospiral LPS while the untreated control was maintained for comparison. Protein was extracted from cell lysates and Western blotting was performed. To confirm the successful knockdown of ORAI1, the membrane was probed with an anti-ORAI1 antibody (1:1000, Santa Cruz Biotechnology). Anti-pCREB (1:1000, Cell Signaling Technology), Anti-CREB (1:1000, Cell Signaling Technology), anti-MIF (1:1000), Invitrogen), and anti-GAPDH (1:1000, Cell Signaling Technology) antibodies were used to determine the impact of ORAI1 KD in CREB mediated MIF expression.

6.1.8. Statistical analysis

Data from triplicate experiments were quantified and expressed as Mean ± SE, n=3. The student's t-test was used to determine the significance of relative expression analysis. The data were computed either with GraphPad Prism version 9.2.0 or SigmaPlot 11.0 software. One-way

ANOVA with subsequent Tukey's test for multiple comparisons was used to compare the LPS treated and untreated controls, and ORAI1 knockdown and SCR-siRNA transfected cells. $P \leq 0.05$ was considered significant in all analyses.

6.2. Results

6.2.1. Leptospiral LPS elevated $[Ca^{2+}]_i$ influx

To measure the changes in $[Ca^{2+}]_i$ dynamics in leptospiral LPS induced THP-1 cells, Fluo-4 staining was performed.

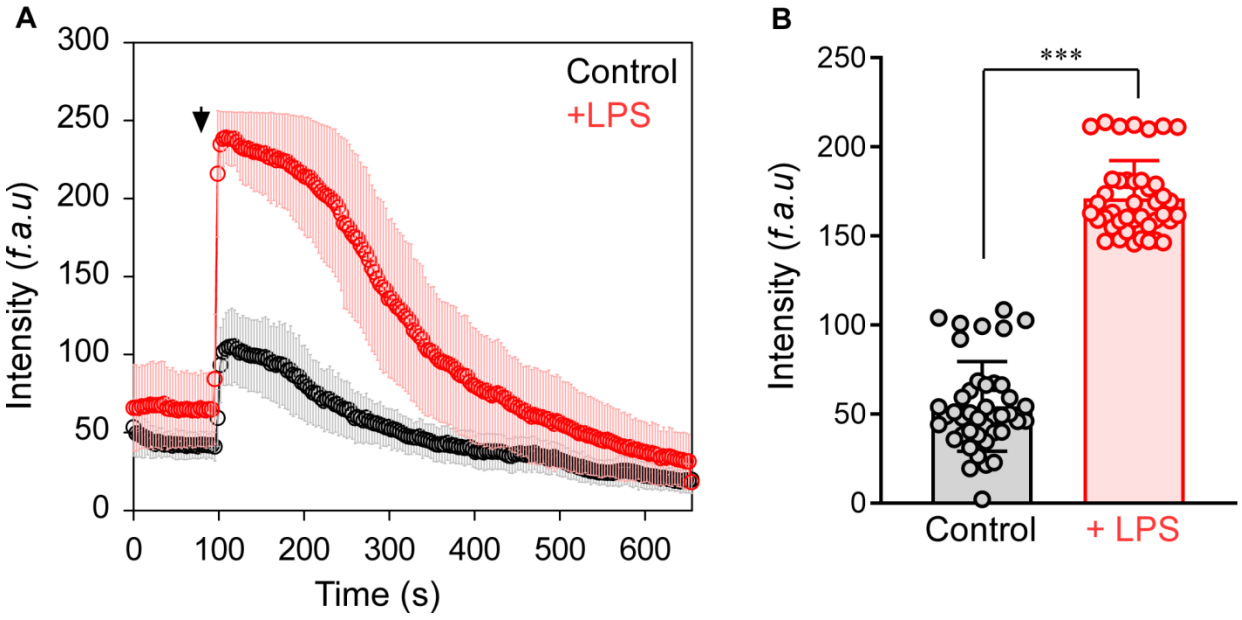

Figure 6.1. Measurement of $[Ca^{2+}]_i$ in leptospiral LPS treated THP-1 cells by Fluo-4 AM staining. A) Time series experiment was performed for 700 seconds. After 15 sec of baseline recording, leptospiral LPS was loaded and the spontaneous changes in $[Ca^{2+}]_i$ was measured at 488 nm excitations. B) The quantification of Fluo-4 fluorescence showed the leptospiral LPS mediated significant increase of $[Ca^{2+}]_i$ influx. n = 3 experiments, ***P<0.001.

CLSM time lap assay revealed that THP-1 cell treated with Leptospiral LPS (1µg) showed the significant $[Ca^{2+}]_i$ influx at 100s which was nearly equal to the ionomycin loaded cells measures with Fluo-4 AM fluorescent intensity. After the baseline imaging of $[Ca^{2+}]_i$ fluorescence, THP-1 cells were induced with leptospiral LPS (1µg, arrowhead), or the positive control ionomycin

(2.5µM, arrowhead) and the significant changes in LPS mediated $[Ca^{2+}]_i$ dynamics were measured and imaged. LPS-induced THP-I cells showed the sustained elevation of $[Ca^{2+}]_i$ influx, while the untreated calcium free HBSS loaded THP-1 cells showed the comparatively reduced $[Ca^{2+}]_i$ influx **(Figure 6.1A and B)**.

6.2.2. Leptospiral LPS increased the protein levels of SOCs

Next, we asked whether the increased $[Ca^{2+}]_i$ is due to the increased functional activity of SOC_s (Store-operated calcium channels) proteins **(Figure 6.2 A and B)**. As suspected, LPS increased

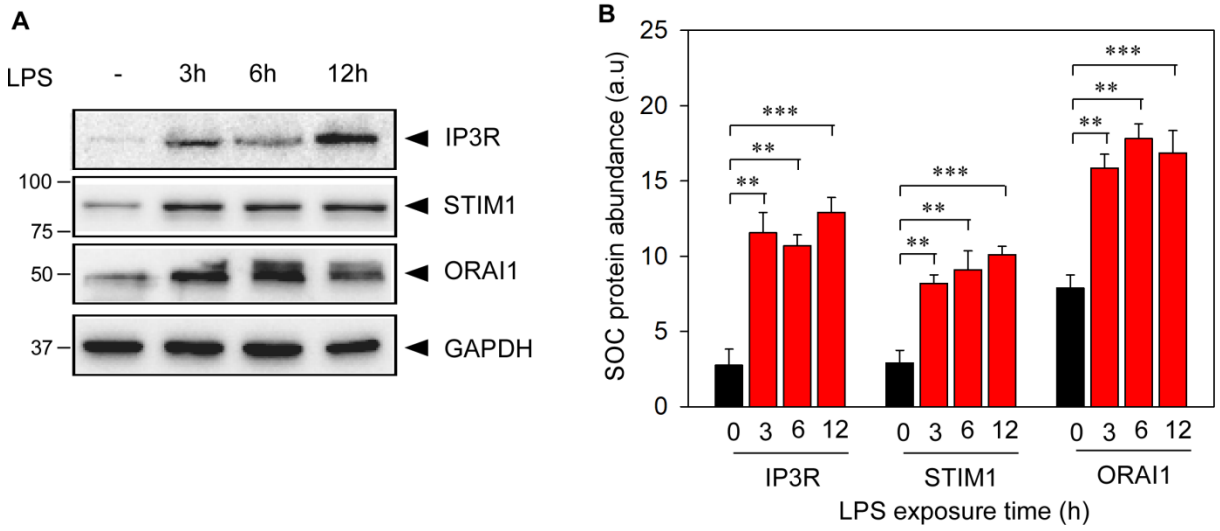

Figure 6.2. Leptospiral LPS mediates the intracellular Ca^{2+} elevation via SOCE. A) LPS mediated SOCE protein expression. Representative Western blot analysis showed increased expression of IP3R, STIM1, and ORAI1 in LPS treated THP- 1 cells with increasing time. B) Quantification of SOCE proteins abundance by densitometric measurement from A. n = 3 experiments, *P<0.05, **P<0.01, ***P<0.001.

the protein levels of two major subunits of SOCs, ORAI1and STIM1, in a time-dependent manner. LPS mediated elevated expression of IP3R, STIM1, and ORAI1 proteins were significantly detected at 3h post LPS treatment and sustained for 12h post treatment. The depleted calcium level in the endoplasmic reticulum was indicated by the upregulation of IP3R expression. The overexpression of STIM1 facilitates the activation of ORAI1 mediated calcium

influx. Thus, leptospiral LPS increases the $[Ca^{2+}]_i$ influx through SOCs by upregulating the expression of IP3R/ORAI1/STIM1.

6.2.3. Leptospiral LPS induction upregulated CREB activation by CAMKII phosphorylation

Because we saw both intracellular Ca^{2+} and MIF expression to be increased in LPS-stimulated THP-1 cells, we next asked whether the increased intracellular Ca^{2+} drives MIF expression.

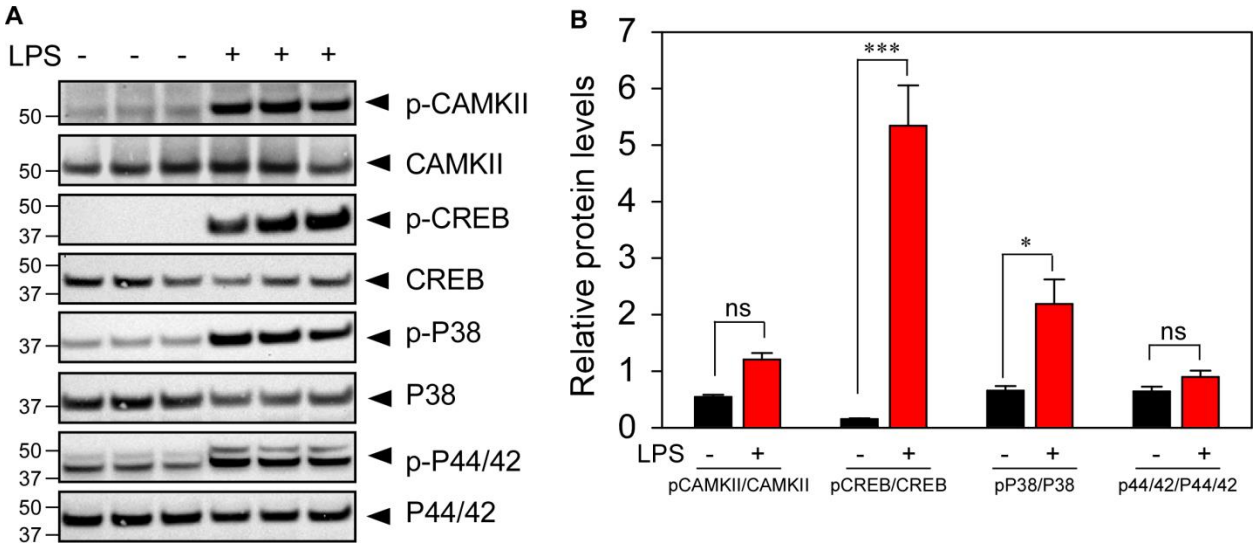

Figure 6.3. Leptospiral LPS stimulates the CREB activation via CAMKII phosphorylation. A) Representative Western blot analysis showed increased phosphorylation of CAMKII, P38, P44/42, and CREB in LPS treated THP-1 cells that corresponds to MIF elevation. B) Quantification of p-CAMKII, p-P38, and p-CREB, pP44/42, and MIF abundance by densitometric measurement from A. n = 3 experiments, *P<0.05, ***P<0.001. ns - no significance.

To validate the role of Ca^{2+} in MIF expression, we performed a Western blot analysis for the upstream intracellular Ca^{2+} dependent cascade for MIF expression. Indeed, in silico analysis revealed that CREB, delta CREB, FOXJ2, p53, and Sp1 are the top transcription factors of MIF gene promoter, predicted by TFBSPred and Qiagen. Based on the pieces of evidence, CREB is one of the crucial Ca^{2+} regulated transcription factors activated via phosphorylation by CAMKII and other protein kinases. So, we investigated the expression pattern of phosphorylated protein

kinases including CAMKII, P38, P44/42, and the phosphorylated transcription factor, CREB. Proteins from cell lysates prepared from LPS treated and untreated THP-1 cells were subjected to Western blotting and revealed that LPS treatment significantly enhanced the phosphorylation rate of CREB, consistent with the phosphorylation of CAMKII, followed by P38 as shown in **Figure 6.3A and B**. The phosphorylation of P44/42 showed no significant difference between untreated and LPS treated cells. The expression levels of pCREB, pCAMKII, p-P38, and p-P44/42 were normalized with the total CREB, CAMKII, P38, and P44/42 respectively. GAPDH was used as a loading control.

6.2.4. Leptospiral LPS promotes the binding interaction of CREB and MIF promoter

To verify that pCREB is bound to the MIF promoter containing CRE^p region located within 50 bp upstream of the transcriptional start site of the MIF gene, we performed chromatin immunoprecipitation assays. The chromatin of THP-1 cells was incubated with an anti-pCREB antibody and anti-NF-κB antibody as a negative control. The purified immunoprecipitated DNA was amplified using the sequences of the primers bind adjacent to CRE^p DNA binding sites. A significantly higher amplification (5-fold) was observed in the DNA immunoprecipitated with anti-pCREB antibody from LPS treated THP-1 cells than in the untreated control cells. There was no amplification of DNA immunoprecipitated with anti-NF-κB antibody. These results confirm the previous investigations (Roger *et al*., 2007) and illustrated that the transcription factor pCREB is bound to the cognate regulatory element, CRE^p region of MIF promoter of THP-1 cells upon leptospiral LPS treatment regulates the MIF gene expression **(Figure 6.4 A)**.

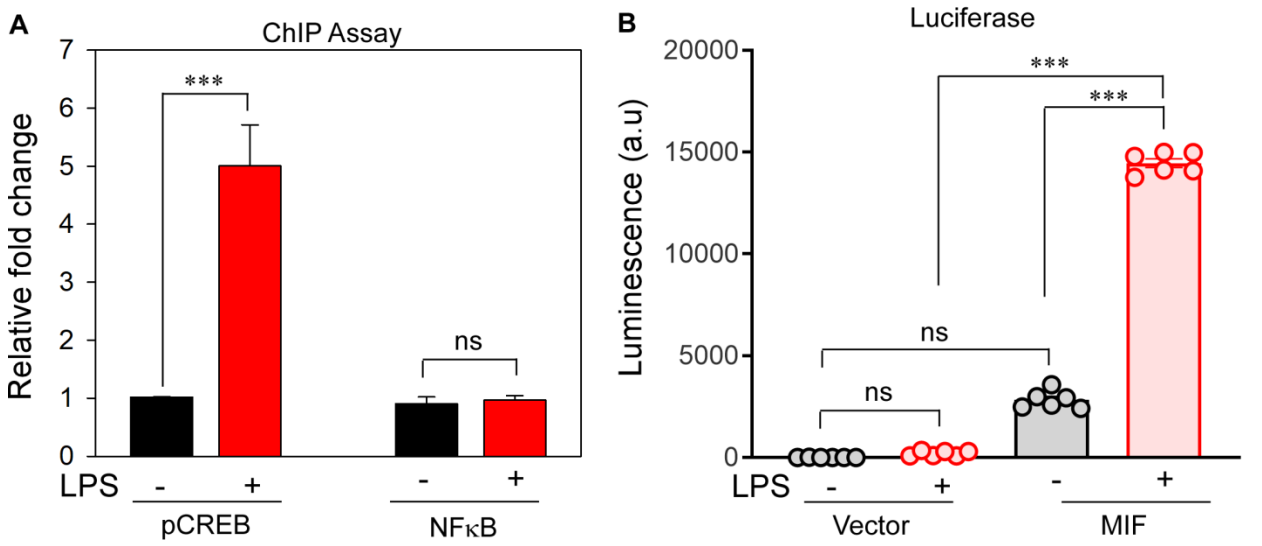

Figure 6.4. LPS-induced binding interaction of pCREB and MIF promoter region upregulates the MIF expression. A) LPS enhanced activation of transcription factor CREB bound to the CRE promoter region of MIF promoter. B) Activated CREB positively regulates the MIF gene promoter activity leading to MIF expression. n = 3 experiments, ***P<0.001. ns - no significance.

6.2.5. CREB, a positive regulator of LPS induced MIF expression

To establish that pCREB contributes to the enhanced MIF expression of LPS induced cells, THP-1 cells were transfected with MIF promoter construct and a luciferase assay was performed. The cells transfected with the MIF gene promoter construct exhibit a 3-fold increased luciferase activity in the presence of leptospiral LPS than the untreated cells. There was no difference in the luciferase activity of control cells. This result strongly suggests that LPS-induced activation of CREB as a transcription factor positively regulates the MIF gene expression **(Figure 6.4 B)**.

6.2.6. ORAI1 knockdown downregulates the CREB activation and MIF gene expression

To further analyze the significance of LPS mediated SOCE in the upregulation of MIF expression via CREB activation, we measured the MIF expression and CREB phosphorylation in ORAI1 KD THP-1 cells treated with or without LPS. **Figure 6.5A and B** evidenced that ORAI1 siRNA transfection significantly ($p \leq 0.01$) downregulates about 70% of ORAI1 expression and

a suitable KD model was constructed. As per **Figure 6.5C and D**, our results sufficiently demonstrated that loss of ORAI1 significantly ($p \leq 0.01$) affects the expression level of MIF in LPS induced THP-1 cells when compared with SCR-siRNA transfected cells. Consistent with downregulated MIF expression, LPS-mediated phosphorylation of CREB was significantly reduced in ORAI1 KD THP-1 cells upon LPS stimulation. Thus, the data sufficiently demonstrated that leptospiral LPS-mediated SOCE contributes to the CREB activation and MIF gene expression.

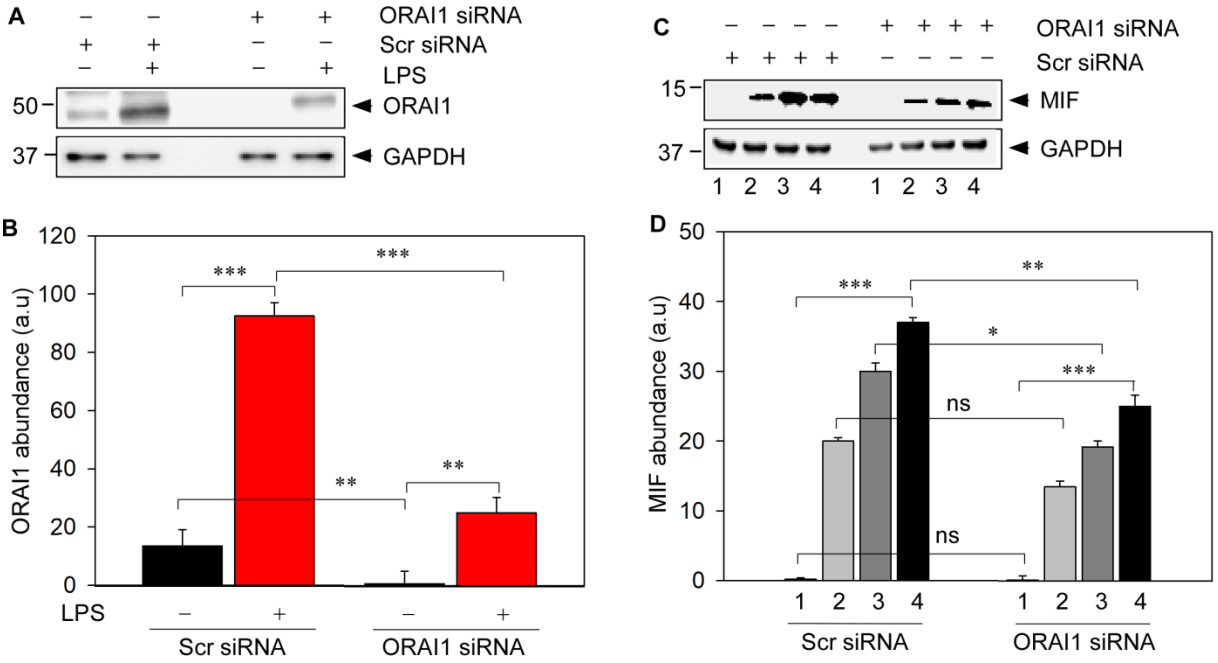

Figure 6.5. Leptospiral LPS-mediated intracellular Ca^{2+} elevation upregulates MIF expression. A) Representative Western blot analysis showed the ORAI1 knockdown. B**)** Quantification of ORAI1 abundance by densitometric measurement from A. C) Representative Western blot analysis showed decreased MIF expression proportional to ORAI1 KD. D) Quantification of MIF abundance by densitometric measurement from C. n = 3 experiments. *P<0.05; **P<0.01; ***P<0.001. ns - no significant

6.3. Discussion

LPS is well known to be the predominant inflammatory stimuli of immune cell activation especially macrophages to overexpress and release MIF (Jovanovik-Krivokuća *et al*., 2016; Lang

et al., 2018; Kudrin *et al*., 2006), however, the mechanism of LPS induced MIF expression was not yet clear. To the best of our knowledge, for the first time, the present investigation proved that LPS-mediated SOCE and calcium signaling is necessary for the transcriptional activation of MIF expression. Leptospiral LPS initiates the aggregation of intracellular signaling proteins to produce cytokines (Park and Lee, 2013). LPS-mediated MIF exhibits an autocrine effect by increasing the protein expression of the signal-transducing receptor molecule of LPS, TLR4 to enhance the host responses to LPS (Roger *et al*., 2003). Klasen *et al*., (2018) described that B lymphocytes secrete the MIF by inducing the upregulation of mRNA and protein expression of the cognate receptor of MIF, CD74 in response to LPS stimulation in a time and dose-dependent manner. The signaling of the MIF/CD74 complex exerts several functions including B cell proliferation and inflammation post LPS exposure.

Most studies speculated that bacterial challenging induced $[Ca^{2+}]_i$ influx, followed by calcium signaling regulated several genes e of inflammatory importance based on the type of content of LPS (Yang *et al*., 2012, Papaioannou et al., 2016). In THP-1 cells, the calcium signaling exhibits the increased release of pro-inflammatory cytokine and other molecules such as interferon, nitric oxide (NO), and inducible nitric oxide synthase (iNOS) (Azenabor *et al*., 2009). Our results revealed that leptospiral LPS stimulation in the human monocytic cell, THP-1 exerts the significant changes in $[Ca^{2+}]_i$ dynamics in a time-dependent manner. In human umbilical vein endothelial cells, LPS-induced overload of $[Ca^{2+}]_i$ causes endothelial cell injury and apoptosis. The calcium influx in HUVECs was regulated by Bruton's tyrosine kinase (Btk)/ Phospholipase C (PLC)γ/ inositol 1,4,5-triphosphate receptor (IP_3R) cascade. Therefore, Btk serves as a mediator of calcium signaling via ORAI1 induced SOCE in LPS stimulated HUVECs (Qiu *et al*., 2021). Apart from LPS, several inflammatory stimuli exert the $[Ca^{2+}]_i$ overload in inflammatory

cell types. In THP-1 cells, lysophosphatidylcholine (LPC) enhanced the cytosolic and mitochondrial Ca^{2+} influx by activating the transient receptor potential Ankyrin 1 (TRPA1) which leads to mitochondrial ROS generation, depolarization of the mitochondrial membrane, cytokine secretion, and cell toxicity and injury (Tian *et al*., 2020).

We found that leptospiral LPS enhances the intracellular calcium influx by SOCs protein (IP3R/ORAI1/STIM1) activation. The upregulation of SOCs protein expression in response to leptospiral LPS was achieved in a time-dependent manner. The IP3R activated calcium depletion in ER and the resulting intracellular calcium influx was observed in LPS treated THP-1 cells. As STIM1 and ORAI1 were identified as calcium sensors and crucial components of calcium release-activated calcium channel (CRAC) respectively, they are considered the two major proteins that activate intracellular calcium entry (Giachini *et al*., 2009). Previous studies revealed that the STIM1 and ORAI1 together are sufficient for the functional activity of SOCs protein. Apart from this, STIM and ORAI proteins are also contributed to non-SOCE $[Ca^{2+}]_i$ influx (Giachini *et al*., 2009, Yang *et al*., 2012).

$[Ca^{2+}]_i$ influx activates the predominant calcium-regulated transcription factor, CREB which controls the expression of numerous genes, and transcription factor EB (TFEB) which regulates the gene expression of cytokines and chemokines. Shanmughapriya *et al*., (2015) found that SOCE activated CREB transcription factor readily binds to mitochondrial Ca^{2+} uniporter (MCU) promoter and upregulates the MCU gene expression to enhance the mitochondrial calcium uptake.

We revealed that LPS stimulated SOCE enhanced the activity of transcription factor of MIF, and CREB via the phosphorylation of CAMKII. The serine residue (Ser^{133}) of CREB, as a major phosphorylation site of CAM kinases (CAMKs), makes CREB an active substrate for CAMKs.

CAMKs are activated by the increased $[Ca^{2+}]_i$ influx (Sheng *et al*., 1991). Some studies found that CAMKIV has a more potential CREB activating ability than CAMKII, however, CAMKIV can only phosphorylate at Ser^{133}, but CAMKII phosphorylates at both sites Ser^{133} and Ser^{142}. Studies have shown that increased intracellular calcium influx activated CAMKs phosphorylate the CREB, which leads to the activation of inflammatory cytokines and lymphokines expressing genes (Sun *et al*., 1994, Yan *et al*., 2016). Consistent with the previous investigations, the present study revealed that $[Ca^{2+}]_i$ influx mediated CREB activation transcriptionally regulates the MIF gene expression during leptospiral LPS exposure.

For the past two decades, the growing evidence on MIF gene upregulation in most the inflammatory diseases are progressing (Gunther *et al*., 2019, Stosic-Grujicic *et al*., 2009). However, the exact molecular regulatory mechanisms involved in MIF gene expression are not well understood. Our study suggested that the knockdown of the ORAI1 channel down-regulates the CREB activation and MIF expression. CREB is one of the major calcium-regulated transcription factors of the MIF gene. Roger *et al*., (2007) demonstrated that exposure to microbial products with THP-1 cells upregulates the transcription factors of MIF, Sp1, and CREB, which overexpresses the MIF gene expression to carry out the innate immune response. Yao *et al*., (2016) found that transcription factor ICBP90 (also known as ubiquitin-like containing PHD ring finger 1 [UHRF1]) was essential for the TLR regulated MIF expression in lymphocytes, monocytes, macrophages, and synovial fibroblasts. ICBP90 exerts the binding interaction with the MIF microsatellite at the site of -794 CATT5-8 and regulates the MIF expression. Altogether, leptospiral LPS mediated elevated $[Ca^{2+}]_i$ influx activates the CREB by CAMKII, followed by pCREB upregulates the MIF expression, therefore, MIF gene expression is transcriptionally regulated by $[Ca^{2+}]_i$ of immune cells. Thus, the data sufficiently

demonstrated that $[Ca^{2+}]_i$ via IP3R/ORAI1/STIM1 mediated SOCE is essential for pCREB mediated MIF expression.

6.4. Conclusion

Our findings indicated that leptospiral LPS mediated Ca^{2+} entry through store-operated Ca^{2+} channels leads to CREB phosphorylation, suggesting that SOCE contributes to the regulation of MIF gene expression. This astonishing data provide a new pattern of concept to the growing shreds of evidence of the molecular mechanism of leptospirosis pathogenesis and the regulation of MIF gene expression. A large number of studies are needed to understand the significance of MIF contribution to inflammatory diseases.

Chapter IV

7. Demonstrate the role of MIF in the pathogenesis of leptospirosis by developing *in vitro* MIF knockdown and *in vivo* MIF blockade strategy

7.1. Materials and methods

7.1.1. Generation of MIF transient knockdown (KD) model

Transient knockdown of MIF was performed using the ON-TARGETplus SMARTpool siRNA (Dharmacon, USA). THP-1 cells grown in 6-well plates were transfected with MIF siRNA using Lipofectamine RNAiMAX transfection reagent (Thermo Scientific, USA) according to the manufacturer's instructions as described previously. Seventy-two hours post-transfection cells were used for the experiments. Dharmacon Non-targeting siRNA/ Scrambled siRNA (SCR-siRNA) duplex (Horizon, Cambridge, UK) was used as control. The transfected experimental model was subjected to Western blotting as described earlier.

7.1.2. MTT assay

To analyze the impact of knockdown of the MIF gene, the transfected cells were treated with different LPS (1 µg) and the percentage of cell proliferation was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. The MIF siRNA and SCR-siRNA transfected THP-1 cells (NCCS, Pune) were seeded in 96-well tissue culture plates at a cell number of 2 x 10^3 cells / well and treated with leptospiral LPS for 12 h, and the MTT assay was carried out as described previously. The absorbance was read at 490 nm by using a microplate reader (Bio-Rad, Hercules, CA, USA). The cytotoxic inhibitory effect of the MIF knockdown model was calculated by the GraphPad prism version 9.2.0.

7.1.3. Confocal Laser Scanning Microscopy

SCR-siRNA and MIF KD THP-1 cells were grown in a complete medium and plated on a glass slide coated with Cell Tak (Sigma-Aldrich, St.Louis, MO, USA). Cells were cultured for 24 h

until 70% confluent and were then incubated with LPS (1 µg) for 12 h. For cell death measurement, LPS induced SCR-siRNA and MIF siRNA transfected THP-1 cells were stained with propidium iodide (0.5µg/ml) and imaged at 561 nm. Nine additional random fields were chosen for three to four independent experiments and PI-positive cells were quantified. For the analysis of Reactive oxygen species (ROS) production, LPS induced MIF knockdown and control cells were stained with 10 µM of Dihydroethidium (DHE) for 30 mins to detect superoxide anion. Cells were washed and imaged using an excitation of 561 nm and fluorescence intensity was quantified. For the analysis of mitochondrial membrane potential ($\Delta\Psi_m$), LPS induced MIF knockdown and control cells were loaded with Rhodamine 123 and incubated for 30 minutes. The cells were washed twice and resuspended in serum-free medium and imaged at 561 nm and the fluorescence was quantified using ImageJ software.

7.1.4. Cytokine Profiling

Quantitative real-time PCR (qRT-PCR) was performed to confirm the attenuated effects of MIF KD against LPS enhanced inflammatory cytokine expression *in vitro* experimental models. RNA was extracted from LPS treated cell lysates and cDNA was synthesized and qRT-PCR was performed as described previously to demonstrate the expression profiling of TNF-α, IL-1β, IL-4, and IL-10 in MIF and SCR-siRNA transfected cells. The reaction mix (10 µL) was prepared by adding 2X SYBR Green PCR Master Mix, 20 µM of each primer, and 20 ng cDNA). The primer sequences used in this experiment are provided in **Table 5**. The housekeeping gene, GAPDH was used as an internal control.

7.1.5. LD_{50} experimental design

All experimental protocols were approved by the Institutional animal ethical committee (IAEC), Bharathidasan University, Tiruchirappalli (BDU/IAEC/P30/2018). Six to eight weeks-old

BALB/c mice (Immunocompromised) were segregated into 9 groups (N=3) and injected intraperitoneally with *L. interrogans* serovar Autumnalis strain N2 MACS for the determination of the median lethal dose. The range of the inoculum as the injection was 10^8-10^0 leptospires (10-fold dilution) in a final volume of 200µL of PBS. For each dose, 3 mice were infected and monitored at every 12h for clinical outcome and survival until 14 days. Dead animals were dissected; moribund animals and survivors were euthanized to harvest the tissues. Then, the LD_{50} value was calculated.

7.1.6. Inflammatory protein expression analysis

Western blotting was performed as previously described to analyze the expression of MIF and its associated proteins in mice injected with 10^8-10^0 leptospires. The antibodies used for the detection of target proteins were MIF (1:1000, Cell Signaling Technology, ICAM-1 (1:1000, Cell Signaling Technology), VCAM (1:1000, Cell Signaling Technology), p38 MAPK (1:1000, Cell Signaling Technology), p-p38 MAPK (1:1000, Cell Signaling Technology), p44/42 (1:1000, eBioscience), p-p44/42 (1:1000, Cell Signaling Technology), β-actin (1:500, Santa Cruz).

7.1.7. Histopathological analysis

Lung tissues were harvested and fixed in 10% buffered formalin (pH 7) for 24-48 hours based on the diameter of the tissues. Tissues were trimmed for adequate size and orientation. Tissues were dehydrated with increased concentrations of ethanol solutions and cleared the tissues by xylene. The dehydration steps are performed by incubating the tissues as mentioned below: 50% ethanol for 5 min, 70% ethanol for 5 min, 95% ethanol for 5 min, 100% ethanol - 10 minutes for 2 times each, xylene - 10 minutes for 2 times each. The infiltrated tissues were embedded with paraffin wax. Then, the tissue blocks were sectioned at 5µm thickness by Microtome and fixed in microscopic slides. Then the sections were subjected to dewax and rehydration by incubating the

tissues as mentioned below: xylene - 10 minutes for 2 times each, 100% ethanol - 10 minutes for 2 times each, 95% ethanol for 5 minutes, 70% ethanol for 5 minutes, 50% ethanol for 5 minutes, and finally rinse with distilled water. The sections were stained with Hematoxylin for 3 min, weak alkaline solution for 1 min, and eosin counterstain for 45 sec. Then the sections were dehydrated and cleared as mentioned previously and mounted with DPX mount. Finally, Sections were examined by light microscopy at 40X objective. Semi-quantitative lung injury scoring was determined on a scale of 0 to 3 based on the degree of immune cell infiltration, pulmonary hemorrhage, and alveolar congestion.

7.1.8. ISO-1 treatment

Six to eight weeks-old healthy mice of similar age, weight, and sexes were segregated into four groups (n=3). All mice received an initial dose of cyclophosphamide (300 mg/kg of body weight) 24h before treatment. The control group (Group 1) received PBS injections over the course of the experiment. Group II was administered LD50 dose of leptospiral strain intraperitoneally, Group III with PBS+ISO-1 (MIF inhibitor), and Group IV with leptospiral LD50+ISO-1. The mice were injected intraperitoneally with an LD_{50} dose (100 leptospires). Mice were treated with ISO-1 (1mg/kg of body weight; intraperitoneally) or vehicle (3% dimethyl sulfoxide) 30 min before and 6 h after leptospiral injection and then once daily for 3 days. Animals were monitored daily for survival and any signs of lethargy and decreased food and water intake for 5 days. On completion of experiments, mice were euthanized as per IAEC procedures. The excised tissues were collected and stored at -80℃ until use. Western blotting was performed as previously described to analyze the expression of MIF and its associated proteins in this experimental group of animals. The antibodies used for the detection of target proteins were anti-MIF (1:1000, Cell Signaling Technology), TLR-4 (1:500, Biocompare),

ICAM-1 (1:1000, Cell Signaling Technology), anti-VCAM (1:1000, Cell Signaling Technology), anti-pCREB (1:1000, Cell Signaling Technology), anti-CREB (1:1000, Cell Signaling Technology), anti-P38 MAPK (1:1000, Cell Signaling Technology), anti-p-P38 MAPK (1:1000, Cell Signaling Technology), anti-P44/42 (1:1000, eBioscience), anti-p-P44/42 (1:1000, Cell Signaling Technology), anti-β-actin (1:500, Santa Cruz). Histopathological features and cytokine profiling were also evaluated as previously described in this study.

7.1.9. Statistical analysis

Data from triplicate experiments were quantified and expressed as Mean ± SE, n=3. Differences in means among multiple data sets were analyzed using 1-way ANOVA with subsequent Tukey's test for multiple comparisons. The student's t-test was used to determine the significance of relative expression analysis of gene transcript analysis. Kaplan Meier plots were performed by GraphPad Prism version 9.2.0 to quantify the survivability. The difference between the survivability of various groups of animal models was analyzed by a log-rank test. A *P-value* less than 0.05 was considered significant in all analysis. The data were computed either with Graphpad Prism version 9.2.0 or SigmaPlot 11.0 Software.

7.2. Results

7.2.1. MIF knockdown of THP-1 cells

To understand the underlying mechanism of MIF-induced pathogenesis in leptospiral infection, we transiently knocked down MIF in THP-1 cells, and cells transfected with SCR-siRNA were used as control throughout our study. **Figure 7.1A and B** showed the MIF protein expression pattern in MIF knockdown and SCR-siRNA transfected THP-1 cells with leptospiral LPS stimulation. To determine the contribution of MIF in the pathogenesis of leptospirosis, MIF

knockdown THP-1 cells were treated with LPS and investigated the pathological events by compared with SCR-siRNA transfected cells.

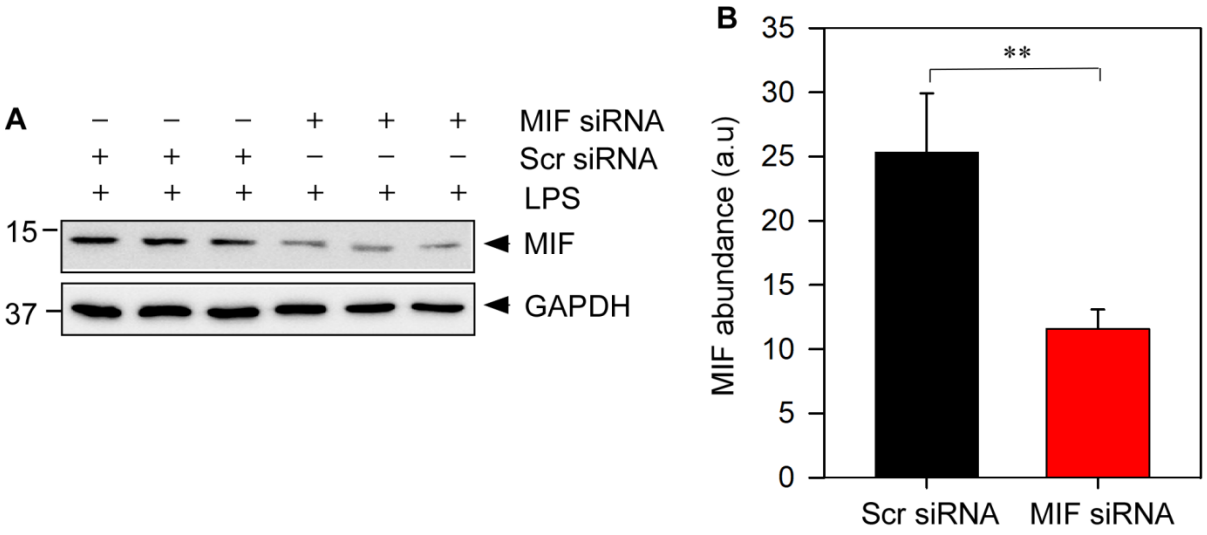

Figure 7.1. Transient knockdown of MIF by siRNA. A) Representative blot showing knockdown of MIF in THP-1 cells. GAPDH was used as a loading control. B) Quantification of MIF abundance by densitometric measurement from A. n = 3 experiments. **P<0.01.

7.2.2. MIF knockdown reduces cytotoxicity and ROS production

To investigate the role of MIF in leptospiral LPS mediated cytotoxicity, an MTT assay was carried out and revealed that *in vitro* MIF knockdown reduced the leptospiral LPS induced cytotoxicity in THP-1 cells, whereas LPS treated wild type cells showed the significantly higher cytotoxicity after 12h of exposure. LPS mediated intracellular ROS stimulated by leptospiral LPS was measured by detecting the fluorescent intensity of Dihydroethidine (DHE) by CLSM. LPS (1µg) stimulation (for 12h) of cells rapidly induced significant ROS in SCR-siRNA transfected cells when compared to MIF knockdown cells **(Figure 7.2A and B)**. Thus, the leptospiral LPS exerts cellular cytotoxicity by inhibiting the metabolic activity and induces oxidative stress by ROS production via activation of NADPH oxidase, whereas *in vitro* MIF knockdown attenuates the elevation of ROS production and cytotoxicity.

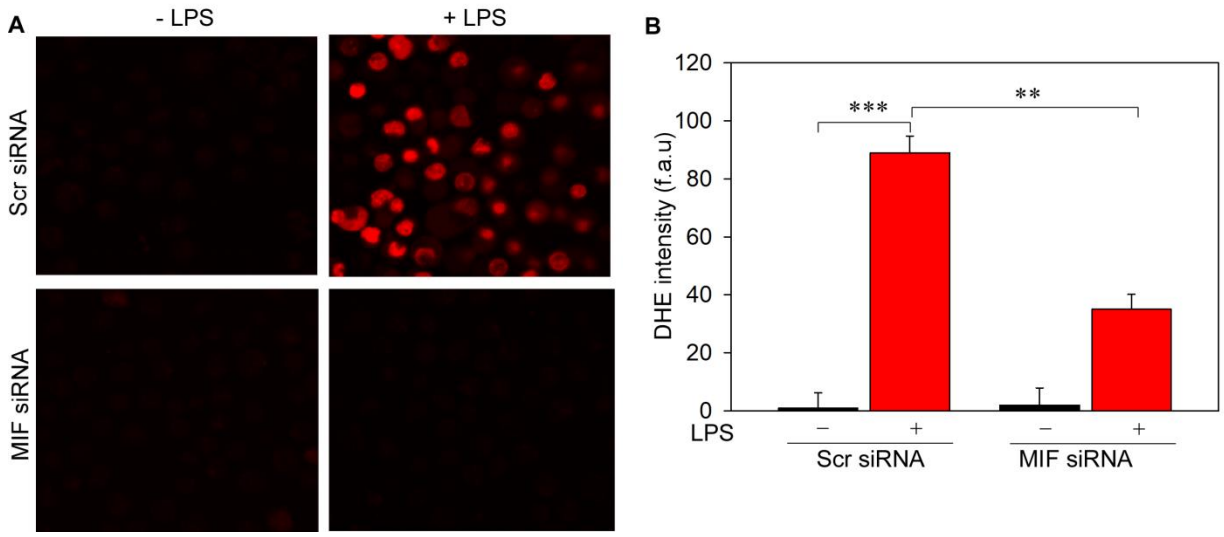

Figure 7.2. Detection of ROS in LPS treated MIF knockdown and control cells by CLSM. MIF knockdown reduced the production of ROS during LPS induction when compared with LPS-induced SCR-siRNA transfected cells. n = 3 experiments. **P<0.01; ***P<0.001.

7.2.3. MIF knockdown reduces cytochrome c release and cell death

To determine the cytochrome c release, we performed membrane fractionation experiments followed by Western blotting in SCR-siRNA and MIF siRNA transfected THP-1 cells treated with or without LPS for 6 h. For this experiment, we collected both mitochondrial and cytosolic fractions of intracellular proteins. In general, cytochrome c resided in mitochondrial space, while during death stimuli it can release from mitochondria to the cytosol by loss of mitochondrial membrane potential leptospiral LPS induced the release of cytochrome c from mitochondrial intermembrane space to the cytosol. In control mice, no significant release of cyt c was observed in cytosolic protein fraction **(Figure 7.3A)**. To analyze the LPS-induced MIF modulated cell death, we stained the SCR-siRNA and MIF siRNA transfected THP-1 cells after exposure to leptospiral LPS with the late-stage apoptotic marker propidium iodide (PI). A significant increase in the number of PI-positive cells was observed after treatment (12h) with LPS which was attenuated in LPS stimulated MIF KD cells **(Figure 7.3B and C)**.

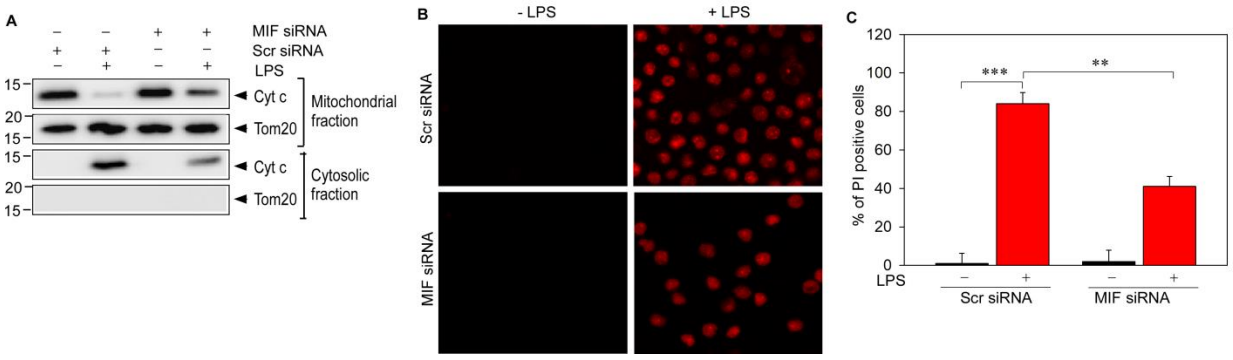

Figure 7.3. Leptospiral LPS induces cytochrome c release and cell death that is attenuated by loss of MIF. A) Representative Western blot for analysis of cytochrome c release by membrane fractionation analysis. The top two strips represent membrane fractions probed with antibodies specific for cytochrome c and Tom20 (loading control), and the bottom two panels represent cytosolic fractions probed with antibodies specific for cytochrome C and Tom20. B) Representative confocal images of SCR-siRNA (top panels) and MIF KD (bottom panels) THP-1 cells stained with PI and stimulated with (right panels) or without LPS (left panels) (1μg/ml) for 6h. C) Quantification of % PI-positive cells. n = 3 experiments. **P<0.01; ***P<0.001.

7.2.4. MIF knockdown attenuates the LPS induced loss of mitochondrial membrane potential ($\Delta\Psi_m$)

To investigate the role of MIF in leptospiral LPS mediated $\Delta\Psi_m$ loss, we performed Rhodamine 123 staining in LPS induced MIF knockdown model. LPS-induced MIF knockdown cells had membrane potential similar to that of SCR-siRNA transfected cells while the LPS induced cells were depolarized significantly (P<0.001). This result indicates that MIF plays a crucial role in mitochondria dysfunction and apoptosis during leptospiral infection **(Figure 7.4A and B)**.

7.2.5. MIF knockdown modifies inflammatory cytokine profile upon LPS stimulation

To verify the role of MIF in LPS-mediated immunomodulation effects, qRT-PCR was performed to record the changes in LPS-mediated inflammatory cytokine profile in MIF knockdown models. The expression patterns showed that MIF knockdown reversed the LPS-induced inflammatory responses of the host. The loss of MIF significantly downregulates the inflammatory cytokine expression, TNF-α, IL-1β, and IL-4 and upregulates the expression of the

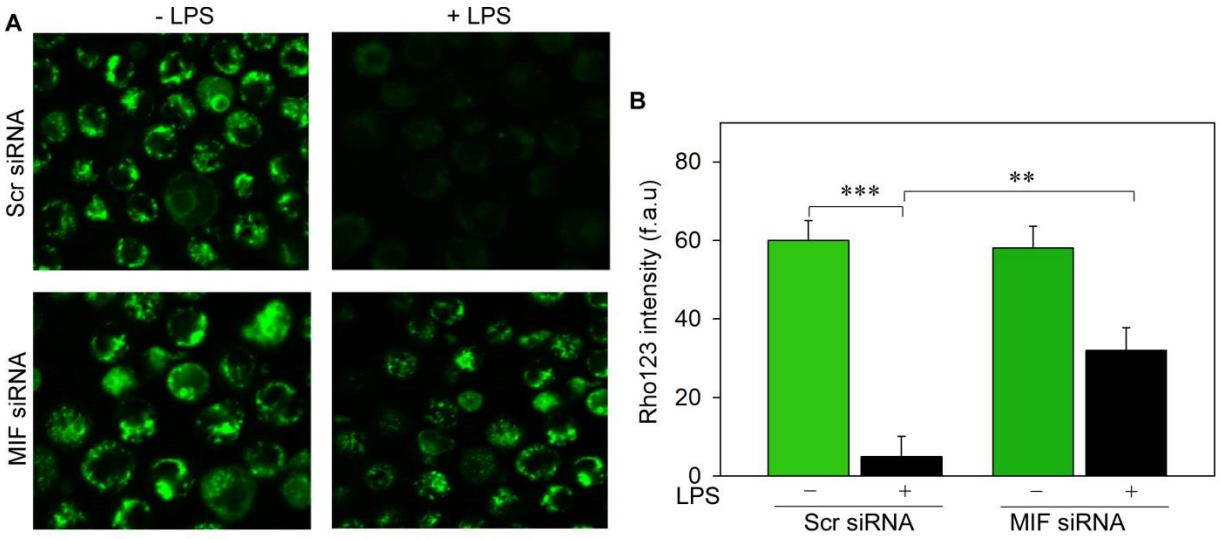

Figure 7.4. Leptospiral LPS induced mitochondrial membrane potential ($\Delta\Psi_m$) loss was reduced by MIF knockdown. MIF siRNA and SCR-siRNA transfected cells were stained with Rhodamine 123 after 12h of LPS treatments showed a significant $\Delta\Psi_{m.}$ B) Quantification of Rhodamine 123 fluorescence intensity. n = 3 experiments. **P<0.01; ***P<0.001.

anti-inflammatory cytokine, IL-10, which contrasted with LPS treated SCR-siRNA transfected control cells **(Figure 7.5)**.

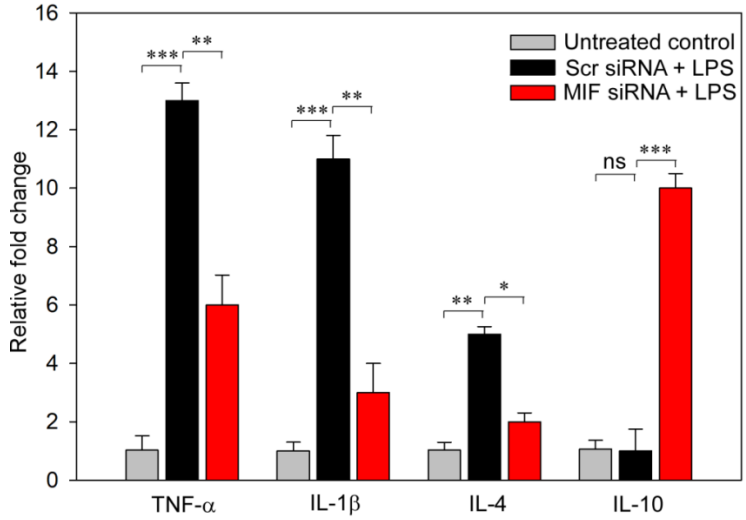

Figure 7.5. Analysis of cytokine profiling in MIF knockdown THP-1 cells. MIF knockdown reduced the expression profile of leptospiral LPS-induced inflammatory cytokine, TNF-α, IL-1β, and IL-4. n = 3 experiments. *P<0.05; **P<0.01; ***P<0.001. ns: non-significant.

The median lethal dose of leptospiral strain was calculated as 100 leptospires in mice. The high dose of leptospires causes earlier death due to its acute toxicity. A survival curve for the experimental design is shown in **(Figure 7.6)**. Fever was a frequently observed symptom in all groups of mice injected with leptospires. Weight loss was also observed in mice injected with 10^5 - 10^1 leptospires. The common behavioral changes observed in infected mice were lethargy and insufficient uptake of feed and water.

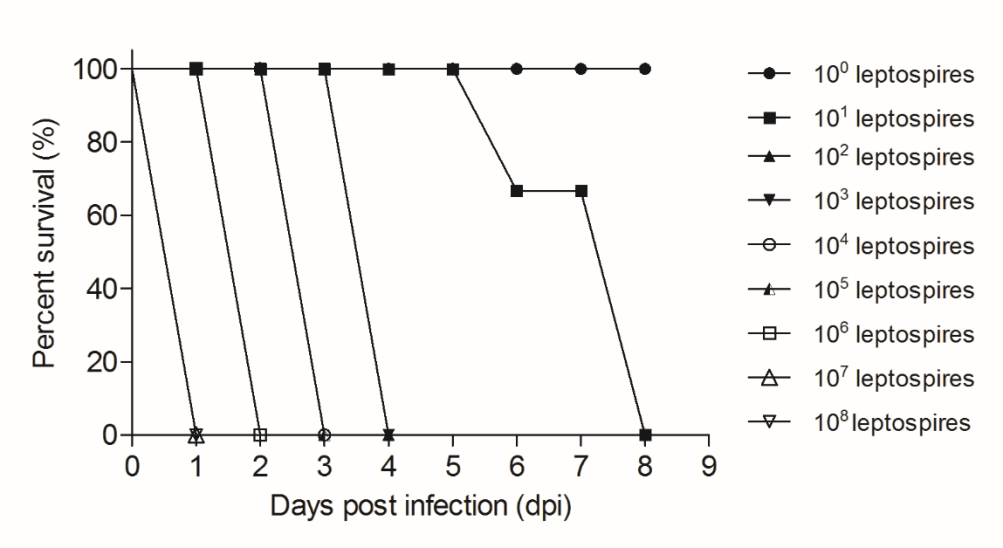

Figure 7.6. LD_{50} dose of leptospires induced lethality of mice. Kaplan Meier survival plots for mice treated with leptospiral starting from 10^0-10^8. 1: 10^1 leptospires, 2: 10^2 leptospires, 3: 10^3 leptospires, 4: 10^4 leptospires, 5: 10^5 leptospires, 6: 10^6 leptospires, 7: 10^7 leptospires, 8: 10^8 leptospires, 9: 10^9 leptospires. n = 3 experiments.

7.2.7. Loss of MIF activity downregulates the expression of inflammatory protein mediators in BALB/c mice

In accordance with *in vitro* analysis that revealed Leptospiral infection induced the MIF expression, here we evaluated the mediators involved in inflammatory pathways that upregulate the MIF expression. As previously evidenced that MIF is a transcriptionally regulated protein and the interrelation of calcium influx and MIF expression, here we analyzed the activation of calcium-regulated transcription factor, CREB, and some protein kinases. For this analysis, we

evaluated the expression profiling of MIF, TLR4, ICAM-1, VCAM, pCREB, CREB, pP38, P38, pP44/42, and P44/42. *In vivo* studies revealed that leptospiral infection enhanced the TLR4, ICAM-1, VCAM, pCREB, pP38, and pP44/42 expression in the LD_{50} of leptospiral dose. The present investigations show that leptospiral infection undergoes enhanced expression of adhesion molecule ICAM-1, p-P38, and p-P44/42 in $\geq 10^2$ leptospires infected mice lung tissues **(Figure 7.7A),** and inhibition of MIF activity significantly attenuates the expression of protein mediators of inflammatory pathways **(Figure 7.7B).**

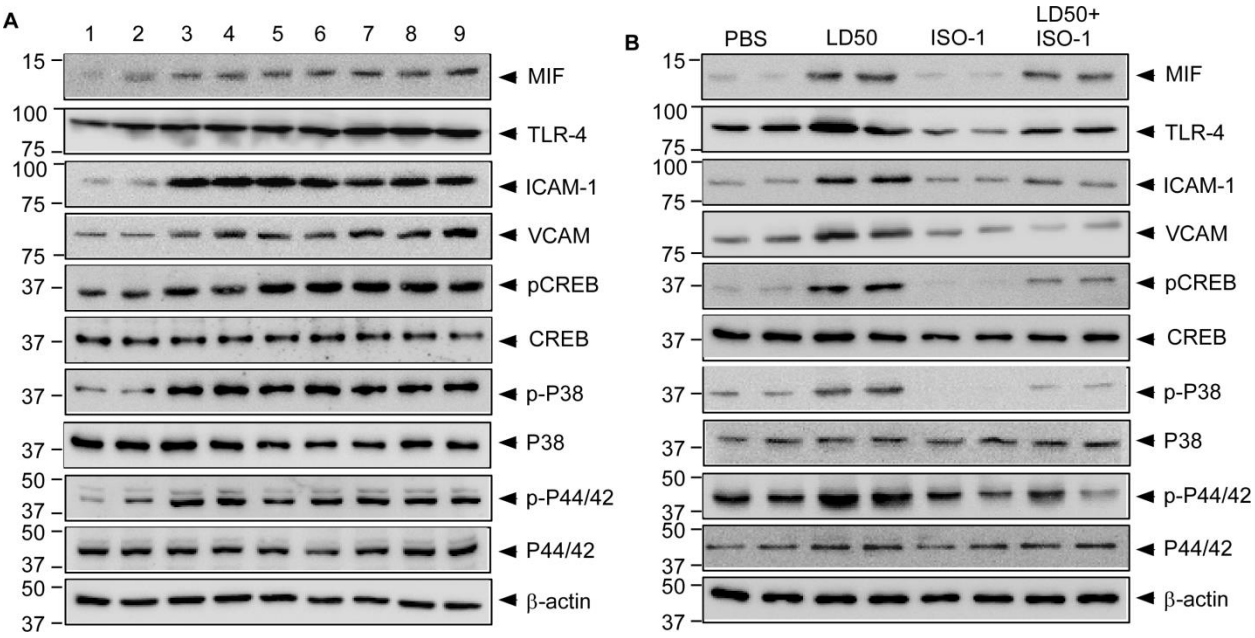

Figure 7.7. Inhibition of MIF attenuates the LPS-mediated overexpression of inflammatory mediators. A) Representative western blotting image of MIF, TLR4, ICAM-1, VCAM, pCREB, CREB, p-P38, P38, p-P44/42, P44/42, and β-actin. B) Representative western blotting image of MIF, TLR4, ICAM-1, VCAM, pCREB, CREB, p-P38, P38, p-P44/42, P44/42, and β-actin. n = 3 experiments.

7.2.8. ISO-1 ameliorates the histopathological changes of lung tissue in leptospires infected mice

The present histopathological report found that *L. interrogans* serovar Autumnalis strain N2 (LD50) causes severe lung injury in mice in a dose dependent manner. Histological pieces of

evidence of lung injury in *Leptospira* infected mice tissues were evaluated by multiple histological features,

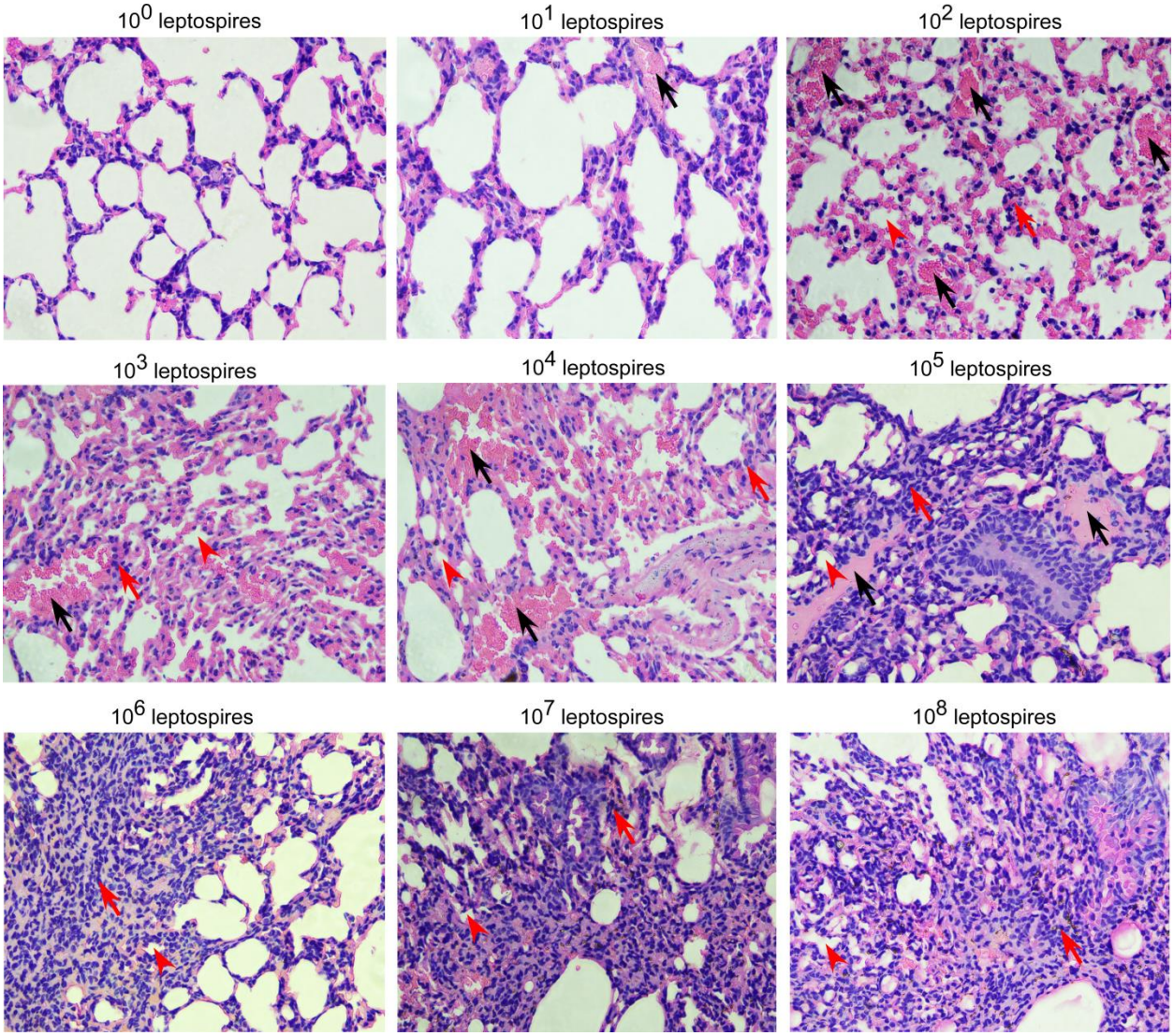

Figure 7.8. MIF antagonist reduced the leptospires (LD_{50}) and mediates histopathological alterations. Histopathological features in leptospiral infected lung tissues. Light microscopic observation of H & E staining tissues. Histological alterations including immune cell infiltration (indicated by red arrow), and hemorrhage (Black arrow) were predominant in $\geq 10^2$ leptospires injected mice and alveolar congestion (Red arrowheads) was in $\geq 10^3$. N= 3 experiments.

such as immune cell infiltration, hemorrhage, and congestion of alveolar septa. Immune cell infiltration was characterized by the entry of polymorphonuclear leukocytes such as neutrophils, eosinophils, and basophils into the alveolar space. The sequestration of erythrocytes in both

alveolar space and septa indicates intra-alveolar hemorrhage. Mild to the severe collapse of alveolar septa pointed out the alveolar congestion. The immune cell infiltration in the alveoli of the lung causes excessive inflammation and tissue damage. The entry of leptospires into the lungs affects the alveolar septal leading to the thickening and collapse of septa.

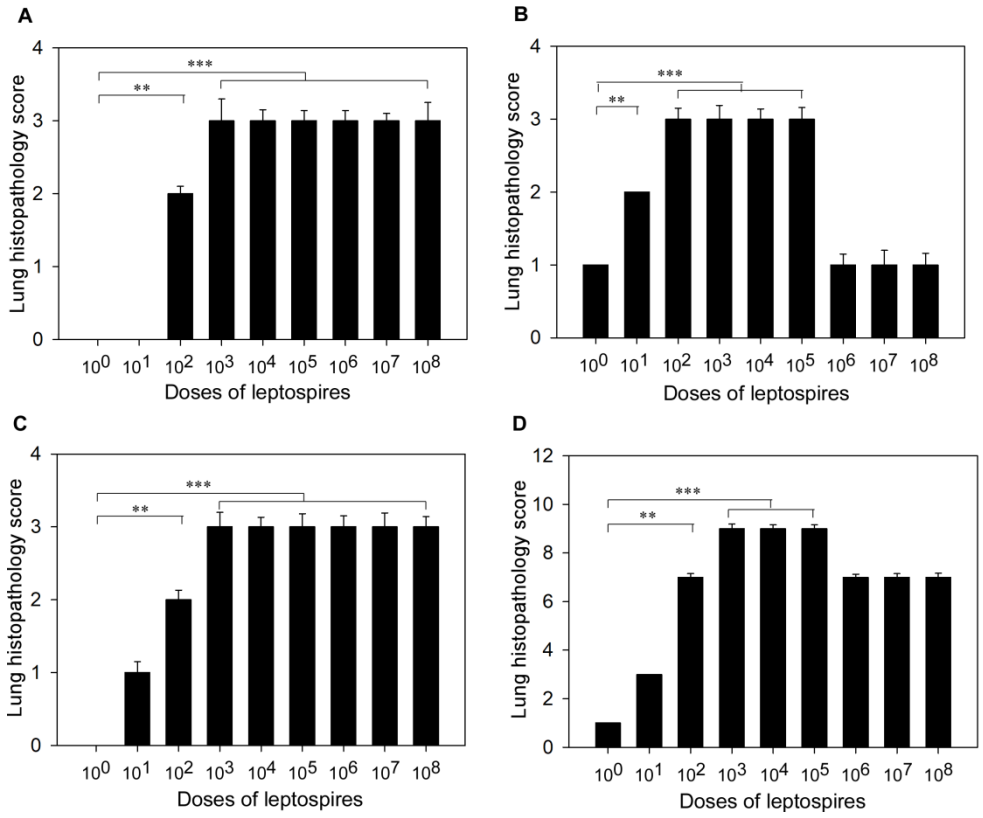

Figure 7.9. Leptospiral LPS mediated lung injury score in BALB/c mice. Semi-quantitative lung injury score of several histological features including Immune cell infiltration A), Hemorrhage B), and Alveolar congestion C). D) Overall lung injury score. Score 0: No features observed, Score 1: Mild, Score 2: Moderate, Score 3: Severe. n = 3 experiments. **P<0.01; ***P<0.001.

The accumulation of red blood cells is due to the damage to blood vessels and it causes both alveolar and septal hemorrhage. In contrast to PBS injected control mice, histopathological signs were significantly increased in leptospires injected mice, which were comparatively inhibited by the administration of ISO-1. By light microscopy, Immune cell infiltration and hemorrhage were

highly noticed in $\geq 10^2$ leptospires infected mice tissues and alveolar congestion was predominant in $\geq 10^3$ leptospires infected mice tissues.

The lungs of control mice showed no pathological evidence but usually few alveolar macrophages are present in the septa **(Figure 7.8)**. The overall histological scoring of the infected lung was 9 **(Figure 7.9A-D).** Next, we examined the effect of the MIF inhibitor, ISO-1 on the leptospires (LD50) caused lung injury. ISO-1 significantly alleviated the histological alterations in lung sections of leptospires infected mice by exhibiting significantly fewer histopathological signs. Inflammatory cell infiltration and alveolar wall thickening in lung tissues were markedly decreased in leptospires infected mice group following administration of MIF inhibitor.

The overall histopathological score of lung injury was obviously reduced in the ISO-1 administered infected group than leptospires alone infected group. This data provided strong evidence for the participation of MIF in lung injury during leptospiral infection **(Figure 7.10A)**. The lung injury scoring system was scaled as 0,1,2,3 based on the degree of histopathological features including normal, mild, moderate, and severe respectively (**Figure 7.10B).**

7.2.9. Inhibition of MIF downregulates inflammatory cytokines *in vivo*

Because we observed increased MIF expression in leptospires treated mice, we then asked whether increased MIF impacts the inflammatory cytokines profile during leptospiral infection. We measured TNF-α, IL-1β, IL-4, and IL-10 gene expression in leptospires infected mice with the dose from 10^0 to 10^8. Leptospiral stimulation and MIF induction significantly increased TNF-α, IL-1β, and IL-4 mRNA levels in BALB/c mice, whereas IL-10 was observed to be downregulated in a dose-dependent manner **(Figure 7.11A-D)**.

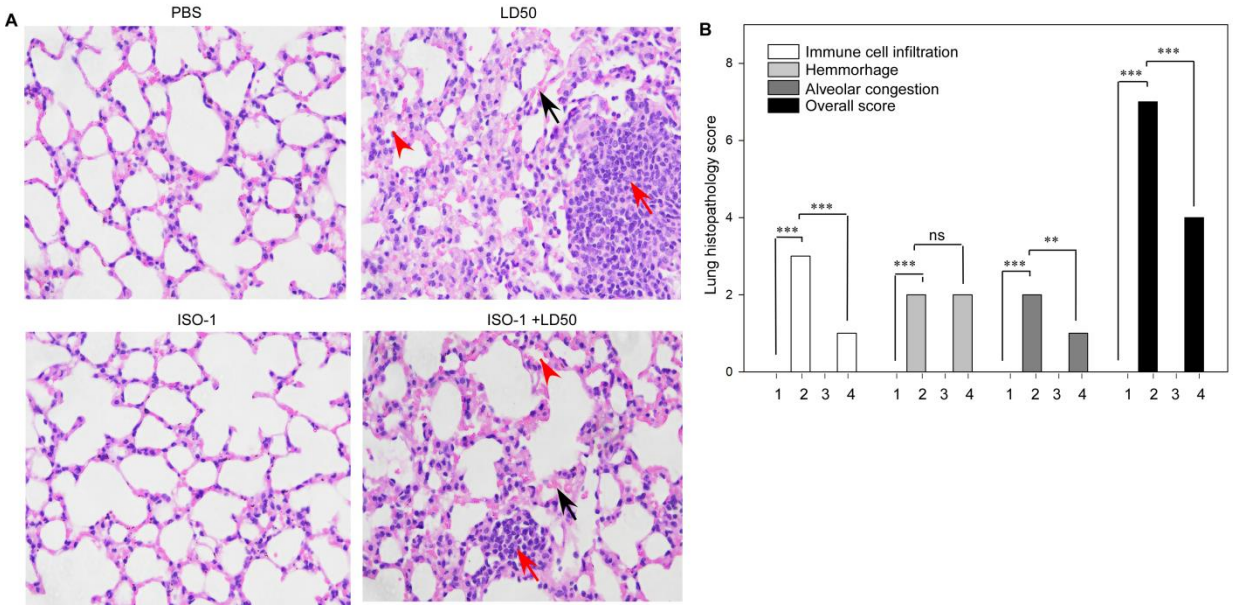

Figure 7.10. MIF inactivation reduced the LPS stimulated histopathological alterations. A) Light microscopic observation of H & E staining tissues. Histopathological features are abundant in LD_{50} dose of leptospires injected mice whereas ISO-1 significantly decreased the histopathological changes in mice lung tissues. B) Semi-quantitative lung injury score of several histological features including Immune cell infiltration, Hemorrhage, Alveolar congestion, and the overall lung injury score. 1: Mice injected with PBS as control, 2: Mice injected with *Leptospira interrogans* serovar Autumnalis strain N2 MACS (LD_{50}), 3: Mice injected with ISO-1 alone, 4: Mice injected with ISO-1 and LD50 of leptospires. Score 0: No features observed, Score 1: Mild, Score 2: Moderate, Score 3: Severe. n = 3 experiments. **P<0.01; ***P<0.001.

Additionally, inhibiting MIF by ISO-1 increased IL-10 mRNA levels approximately two-fold. The increased IL-10 mRNA levels significantly decreased the pro-inflammatory cytokine TNF-α mRNA levels. Thus, we hypothesized blockade of MIF to be protective during leptospiral infection through upregulation of IL-10 expression (**Figure 7.12A**).

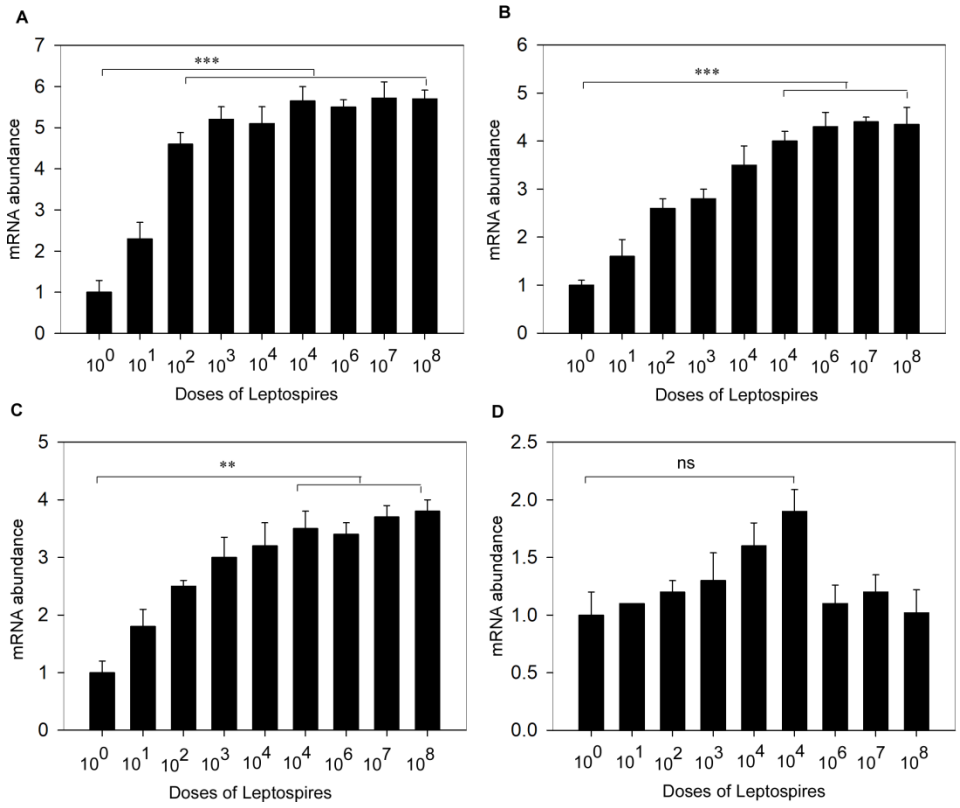

Figure 7.11. Leptospiral infection induces the expression of inflammatory cytokines expression. Quantification of mRNA abundance of TNF-α (A), IL-1β (B), IL-4 (C), and IL-10 (D) in mice injected with leptospiral dose starting from 10^0-10^8. n = 3 experiments. **P<0.01; ***P<0.001. ns = no significance.

7.2.10. Blockade of MIF prevents leptospires induced lethality in mouse models

Due to our observation that overexpression of MIF and MIF mediated inflammatory cytokine in leptospires injected mice, we next chose to investigate the contribution of MIF to lethality rate. The survivability of mice with different doses of leptospires was analyzed. The survival rate of mice was decreased gradually due to the increased dose of leptospires (10^0 to 10^8) in mice **(Figure 7.6)**.

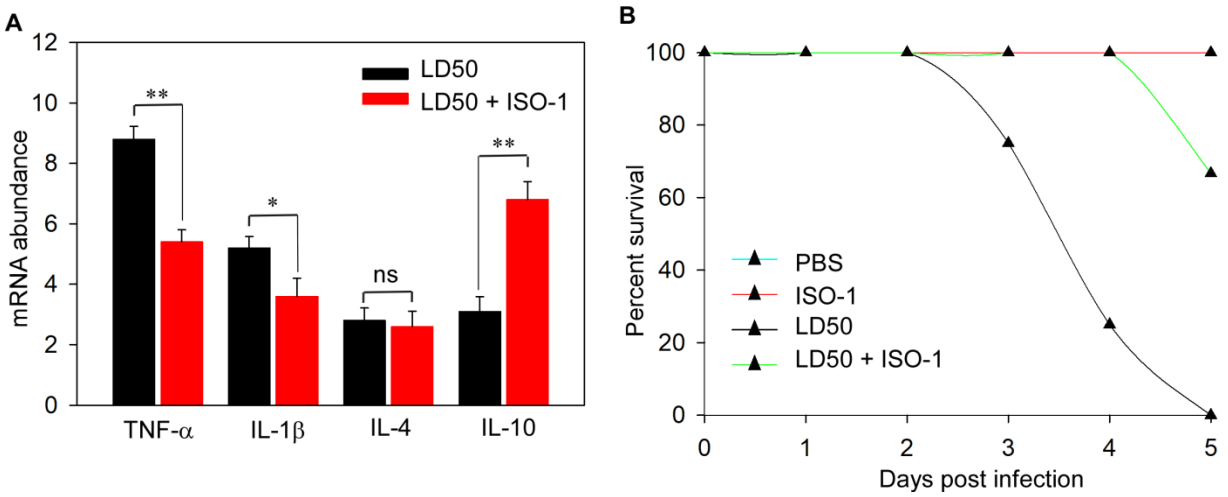

Figure 7.12. Inhibition of MIF attenuates the pathogenic effects of leptospiral infection *in vivo*. MIF inhibition protects the mice from lethality and downregulates the expression of proinflammatory cytokine and other inflammatory mediators. A) Quantification of mRNA abundance of inflammatory cytokines including TNF-α, IL-1β, IL-4, and IL-10 in leptospires treated mice with or without ISO-1 injection. B) Kaplan Meier survival plot for mice treated with PBS, ISO-1 (1mg/kg body weight of mice), LD50 of leptospires, LD50 with ISO-1. n = 3 experiments. *P<0.05; **P<0.01; ***P<0.001.

To determine the promotional role of MIF in the lethality of mice, inactivation of MIF was carried out by ISO-1 treatment and analyzed the survivability of mice. Groups I, II, III, and IV have 100%, 25%, 100%, and 66% of survivability, respectively. As per our results, the Blockade of MIF with ISO-1 prevented the leptospiral infection-induced lethality in group IV mice by increasing the survivability from 25% to 55%. Because we observe a preserved survivability among ISO-1-treated animals, MIF-mediated leptospiral pathogenesis is very evident **(Figure 7.12B)**.

7.3. Discussion

In this investigation, we explored the potential role of MIF in the pathogenesis of leptospirosis by using *in vitro* MIF knockdown THP-1 model and *in vivo* MIF inhibited mice model. *In vitro* experimental models showed significantly higher cytotoxicity, mitochondrial membrane

potential loss, increased ROS production, cytochrome c release, and cell death. The virulence factors of leptospires including lipopolysaccharide, glycolipoprotein, hemolysin sphingomyelinase, and phospholipases exhibit the hallmarks of cytotoxic, hemolytic, and apoptotic activity such as elevated inflammatory cytokine expression, cell membrane damage, leakage of LDH, chromatin condensation, increased production of ROS and loss of mitochondrial membrane potential (Isogai *et al*., 1998, Lee *et al*., 2002,). But the exact mechanism of pathogenesis and the reason for disease heterogeneity were not yet well understood. Leptospirosis has been reviewed as an invasive infectious disease and systemic inflammatory response syndrome. The host immune system triggers the inflammatory responses such as increased production and release of inflammatory cytokines during leptospiral infection to get rid of the pathogen. But the elevated inflammatory responses stimulate the destructive immune mechanisms which contribute to the tissue lesions and damage (Che *et al*., 2019).

Over the decades, MIF has been considered one of the captivating inflammatory cytokines that acts as a key mediator of the pathogenesis of several inflammatory diseases. The autocrine activity, isomeric enzymatic activity, and pro-inflammatory potential of MIF drive the disease progression and severity. Bozza *et al*., (1999) illustrated that MIF triggered the bacterial LPS mediated lethality, neutrophil infiltration, increased inflammatory cytokine expression, TNF-α, IL-6, IL-12, and higher nitric oxide production, as well as MIF counter, regulates the activity of anti-inflammatory dexamethasone. $MIF^{-/-}$ mice showed diminished endotoxic effects in the septic shock experimental model. The higher level of serum or tissue MIF levels is involved in several respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis, cystic fibrosis, and pulmonary hypertension. Astonishingly, recent studies proved that MIF antagonists, ISO-1 significantly decreased the immune cell infiltration, vascular

leakage, and migration of endothelial progenitor cells (Le Hiress *et al*., 2015, Zis *et al*., 2015). A pre-clinical study on experimental autoimmune encephalomyelitis (EAE) demonstrated that MIF enhances the inflammatory demyelination, whereas the MIF and/or its homolog D-DT knockout mice displayed the reduced stimulation and progression of EAE (Benedek *et al*., 2017).

Our study suggested that the LD50 of *Leptospira interrogans* serovar Autumnalis strain N2 was 100 leptospires in 6-week old mice model. Although mice were resistant to developing a leptospiral infection, the immunocompromised mice infected with mouse-adapted challenge strain were suitable to develop the sublethal leptospiral infection. Indeed, the mice model has proven efficient to illustrate the interaction between host immunity and pathogen, studying the responses of the host immune system to leptospires, and exploring the contribution of TLR-4 in *Leptospira* mediated pathogenic mechanisms and the consequent pathophysiology of inflammation (Gomes-Solecki *et al*., 2017). MIF inactivation by antagonists (ISO-1) improves the survival rate and decreases the disease progression and severity of infected mice (Al-Abed and VanPatten, 2020). In the present investigation, we found that leptospiral infection enhanced the apoptotic cell death, mitochondrial membrane damage, expression of inflammatory mediators which regulate the pathogenesis of diseases such as adhesion molecules, ICAM and VCAM, protein kinases, P38 and P44/42, and overexpression of inflammatory cytokines, TNF-α, IL-1β, and IL-4. MIF knockdown and MIF inactivation reduced the pathophysiological effects of experimentally induced leptospirosis.

Chuang *et al*. (2011) described that MIF plays a pathogenic role in dengue haemorrhagic fever (DHF) and MIF knockdown significantly reduced the vascular permeability of human endothelial cell line (HMEC-1) by decreasing the production of cytokines and soluble inflammatory mediators such as interleukins, monocyte chemoattractant protein -1 (MCP-1) and

matrix metalloproteinase (MMP). MIF knockout mice challenged with LPS attenuate the endotoxin stimulated cardiac dysfunction by reducing the activation of inflammatory mediators, p-JNK (c-Jun N-terminal kinase), p-ERK (Extracellular signal-regulated kinase), and endoplasmic reticulum stress marker IRE1a (inositol- requiring enzyme 1 alpha), Gadd (growth arrest- and DNA damage-inducible gene) 153, Grp (glucose regulated protein) 78, and eIF2a (Eukaryotic translation initiation factor 2a) (Zhang *et al*., 2019). Consistent with the mice model, MIF shRNA transfected knockdown HUVECs showed the downregulated expression of p-JNK and p-ERK. JNK, ERK, and P38 are the most important family members of MAPK (mitogen-activated protein kinase) (Zhang *et al*., 2018). Our investigations clearly indicated that the elevated inflammatory responses cause immune cell infiltration, haemorrhage, and tissue damage in *Leptospira* injected mice, which was significantly reduced in ISO-1 injected leptospirosis mice model.

As MIF is a multifaceted immunomodulatory cytokine, it exerts several fundamental physiological processes of cells. Therefore, the disruption of the MIF gene by knockout can cause apoptosis and neuronal loss in some cell types (Zhang *et al*., 2014). MIF homolog, D-DT (MIF-2) acts as MIF in inflammation and the mechanism of disease progression. Ochi *et al*., (2017) investigated the contribution of D-DT in ischemia/–reperfusion-induced kidney injury in a murine model and found the functional similarity in diseases. Pasupuleti *et al*., (2014) demonstrated that DD-T and MIF displayed a significant functional overlap in the regulation of cell survival, tumorigenesis, and migration of tumor cells and endothelial cells. The dual inhibition provides the potential inhibition of MIF signaling.

MIF drives the inflammatory disease progression and leads to disease severity and a high fatality rate. Our study revealed that MIF inhibition by ISO-1 protects the mice from *Leptospira*-

mediated lethality. Acute pancreatitis (AP) induced $MIF^{-/-}$ mice showed a higher percentage of survival rate till 72h post-induction and significantly diminished inflammatory responses and pancreatic tissue damage (Zhu *et al*., 2020). $MIF^{-/-}$ macrophages have protected from the LPS-induced apoptosis by reducing the nitric oxide stimulated p53 accumulation, arachidonic acid metabolism, and diminished pro-inflammatory responses (Mitchell *et al*., 2002). MIF inhibitor, ISO-1 treated mice also repaired the effects of induced acute pancreatitis. MIF inhibitors have specifically bound to the catalytic site and inhibit the tautomerase activity of mice and inhibit the cytokine proinflammatory activities (Stosic-Grujicic *et al*., 2009, Cheng *et al*., 2009). Our data sufficiently evidenced that MIF plays a pivotal role in pathogenic events of leptospirosis.

7.4. Conclusion

The LD_{50} of *L. interrogans* serovar Autumnalis strain N2 was calculated as 100 leptospires in mice. The median lethal dose of leptospiral infection causes severe lung injury in mice in a dose-dependent manner with the significantly higher histological scoring (Score 9) of lung tissues. The histological alterations including immune cell infiltration, hemorrhage, and congestion of alveolar septa were predominant in $\geq 10^3$ leptospires infected mice. The proteins involved in inflammatory pathways (MIF, TLR-4, ICAM-1, VCAM, pCREB, CREB, P38, p-P38, P44/42, p-P44/42) and calcium-dependent CREB and pCREB are significantly expressed in leptospires injected mice in a dose-dependent manner. MIF inhibition enhanced the considerable reduction in histological scoring and inflammatory protein expression in infected lung tissues. Therefore, MIF will be a promising diagnostic marker for leptospirosis.

Chapter V

8. Understand the role of MIF gene polymorphism (-173G/C SNP) in the severity of leptospirosis

8.1. Materials and Methods

8.1.1. Study site, duration, and Study design

An observational case-control study was conducted in the duration of August 2019 to January 2020 at Mahatma Gandhi memorial government hospital, GVN multispecialty hospital, Tiruchirappalli, and Rajiv Gandhi memorial general hospital, Chennai, Tamil Nadu, India. The geographical location of the study area is 10°48′18″ N latitude and 78°41′08″ E longitude and the temperature ranges from 36ºC to 41 ºC. This study was carried out i) to examine the contribution of host MIF gene polymorphisms -794 $CATT_{5-8}$ microsatellites and -173 G/C SNP in the determination of disease susceptibility and severity in leptospirosis patients, and ii) to investigate the correlation between the MIF gene polymorphisms and the elevated MIF gene expression to find the fact behind the heterogeneity of leptospirosis disease outcome. The genotypic and allelic frequencies of MIF gene polymorphisms were compared in leptospirosis and healthy control subjects.

8.1.2. Study subjects

The samples collected by hospital-based surveillance were segregated into three groups; the first group consists of 64 patients with mild and non-hospitalized leptospirosis cases, the second group consists of 22 patients with severe and hospitalized cases of leptospirosis, and the third group consists of 80 healthy control subjects recruited from the population of same geographical settings matched for gender and age (± 5 years). The healthy controls with a history of fever in the last 2 weeks had removed from the study.

8.1.3. MAT assay and IgM ELISA

To confirm the leptospirosis in study subjects, we performed the MAT assay by using a panel of 12 live leptospiral serovars as described previously in the Materials and Methods section 4.1.4. A titer of ≥1:80 and ≥50% of agglutination was considered as seropositivity for MAT assay. To further confirm the leptospiral infection in study subjects, we carried out IgM ELISA as described previously in the Materials and Methods section 4.1.6 by measuring the IgM-based early host immune response to leptospiral infection.

8.1.4. Extraction of genomic DNA

Genomic DNA was extracted from the peripheral blood samples collected from all the study subjects. DNA was extracted by using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) as per the manufacturer's instructions. In brief, the peripheral blood was diluted with PBS to the final volume of 220µL. 20µL of proteinase K and 500µL of AL buffer were added to each sample, mixed well, and incubated at 56°C for 10 minutes. After ethanol precipitation, the lysed blood cells were transferred to the spin column and centrifuged at 8000rpm for 1 minute. Then, washed the membrane with 500µL of AW1 buffer, followed by 500µL of AW2 buffer, and discarded the flow-through. Finally, the DNA was eluted from the column by using a 50µL AE buffer. The extracted DNA samples were stored at -20°C for further use.

8.1.5. Genotyping of MIF gene promoter -173G/C SNP

To determine the functional single nucleotide polymorphism (-173G/C) in the MIF promoter region, we executed Polymerase chain reaction- Restriction fragment length polymorphism (PCR-RFLP) for all DNA samples of study subjects. The reaction mixture containing 50µl of reaction mixture contained approximately 50 ng of purified DNA, 0.1 µM of each Forward and reverse primer, 250 µM of each dNTPs (Thermo Scientific, Waltham, MA, USA), 0.5 U of

Dream Taq DNA polymerase (Thermo Scientific, Waltham, MA, USA), Dream Taq buffer 1X. Amplification was performed in a thermal cycler (Eppendorf, Germany). The polymerase chain reaction conditions are as follows, the cycle of 95°C for 10 min, 35 cycles of 95°C for 45 sec, 62°C for 45 sec, 72°C for 1 min, and 1 cycle of 72°C for 7 min. The details of primers used in this experiment were listed in **Table 8.1**. The amplified product was visualized by 1.5% agarose gel and digested with FastDigest *Alu*I restriction endonuclease enzyme (Thermo Scientific, Waltham, MA, USA) at 37°C for 1 h, followed by incubating at 65°C for 10 minutes for enzyme inactivation. The digested DNA fragments were separated by using 6% Native- Polyacrylamide gel and stained with Ethidium bromide dye. The banding patterns of different genotypes are as follows, genotype GG has 2 bands of 268 and 98bp size, genotype GC has 3 bands of 205, 98, and 63bp size, and genotype CC has 4 bands of 268, 205, 98, and 63bp in size.

Table 8.1. List of primers used in this study

Gene	Primer sequence
Human MIF -173 G/C SNP	FP: 5'-CAGTGCGTGTCGTGGAGT-3'
	RP: 5'-GGGTGAGAACTGAATTCCA-3'
Human MIF	FP: 5'-CGCAGAACCGCTCCTACAG-3'
	RP: 5'-GGAGTTGTTCCAGCCCACAT-3'
Human TNF-α	FP: 5'-AGAGGGAAGAGTTCCCCAGGAC-3'
	RP: 5'-TGAGTCGGTCACCCTTCTCCAG-3'
Human IL-1β	FP: 5'-CCAGCTACGAATCTCGGACCACC-3'
	RP: 5'-TTAGGAAGACACAAATTGCATGGTGAAGTCAGT-3'
Human IL-4	FP: 5'-CTGCAAATCGACACCTATTA-3'
	RP: 5'-GATCGTCTTTAGCCTTTC-3'
Human IL-10	FP: 5'-ATGCCCCAAGCTGAGAACCAAGACCCA-3'
	RP: 5' TCTCAAGGGGCTGGGTCAGCTATCCCA-3'
Human GAPDH	FP: 5'-AACGACCCCTTCATTGAC-3'
	RP: 5'-TCCACGACATACTCAGCA -3'

8.1.6. Quantitative real-time PCR analysis

To analyze of the regulatory role of (-173G/C) polymorphic genotype in MIF gene expression and other inflammatory cytokine expression was determined by performing qRT-PCR analysis as mentioned previously in Materials and Methods section 5.1.7. The details of primer sequences used in this experiment were listed in **Table 8.1.**

8.1.7. MIF ELISA

The serum was separated from the collected peripheral blood samples of all patients and control subjects. To assess the serum MIF levels of study subjects with different MIF -173 (G/C) polymorphic genotypes, MIF ELISA was performed by using a Human MIF ELISA kit (Sigma-Aldrich, St. Louis, Mo, USA) as per the manufacturer's instructions. All the samples were loaded in triplicates.

8.1.8. Statistical analysis

All the triplicate experiments were quantified and expressed as Mean ± Standard Deviation. The cut-off value was calculated by mean ± 2 (Standard deviation) of controls. The data was measured with SigmaPlot 11.0 software. Pearson's Chi-Square test was performed to analyze the Hardy-Weinberg equilibrium for MIF gene polymorphisms. The genotype and allele frequencies were determined by direct counting and the difference between the distribution of genotypes and alleles was calculated by the Chi-Square test. The measurement of odds ratio and 95% confidence interval were used to analyze the risk of MIF polymorphism associated with leptospirosis. Pearson correlation coefficient analysis was performed to estimate the associations between polymorphism and serum MIF. For all tests, a P-value ≤ 0.05 was considered statistically significant.

8.1.9. Ethical compliance

The complete study protocol was approved by the Institutional Ethical Committee (IEC No: DM/2014/101/51) at the Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. All the study subjects signed the informed written consent form after the explanation of the goal of this present study. If study subjects were minors, their surrogates signed the written consent form.

8.2. Results

8.2.1. Baseline features of study subjects

A total of 86 leptospirosis cases and 80 healthy control subjects were evaluated in this study. MAT assay determined the seropositive of leptospirosis with the titre of 1:80 to 1:2560. The highly infecting serovars were Autumnalis (43%), Australis (29%), and Icterohaemorrhagiae (17.5%).

Table 8.2. Demographic, clinical, and serological characteristics of leptospirosis patients and healthy control subjects

Variables	Leptospirosis patients		Healthy controls
	Hospitalized patients	Non- Hospitalized patients	
Sex Female (%) Male (%)	 60 40	 47.4 52.6	 45 55
Age Range (years) Mean (years) ± S.D	 8 - 53 38.6±15.9	 6 - 68 32±16.2	 7 - 65 33.2±15.8
Duration of disease Range (days) Mean (days) ± S.D	 12 - 25 19 ± 4.1	 4 - 10 6.6 ± 1.5	 - -
Serology MAT titres Range IgM ELISA titre	 (1:80 - 1:2560) 1:100	 (1:80 - 1:2560) 1:100	 - -

The mean age of leptospirosis confirmed hospitalized and non-hospitalized cases and healthy control subjects were 38.6±15.9, 32±16.2, and 33.2±15.8 years old, respectively. The gender distribution among leptospirosis cases was 45% female and 55%, male. There was no noteworthy difference was found between the studied groups with respect to age and sex. The mean difference in disease duration between hospitalized and non-hospitalized patients was found to be 12.4. The clinical and demographic characteristics of the study subjects are summarized in **Table 8.2.**

8.2.2. MIF gene polymorphism on leptospirosis susceptibility and severity

To determine the influence of MIF gene polymorphism on leptospirosis susceptibility and severity, genotypic and allelic frequencies of MIF polymorphism, -173G/C SNP were analyzed in leptospirosis cases and healthy controls. For both the leptospirosis and healthy control subjects, the distribution of MIF polymorphisms adopted the Hardy-Weinberg equilibrium (P=1). When we analyzed the distribution of MIF polymorphic genotypes of -173G>C between leptospirosis patients and healthy control groups, we observed that MIF -173G>C SNP had a significant difference in genotype frequency among groups.

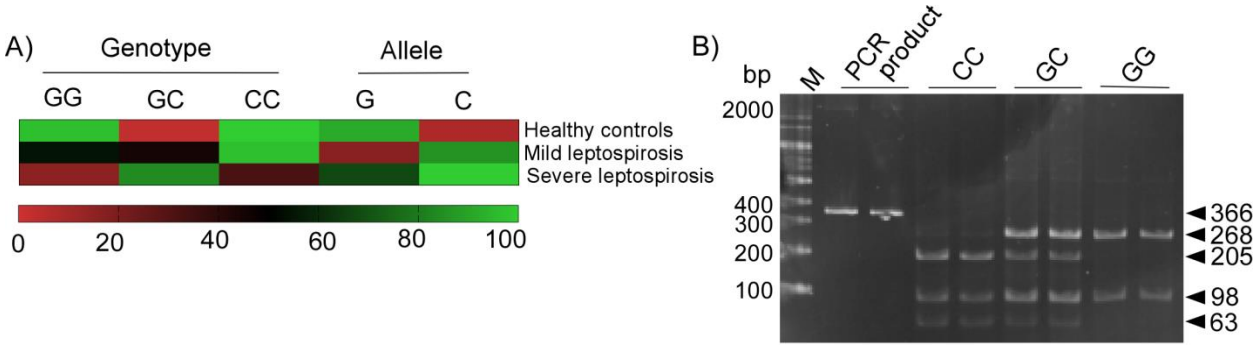

Figure 8.1. Analysis of MIF -173 G/C genotype distributions among study subjects. A) Genotypes (GG, GC, and CC) and alleles (G and C) frequency of MIF -173G/C polymorphism were observed in leptospirosis patients and healthy control subjects. Heatmap showed that C allele-associated genotypes (GC and CC) were highly observed in leptospirosis cases whereas

GG genotype was frequently distributed in healthy controls. B) Representative image of MIF -173 G/C SNP genotype analysis by polyacrylamide gel electrophoresis.

Table 8.3. Distribution of genotype and allele frequency of MIF -173 SNP and association with susceptibility and severity of leptospirosis.

Genotype	Healthy controls n=80, Frequency (n)	Leptospirosis (n = 86)					
		Severe and hospitalized (n = 22)			Mild and non-hospitalized (n = 64)		
		Frequency (n)	OR (95% CI)	P-value	Frequency (n)	OR (95% CI)	P-value
GG	0.92 (74)	0 (0)	0.12 (0.007-2.32)	=0.16	0.14 (9)	0.01 (0.004-0.03)	<0.0001
GC	0.08 (6)	0.32 (7)	0.09 (0.03-0.29)	<0.0001	0.83 (53)	59.42 (20.68-170.7)	<0.0001
CC	0 (0)	0.68 (15)	66.42 (12.5-352.74)	<0.0001	0.03 (2)	6.44 (0.3-136.57)	=0.23
Allele							
G	0.97 (80)	0.16 (7)	0.01 (0.002-0.07)	<0.0001	0.55 (61)	0.15 (0.007-3.29)	=0.23
C	0.03 (6)	0.84 (22)	7.7 (0.42-138)	=0.16	0.45 (55)	75.37 (25.33-224.25)	<0.0001

No deviation from Hardy-Weinberg equilibrium was observed in leptospirosis cases or healthy controls for MIF -173G/C polymorphisms.

The distribution of MIF -173G/C genotype variants GG, GC and CC were 0% (OR: 0.12, 95% CI: 0.007-2.32), 32% (OR: 0.09, 95% CI: 0.003-0.29) and 68% (OR: 66.42, 95% CI: 12.5-352.74) among severe leptospirosis cases and were 14% (OR: 0.01, 95% CI: 0.004-0.03), 83% (OR: 59.42, 95% CI: 20.68-170.7) and 3% (OR: 6.44, 95% CI: 0.3-136.57) in mild leptospirosis while 92%, 8% and 0% in healthy control subjects respectively. GC genotype was more frequently observed in leptospirosis cases than in controls while GG genotype frequency was highly reported in healthy controls. In addition, most the severe leptospirosis cases have a genotype frequency of CC (68%). No GG and CC genotype was found in severe leptospirosis and healthy control subjects, respectively. The frequency of the C allele in severe and mild

leptospirosis patient groups was 84% (OR: 7.7, 95% CI: 0.42-138) and 45% (OR: 75.37, 95% CI: 25.33-224.25) respectively, which is significantly higher than the healthy control subjects (6%). While the frequency of the G allele in severe and mild leptospirosis patient groups was 16% (OR: 0.01, 95% CI: 0.002-0.07) and 55% (OR: 0.15, 95% CI: 0.007-3.29) respectively, which is considerably lower than the healthy control subjects (80%).

With regard to the MIF -173 G/C polymorphism, genotype and allele distributions in leptospirosis and controls are shown in **Table 8.3 and Figure 8.1A**. The representative image of genotype analysis by polyacrylamide gel electrophoresis was presented in **Figure 8.1B**. This result strongly suggests that the distribution of the -173*C allele increases the risk of leptospirosis. The overall and stratified analysis revealed a strong positive correlation between leptospirosis risk and MIF -173G/C polymorphism.

8.2.3. Influence of MIF -173G/C SNP on mRNA and serum MIF levels

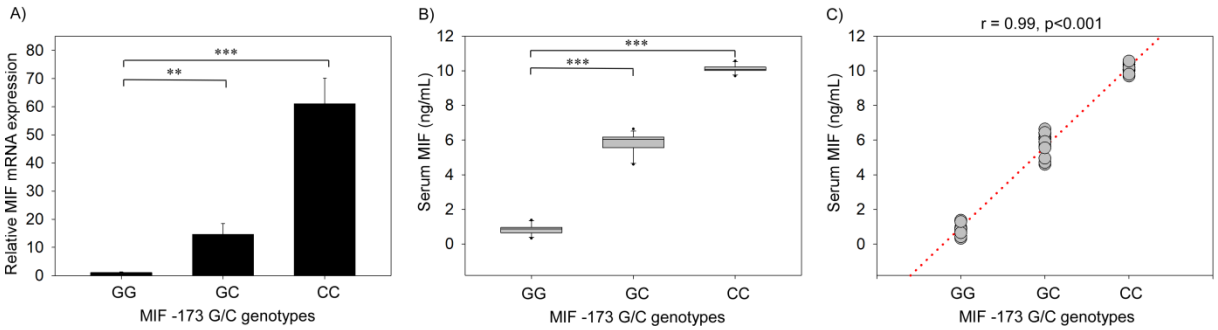

Figure 8.2. Association of MIF -173G/C SNP with MIF mRNA expression and serum MIF levels. A) Relative MIF mRNA expression with respect to -173G/C genotypes, GG, GC, and CC. B) Box plot showed the serum MIF levels in different -173 G/C genotype carriers. Both MIF mRNA expression and serum MIF levels were significantly higher in -173*C allele-associated genotypes GC and CC when compared with GG. C) Pearson correlation coefficient analysis between serum MIF level and MIF gene polymorphism -173G/C SNP. This analysis showed that elevated serum MIF level was positively correlated with -173*C allele-associated genotypes GC and CC (r = 0.99, P<0.001). n=3 experiments. **P<0.01, ***P<0.001.

To demonstrate the functional impact of MIF -173G/C polymorphism, we analyzed the MIF mRNA expression and serum concentration between different genotype carriers. The quantitative analysis of MIF RNA expression according to -173G/C SNPs showed that the relative mRNA expression of CC and GC carriers was significantly higher with respect to GG genotype carriers ($p<0.001$) as shown in **Figure 8.2A**. The quantification of serum MIF levels in different allele carriers determined the association between -173G/C SNP of the MIF gene and the production of circulating MIF proteins. The analysis highlighted the significant increase of serum MIF with carriage of CC genotype followed by GC. The mean score of serum MIF was significantly elevated 11-fold in carriers of CC (10.12ng/mL) and 6-fold in GC (5.81ng/mL) of leptospirosis patients with respect to GG (0.86ng/mL) of healthy controls ($p<0.001$) (Table 4). It is also important to note that the carriers of the MIF -173*C allele have significantly higher serum MIF levels than MIF -173G alleles (7.96ng/mL vs 3.34ng/mL, $p<0.001$) **(Figure 8.2B)**.

Table 8.4. Serum MIF levels in study subjects, according to MIF -173 G/C polymorphism

Genotype	**MIF concentration (Mean ± SD)**	**p-value**
GG	0.86 ± 0.3 ng/mL	<0.001
GC	5.81 ± 0.61 ng/mL	
CC	10.12 ± 0.23 ng/mL	
Allele		
G	3.34 ± 2.56 ng/mL	<0.001
C	7.96 ± 2.23 ng/mL	

The most remarkable result to emerge from the data is that C allele related genotypes are positively correlated with serum MIF levels upon leptospiral infection ($r = 0.99$, $p<0.001$) **(Figure 8.2C)**.

8.2.4. MIF -173G/C SNP regulates cytokine profile of leptospirosis patients

Relative mRNA expression of inflammatory cytokines TNF-α, IL-1β, and IL-4 and anti-inflammatory cytokine IL-10 with respect to -173G/C genotypes, GG, GC, and CC was analyzed by qRT-PCR **(Figure 8.3)**.

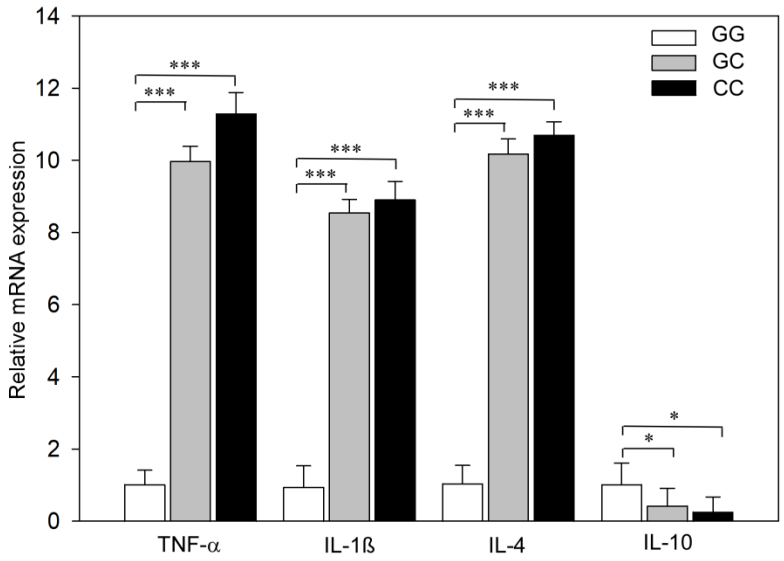

Figure 8.3. MIF -173G/C SNP regulated cytokine expression during leptospirosis. Relative mRNA expression of TNF-α, IL-1β, IL-4, and IL-10 with respect to -173G/C genotypes, GG, GC, and CC was determined by qRT-PCR analysis. This analysis showed that TNF-α, IL-1β and IL-4 expression was significantly higher in MIF -173 GC and CC genotype carriers when compared with GG carriers. n=3 experiments. *P<0.05, ***P<0.001.

The results revealed that high MIF expression genotype GC and CC carriers had 10-fold and 11-fold upregulated mRNA expression of TNF-α respectively, whereas 9-fold expression of IL-1β and 11-fold expression of IL-4 when compared to GG carriers. Meanwhile, the anti-inflammatory cytokine IL-10 expression was significantly ($p<0.05$) downregulated in GC and CC genotype carriers. This elevated expression of inflammatory cytokines may drive the disease severity by accelerating other inflammatory responses and organ damage.

8.3. Discussion

The investigations on host genetic factors responsible for the disparate outcomes are of great importance for attaining novel insights into pathogenic mechanisms and developing the potential personalized treatment for high-risk populations. Previous studies revealed that MIF gene promoter polymorphisms are a firm genetic risk factor for the susceptibility and severity of diseases (Garcia-Orozco *et al*., 2020; Awandare *et al*., 2009; Valdés-Alvarado *et al*., 2014; Qin *et al*., 2021). In the present investigation, we found MIF gene polymorphism (-173G/C SNP) was significantly associated with the risk of leptospirosis.

Two functionally significant MIF gene promoter polymorphisms are $CATT_{5\text{-}8}$ microsatellite and G/C SNP. Previous studies have determined that the longer length of CATT repeats of MIF -794 $CATT_{5\text{-}8}$ polymorphism was strongly correlated with disease onset and/ or progression (Liu *et al*., 2018; Savva *et al*., 2016; Leng *et al*., 2020; Shiroeda *et al*., 2010). Regarding MIF -173G/C SNP, previous reports showed that -173 GC and CC genotype carriers have an increased risk of developing diseases than GG genotype carriers. MIF -173*C alleles were found to be correlated with susceptibility to inflammatory polyarthritis but not associated with disease progression and severity (Barton *et al*., 2003). The host genetic factors have not only determined the outcome of disease but also the outcome of treatment, so the consideration of host genetic or epigenetic signatures is of great importance in disease management (Johnson *et al*., 2008). For the first time, we conducted a case-control study to investigate the association of MIF -173G/C gene polymorphism with susceptibility and severity of leptospirosis. The results of the present study are the first demonstration of the relationship between the outcome of leptospirosis and MIF polymorphisms. During outbreak situations, the typing of MIF -173G/C SNP may be instructed to identify the patients at increased risk in order to provide personalized and/ or prophylactic

treatments. Thus, the identification of MIF -173G/C variants of leptospirosis patients could be a better severity predictor of leptospirosis.

MIF has been reported to be elevated in the serum of patients with inflammatory or autoimmune diseases. Based on the genetic location, the genetic variants alter the gene expression. The SNPs within the transcriptionally regulatory elements can modify the mRNA expression of a gene; SNPs within genes can alter splicing and translation; SNPs within coding sequence can modify the activity of the protein (Robert and Pelletier, 2018). The G to C allele transition in MIF -173G/C polymorphism can enhance the binding of the transcription factor, activator protein-4 (AP-4) to increase the mRNA and serum level of MIF (Donn *et al*., 2001).

In addition, to analyze the correlation between the MIF -173G/C polymorphism and the MIF gene expression, we performed cytokine profiling to evaluate the MIF -173G/C SNP regulated immune responses in leptospirosis patients. The upregulated MIF expression stimulates the production of inflammatory cytokines by downregulating the anti-inflammatory cytokine. Previous investigations revealed that macrophage MIF counter-regulates the anti-inflammatory activity of glucocorticoids and promotes the secretion of pro-inflammatory cytokines, TNFα, IL-1β, IL-6, and IL-8 upon LPS stimulation (Calandra *et al*., 1995). The elevated circulated levels of inflammatory cytokines cause cell toxicity, tissue damage leads to disease severity (Gupta *et al*., 2020).

The most striking result of this study states that leptospirosis patients carrying at least one C allele at -173G/C SNP also had an increased risk of developing severe leptospirosis and significantly higher serum MIF than those with the G allele. The primary requirements of the research on diagnostic, prophylactic, and therapeutic approaches to leptospirosis are a better understanding of the disease process. Also, as a part of this effort, we conducted this case-control

study to provide new insights into leptospirosis pathogenesis and help to identify the patients at increased risk.

8.4. Conclusion

The evidence from this study intimates that MIF gene promoter polymorphism -173G/C SNP might be contributing to the genetic risk of leptospirosis susceptibility and severity and the upregulation of MIF gene expression and serum MIF levels. Thus, the MIF -173G/C genotyping of leptospirosis patients may be helpful to guide and stratify the leptospirosis patients to institute prophylaxis and personalized treatment under outbreak situations. These results also showed the significance of considering MIF gene polymorphisms to perceive the intended mechanisms of host immune response to leptospirosis and extend the knowledge of the role of host genetic factors in developing disease susceptibility and severity.

Chapter VI

9. Screening and evaluation of chemical compound inhibitors for the anti-MIF activity as the drug for leptospirosis

9.1. Materials and Methods

9.1.1. Chemical compounds

17 test compounds were selected for the screening of anti-MIF activity. The general properties of reference and test compounds were listed in **Table 9.1**. ISO-1, the first Food, and drug administration (FDA) approved MIF inhibitor was employed as a reference compound. The test compounds comprise essential tautomerase inhibition classes of 10 chemical compounds which are derivatives of isoxazoline (C1, C2), plant derived isoflavone (C3) and quinone (C4), phenyl pyrimidine (C5), quercetin (C6, C7), phenyl pyruvate (C8), cinnamates (C9), and acetylic compound (C10), and 7 already existing clinically approved drugs (C11-C17) with anti-inflammatory and antibiotic activity for repurposing of old drugs as novel MIF antagonists. The reference and test compounds were purchased from Sigma Aldrich chemicals private limited.

Table 9.1. Properties of reference and test compounds of anti-MIF activity

S.No.	Chemical compound	Empirical Formula	Molecular weight
Reference compound			
R1	(S,R)-3-(4 hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1)	$C_{12}H_{13}NO_4$	235.24
Test compounds			
C1	N-(5-Chloro-2,4-dimethoxyphenyl)-N′-(5-methyl-3-isoxazolyl)-urea	$C_{13}H_{14}ClN_3O_4$	311.72
C2	6-Fluoro-2- *o*-tolylbenzo[d] isothiazol-3(2H)-one (NADPH Oxidase Inhibitor VII)	$C_{14}H_{10}FNOS$	259.3
C3	3-(2,4-Dihydroxyphenyl)-7-hydroxy-5-methoxy-6-(3-methyl-2-buten-1-yl)-2H-1-benzopyran-2-one (Glycycoumarin)	$C_{21}H_{20}O_6$	368.35
C4	Tetrafluoro-1,4-benzoquinone	$C_6F_4(=O)_2$	180.06
C5	4-Amino-5-(4-fluoroanilino)-pyrazolo[3,4-d]pyrimidine (MNK-1 inhibitor)	$C_{11}H_9FN_6$	244.23
C6	(2R,3R)-Dihydroquercetin	$C_{15}H_{12}O_7$	304.25
C7	Quercetin 7-methyl ether	$C_{16}H_{12}O_7$	316.26

C8	Phenyl-d_5-pyruvic acid	$C_9D_5H_3O_3$	169.19
C9	Ethyl 4-nitrocinnamate	$O_2NC_6H_4CH=CHCO_2C_2H_5$	221.21
C10	Methyl 11-bromoundecanoate	$Br(CH_2)_{10}CO_2CH_3$	279.21
C11	6-((4,4-dimethylcyclohexyl)methyl)-4-hydroxy-3-phenylpyridin-2(1H)-one (InhA inhibitor)	$C_{20}H_{25}NO_2$	311.42
C12	2-Phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen)	$C_{13}H_9NOSe$	274.18
C13	N-(7-(Methylsulfonamido)-4-oxo-6-phenoxy-4H-chromen-3-yl)formamide (Iguratimod)	$C_{17}H_{14}N_2O_6S$	374.37
C14	2-Methyl-1-[2-(1-methylethyl)pyrazolo[1,5-a]pyridin-3-yl] 1-propanone (Ibudilast)	$C_{14}\ H_{18}\ N_2O$	230.31
C15	4-Pyridinecarboxylic acid hydrazide (Isoniazid)	$C_6H_7N_3O$	137.14
C16	Rifapentine	$C_{47}H_{64}N_4O_{12}$	877.03
C17	2-(3-Chloro-2-hydroxyphenyl)-5-hydroxy-3,7,8-trimethoxy-4H-1-benzopyran-4-one (Chlorflavonin)	$C_{18}H_{15}ClO_7$	378.76

9.1.2. Dopachrome tautomerase assay

A spectrophotometric assay was performed to detect the MIF inhibition property of compounds by analyzing the tautomerase activity of MIF. This assay was performed as per the earlier report (Healy *et al*., 2011) with slight modification. 250µL of 12 mM L-3,4- dihydroxyphenylalanine methyl ester and 250µL of 24mM sodium periodate (Sigma-Aldrich, St. Louis, MO, USA) were added and allowed to oxidize to form L-dopachrome methyl ester. The oxidized substrate was added with 9.5 mL of reaction buffer (50mM Bis-Tris, 1mM EDTA, pH 6.2) and incubated at room temperature for 5 minutes in the dark. To test the anti-MIF activity of compounds, different concentrations (500, 250, 100, 50, 25, 10, 5, 2.5, 1 µM) of test compounds or reference MIF inhibitors were added to 96 well plates containing MIF solution (50nM) and incubated for 20 minutes at room temperature. Control was also maintained with DMSO. ISO-1 was employed as a positive control. 100µL of reaction buffer was added to each well and incubated for 2 minutes at dark. Read the plate at 475nm and measure the decrease in absorbance. Analysis of each concentration of all compounds was triplicated. The half maximal inhibitory concentration (IC_{50}) value was determined for nine different concentrations of each compound. The percentage of

MIF inhibition was assessed by using the formula mentioned below,

$$\text{MIF activity (\%)} = \frac{100 - (100 \times (\text{Test compound value} - \text{Average of blank control}))}{(\text{Average of DMSO control} - \text{Average of blank control})}$$

Then, the IC_{50} value of compounds was determined by using SigmaPlot software.

9.1.3. MTT assay

To determine the cytotoxic effects of MIF inhibitory compounds, an MTT assay was performed as mentioned previously in materials and method section 5.1.4. Compound C2, C5, C6, C7, C11, C12, C13, C14, C15, C16, and C17 were tested with THP-1 cells for cytotoxicity. PBS, DMSO, and Triton X-100 were employed as a negative control, vehicle control, and positive control of cytotoxicity.

9.1.4. Cell culturing, LPS induction, and MIF inhibitor treatment *in vitro*

THP-1 cells were grown in 6 well plates at the concentration of 5×10^5 cells per well and treated with leptospiral LPS (1µg/ml). To verify the therapeutic role of MIF inhibitor in leptospirosis, THP-1 cells were pretreated with MIF inhibitor (10 µg) at 12h before LPS induction. All the treatments were performed in triplicates. The treated cells were pelleted for further analysis.

9.1.5. Inflammatory protein expression analysis

Cell lysates were prepared from treated cells and subjected to Western blotting as previously described in Materials and method section 7.1.6 to analyze the expression of inflammatory mediators in *in vitro* experimental models. The antibodies used to detect the protein targets are ICAM-1 (1:1000, Cell Signaling Technology), p38 MAPK (1:1000, Cell Signaling Technology), p-p38 MAPK (1:1000, Cell Signaling Technology), p44/42 (1:1000, eBioscience), p-p44/42 (1:1000, Cell Signaling Technology), β-actin (1:500, Santa Cruz).

9.1.6. Quantitative Real-time PCR analysis

RNA was extracted from treated cells and reverse transcribed into cDNA. qRT-PCR was performed as mentioned previously in Materials and methods section 5.1.7. The primer sequences used in this experiment are provided in **Table 5**. The housekeeping gene, GAPDH was used as an internal control.

9.1.7. Confocal Laser Scanning Microscopy

THP-1 cells were grown in a complete medium and plated on a glass slide coated with Cell Tak. Cells were cultured for 24 h until 70% confluent and were then pretreated with MIF inhibitor and incubated with LPS (1 µg) for 12 h. For cell death measurement, analysis of ROS production, and $\Delta\Psi_m$ loss, we performed PI, DHE, and Rhodamine 123 staining respectively as described previously in materials and methods section 7.1.3. The fluorescent intensity was quantified by using ImageJ software.

9.1.8. BALB/c mice and treatment of MIF inhibitor

All experimental protocols were approved by the Institutional animal ethical committee (IAEC), Bharathidasan University, Tiruchirappalli (BDU/IAEC/P30/2018). Six to eight weeks-old BALB/c mice (Immunocompromised with cyclophosphamide) were segregated into 4 groups (N=5). The control group (Group 1) received PBS injections over the course of the experiment. Group II was administered LD50 dose of leptospiral strain intraperitoneally, Group III with PBS+ MIF inhibitor, and Group IV with leptospiral LD50+MIF inhibitor. To investigate the therapeutic role of a novel MIF inhibitor in *in vivo* model, mice were treated with MIF inhibitor (5mg/kg of body weight; intraperitoneally) or PBS 30 min before and 6 h after leptospiral injection and then once daily for 3 days. Animals were monitored daily for survival and any signs of lethargy and decreased food and water intake for 5 days. On completion of experiments,

mice were euthanized as per IAEC procedures. The excised tissues were collected and stored at -80℃ until use.

9.1.9. Analysis of inflammatory cytokine expression and histopathology

To analyze the role of MIF inhibitors as the regulators of inflammation in *in vivo* leptospirosis experimental model, we performed Western blotting, qRT-PCR analysis, histopathological analysis, and survivability analysis. Inflammatory cytokine profile was determined by qRT-PCR as mentioned previously in materials and methods 5.1.7 respectively. Histopathological features of lung tissues were examined by H & E staining as described earlier in materials and methods section 7.1.7.

9.1.10. Biocompatibility assays

To determine the biocompatibility of MIF inhibitor to human cells, we performed MTT assay (as mentioned in 5.1.4), PI staining (as mentioned in 7.1.3), and hemocompatibility assay. For MTT assay, THP-1 cells were treated and incubated with different concentrations (1, 10, 25, 50, 75, 100, and 250 μM of MIF inhibitor. For PI staining, cells were treated with 1, 10, 50, 100, and 250 μM of MIF inhibitor. Further, the haemolytic activity was measured by hemocompatibility assay (Ilangovan *et al.*, 2017). The study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (Ref No. DM/2014/101/54). The consent form was obtained from healthy volunteers before the collection of blood samples. Fresh blood was collected from healthy individuals in sterile EDTA vacutainers and the RBCs were isolated by centrifugation at 1500 rpm for 10 min at 4ºC. The erythrocytes were washed with 1X sterile phosphate buffered saline several times until the supernatant was clear and then resuspended with 3mL of PBS. For RBC lysis analysis, 100μL of erythrocyte suspension was added to Ibudilast MIF inhibitor at a various concentrations (1, 10, 25, 50, 75, 100, and 250 μM) dissolved

in 500µL of PBS suspension and incubated at 37ºC for 4h. After the centrifugation at 12000 rpm, the haemoglobin concentration in the supernatant was quantified by measuring absorbance at 570 nm by a microplate reader (Bio-Rad, USA). All the samples were prepared in triplicate. The percentage of hemolysis was calculated as follows:

$$\text{Hemolysis (\%)} = \frac{\text{Sample absorbance - Negative control}}{\text{Positive control - Negative control}} \times 100$$

9.1.11. Statistical analysis

Data from triplicate experiments were quantified and expressed as Mean ± SE, n=3. Differences in means among multiple data sets were analyzed using 1-way ANOVA with subsequent Tukey's test for multiple comparisons. The student's t-test was used to determine the significance of relative expression analysis of gene transcript analysis. Kaplan Meier plots were performed by GraphPad Prism version 7.0 to quantify the survivability. The difference between the survivability of various groups of animal models was analyzed by a log-rank test. *P*- value less than 0.05 was considered significant in all analysis. The data were computed either with Graphpad Prism version 9.2.0 or SigmaPlot 11.0 Software.

9.2. Results

9.2.1. Selected chemical compounds inhibit the tautomerase activity of MIF

A number of chemical compounds and drugs were screened for anti-MIF activity by performing the *in vitro* dopachrome tautomerase activity assays to determine the value of 17 selected compounds. The IC_{50} value of each chemical compound was reported in **Table 9.2**. As the standard MIF inhibitor, ISO-1 showed a strong inhibition (61.26%) on MIF tautomerase activity. We found that IC_{50} of compound C14 (9.5 ± 5.6 µM) was significantly similar to the standard reference compound ISO-1 (6.2 ± 3.8 µM). Compound C15, C2, C5, C17, C16, C12, C8, C13, C6, C11, and C7 exhibited the IC_{50} of MIF tautomerase activity at the working

Table 9.2. IC_{50} of novel MIF inhibitors

Chemical compounds	**IC_{50} (µM)**
R1	**6.2 ± 3.8**
C1	Nil
C2	62 ± 8.6
C3	Nil
C4	Nil
C5	81 ± 5.6
C6	328 ± 1.6
C7	405 ± 4.3
C8	150 ± 2.8
C9	Nil
C10	Nil
C11	372 ± 5.8
C12	89 ± 9.3
C13	164 ± 1.6
C14	**9.5 ± 5.6**
C15	55 ± 4.6
C16	89 ± 7.6
C17	85 ± 2.1

concentration (µM) of 55 ± 4.6, 62 ± 8.6, 81 ± 5.6, 85 ± 2.1, 89 ± 7.6, 89 ± 9.3, 150 ± 2.8, 164 ± 1.6, 328 ± 1.6, 372 ± 5.8 and 405 ± 4.3 respectively whereas C1, C3, C4, C9 and C10 have no inhibition on tautomerase activity of MIF. The cytotoxicity of THP-1 cells treated with MIF inhibitors revealed that compound C14 showed no significant cytotoxicity (3.2%) when compared with other MIF inhibitors (>20%) **(Figure 9.1)**. Thus, compound C14, Ibudilast acts as a potential MIF inhibitor which is highly compatible with human cells at a concentration (9.5 µM), which showed the potential MIF inhibition. As per the MIF inhibition and cytotoxicity assays, the potent MIF inhibitor Ibudilast was subjected to *in vitro* and *in vivo* analysis to determine the therapeutic potential by examining its antagonistics on leptospirosis mediated inflammation and severity.

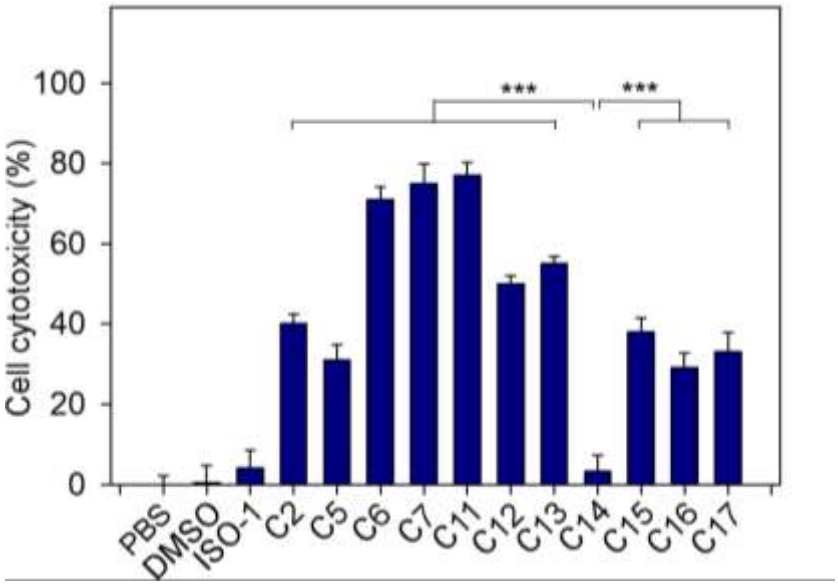

Figure 9.1. MTT cell proliferation assay. The representative graph indicates the cytotoxic effects of all MIF inhibitors. n = 3 experiments. ***P<0.001.

9.2.2. Ibudilast reduces the LPS mediated inflammatory protein expression and cytokine expression

To determine the efficiency of MIF inhibitor Ibudilast in attenuation of leptospirosis associated inflammatory responses, we evaluated the LPS-mediated overexpression of inflammatory pathway protein mediators and cytokines by performing the Western blotting and qRT-PCR analysis. *In vitro* analysis revealed that compound C14 was significantly reduced the inflammatory responses of leptospirosis by downregulating the LPS mediated expression of ICAM, and the decreased phosphorylation of p38 and p44/42 MAPK **(Figure 9.2A)**. Indeed, MIF inhibitor Ibudilast diminished the leptospiral LPS mediated immunomodulatory effects, by down-regulates the inflammatory cytokine expression, TNF-α, IL-1β and IL-4 and upregulates the expression of anti-inflammatory cytokine, IL-10 **(Figure 9.2B)**.

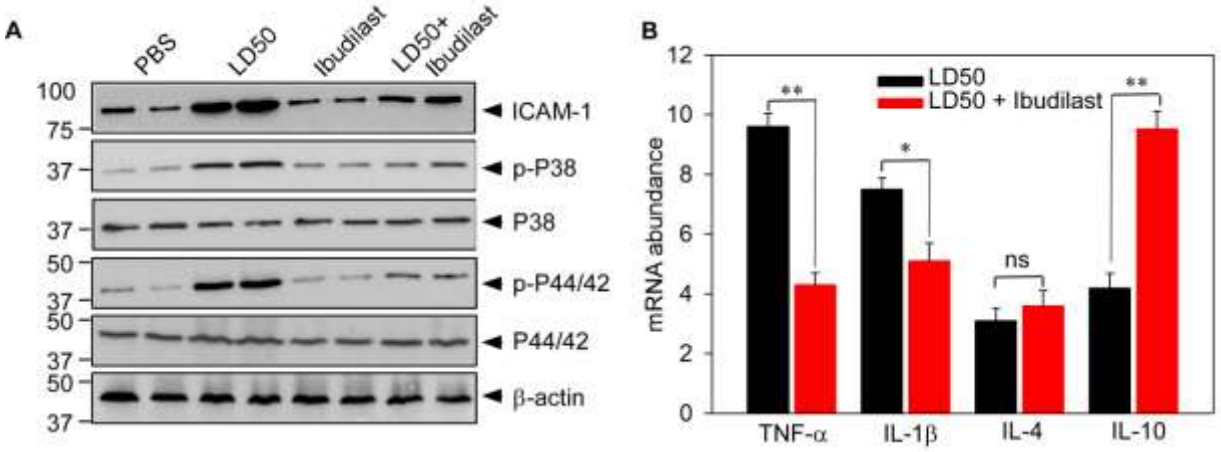

Figure 9.2. Analysis of LPS mediated inflammatory mediators and cytokine expression. Inflammatory mediators such as ICAM-1, P38 MAPK, and P44/42 MAPK and cytokines TNF-α, IL-1β, IL-4, and IL-10 by Western blotting and qRT-PCR respectively. n = 3 experiments. *P<0.05; **P<0.01.

9.2.3. Role of MIF inhibitor in LPS mediated cell death, $\Delta\Psi_m$ loss, and ROS production

In general, leptospiral LPS stimulation significantly increased ROS production, loss of $\Delta\Psi_{m,}$ and apoptotic cell death in THP-1 cells. To analyze whether the activity of the MIF inhibitor was influenced by LPS-mediated cellular effects, we performed PI staining **(Figure 9.3)**, Rhodamine 123 staining **(Figure 9.4),** and DHE staining **(Figure 9.5)**. Based on the observations of CLSM experiments, this study revealed that the percentage of LPS mediated cell death was significantly declined in Ibudilast treated THP-1 cells. Rhodamine 123 stainings found that LPS mediated drastic change in $\Delta\Psi_m$ loss and ROS production was significantly reduced by the treatment of MIF inhibitor.

9.2.4. Ibudilast ameliorates the histopathological changes of lung tissue in leptospires infected mice

We examined the effect of MIF inhibitor, Ibudilast on the LPS caused lung injury by H & E staining**.** Leptospiral infection enhanced the alveolar hemorrhage, immune cell infiltration and

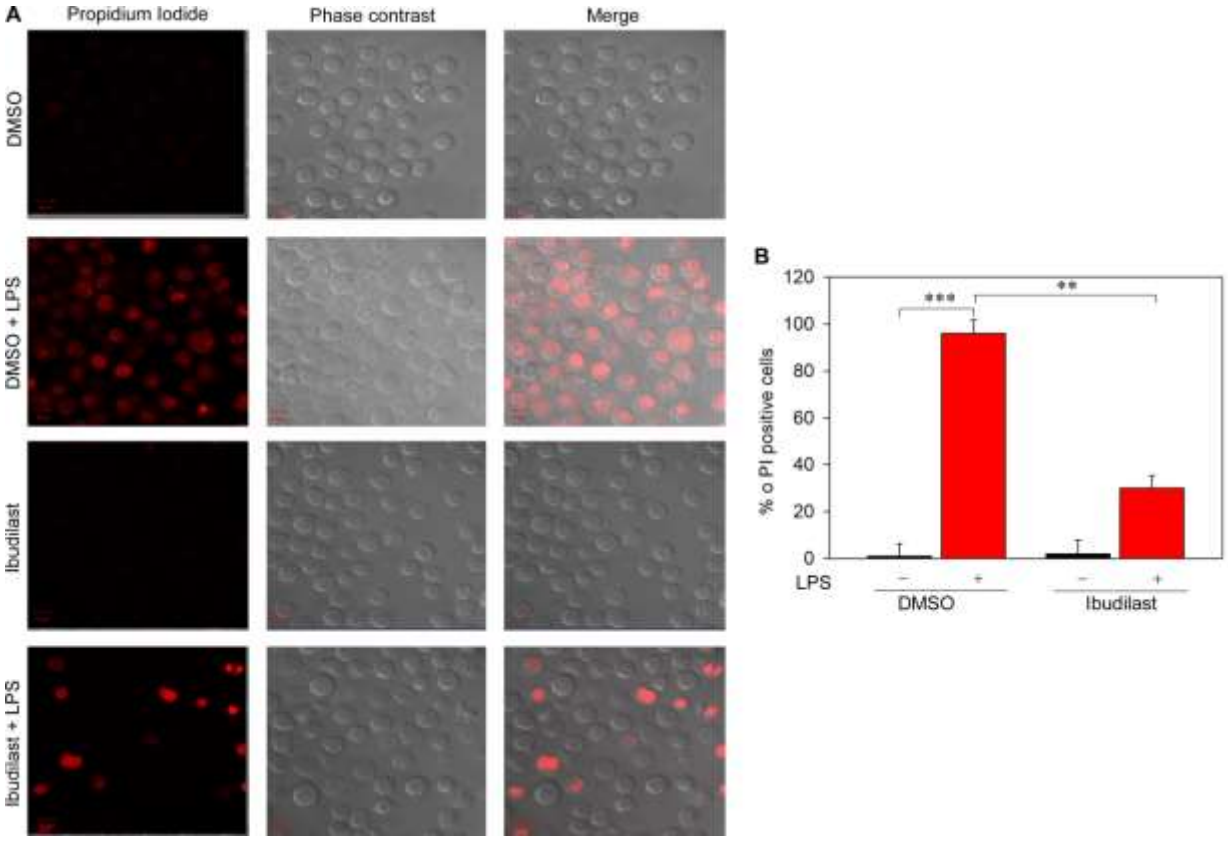

Figure 9.3. Cell death assay by PI staining. A) Representative confocal images of DMSO and MIF inhibitor pre-treated THP-1 cells were stained with PI and stimulated with or without LPS (1µg/ml) for 6h. B) Quantification of % PI-positive cells. n = 3 experiments. **P<0.01; ***P<0.001.

alveolar congestion in lungs of BALB/c mice. The overall histological scoring of the infected lung was very high **(Figure 9.6).** The lungs of control mice showed no pathological evidence but usually few alveolar macrophages are present in the septa. MIF inhibitor significantly alleviated the histological alterations in lung sections of leptospires infected mice by exhibiting significantly fewer histopathological signs. The extensive rate of hemorrhage, Inflammatory cell infiltration, and alveolar wall thickening in lung tissues was markedly decreased in leptospires infected mice group following administration of MIF inhibitor. The morphological changes and appearance of organs in LPS treated mice were significantly varied.

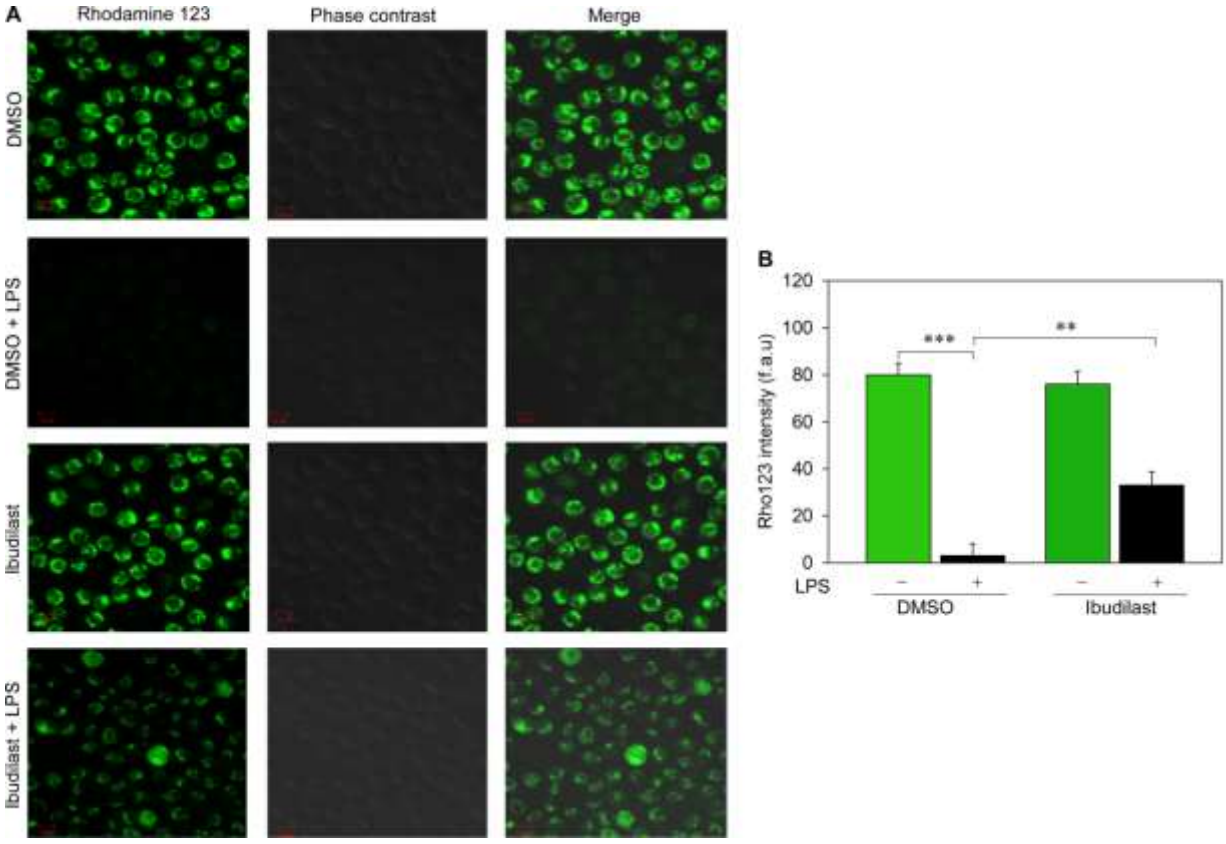

Figure 9.4. Analysis of $\Delta\Psi_m$ loss by Rhodamine 123 staining. A) MIF inhibitor pretreated THP-1 cells were stained with Rhodamine 123 after 12h of LPS treatment, which showed the significant reduction of $\Delta\Psi_m$ loss by novel MIF inhibitor. B) Quantification of Rhodamine 123 fluorescence intensity. n = 3 experiments. **P<0.01; ***P<0.001.

9.2.5. MIF inhibitor downregulates the inflammatory cytokines *in vivo*

MIF inhibition increased the anti-inflammatory cytokine IL-10 mRNA levels approximately two-fold. The increased IL-10 mRNA levels significantly decreased the pro-inflammatory cytokine TNF-α mRNA levels **(Figure 9.7A)**. Thus, we hypothesized blockade of MIF by Ibudilast to be protective during leptospiral infection through upregulation of anti-inflammatory responses.

9.2.6. Blockade of MIF prevents leptospires induced lethality in mouse model

Due to our observation that overexpression of MIF and MIF mediated inflammatory cytokine in leptospires injected mice, we next chose to investigate the contribution of MIF to lethality rate.

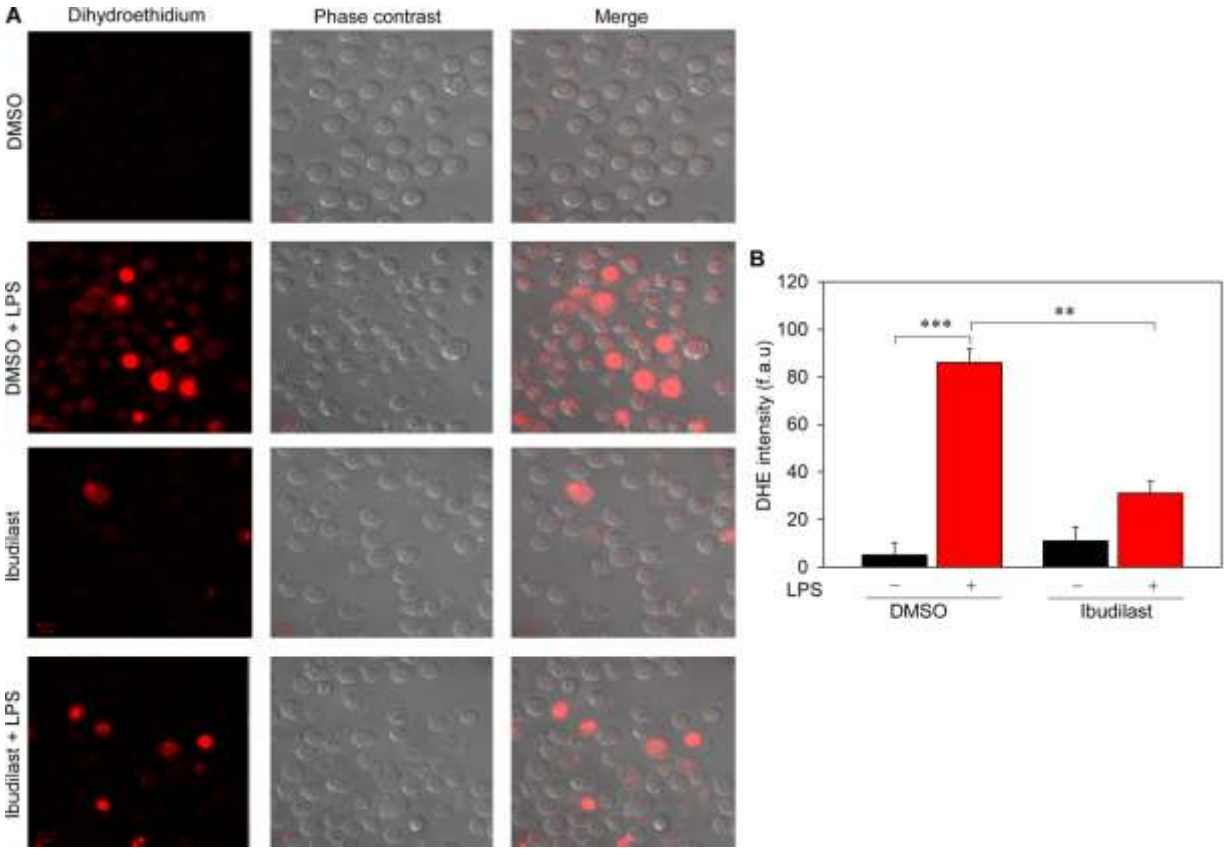

Figure 9.5. Detection of ROS production by DHE staining. A) Representative CLSM images showed that MIF inhibitor reduced the production of ROS during LPS induction when compared with LPS induced control cells. B) Quantification of DHE fluorescence intensity. n = 3 experiments. **P<0.01; ***P<0.001.

To determine the promotional role of MIF in the lethality of mice, inactivation of MIF was carried out by Ibudilast treatment and analyzed the survivability of mice. Group I, II, III, and IV have 100%, 100%, 25%, and 66% of survivability respectively. As per our results, blockade of MIF with Ibudilast prevented the leptospiral infection induced lethality in group IV mice by increasing the survivability from 25% to 66% **(Figure 9.7B)**. Because we observe a preserved survivability among MIF inhibitor-treated animals, Ibudilast may play a key role in MIF-based therapeutic approaches to leptospirosis by declining the inflammatory responses and decreasing the risk of mortality.

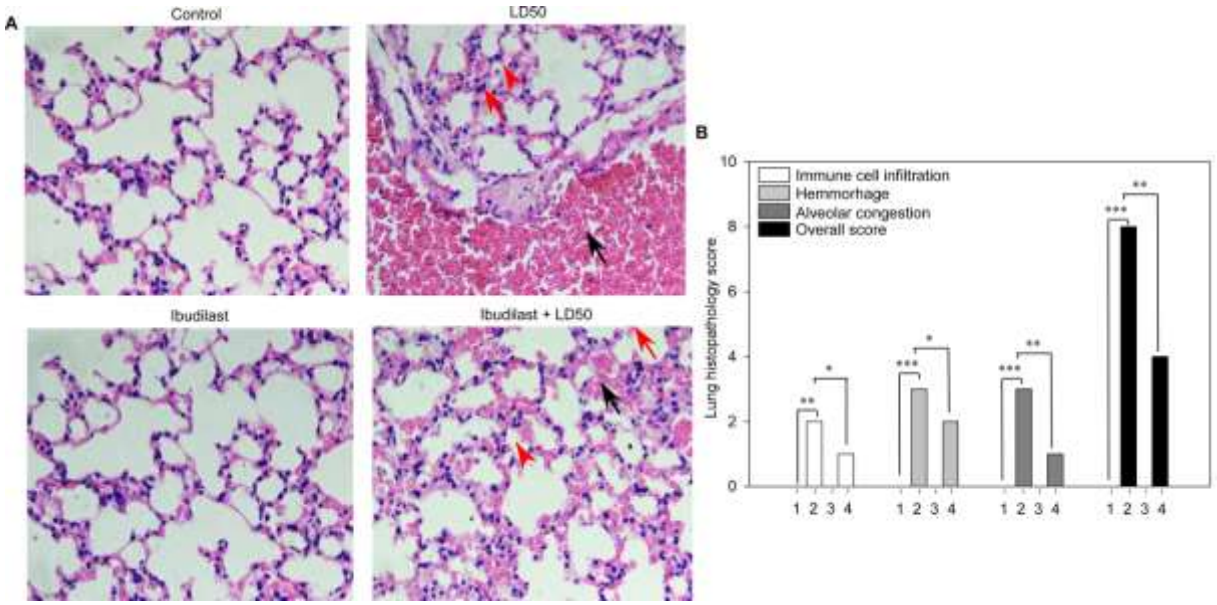

Figure 9.6. MIF inhibitor reduced the leptospiral LPS stimulated histopathological alterations. Light microscopic observation of H & E staining tissues. Histopathological features are abundant in LD50 dose of leptospires injected mice whereas ISO-1 significantly decreased the histopathological changes in mice lung tissues. **B)** Semi-quantitative lung injury score of several histological features including Immune cell infiltration, Hemorrrhage, Alveolar congestion, and the overall lung injury score. 1, 2, 3, and 4 indicate the *in vivo* experimental model group I, II, III, and IV. Score 0: No features observed, Score 1: Mild, Score 2: Moderate, Score 3: Severe. n = 3 experiments. *P<0.05; **P<0.01; ***P<0.001.

9.2.7. Assessment of cytotoxic activity of Ibudilast

For developing the novel drug, there is a need to evaluate its compatibility to host cells. An *in vitro* cytotoxicity effect of ibudilast was analyzed in cultured THP-1 cells by MTT assay. This is a colorimetric assay based on the conversion of yellow tetrazolium MTT into insoluble (E,Z)-5-(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan) formazan by cellular NAD(P)H-dependent oxidoreductase for the assessment of cell metabolic activity. The results of biocompatibility assays highlighted that Ibudilast has no significant cytotoxicity even at the higher concentration (≤50μM) and also exhibited about 40% of cytotoxic effect at the extremely higher concentration (100μM), when compared with the positive control, 0.1% Triton X-100. The observation of the cytotoxic effect of Ibudilast was shown in **(Figure 9.8A).** Thus, the MIF inhibitor has a dose-

dependent effect on cytotoxicity on THP-1 cells, and only at the concentration of ≥100μM causes the significant cytotoxicity.

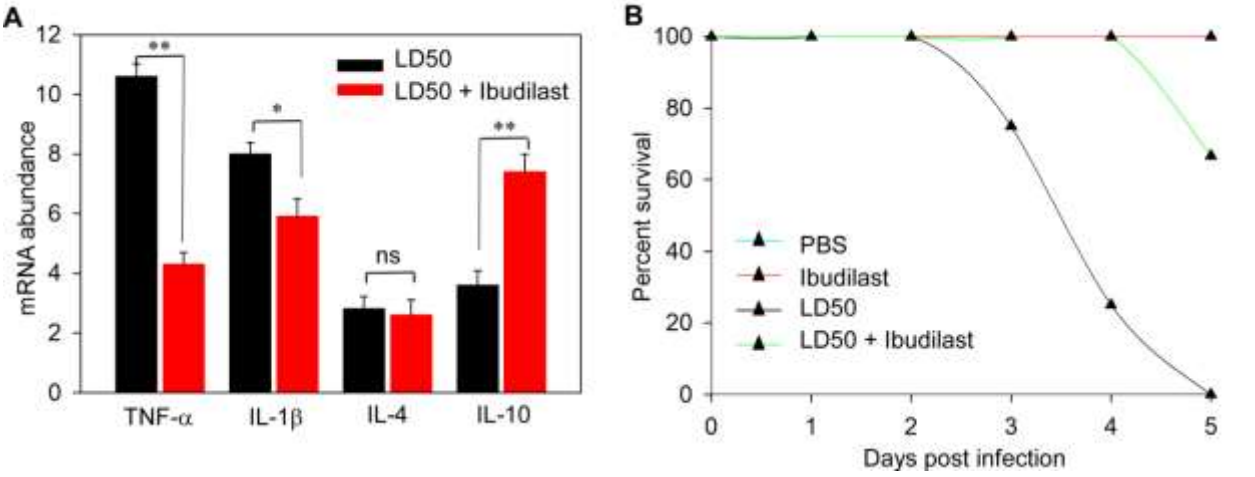

Figure 9.7. Ibudilast attenuates the pathogenic effects of leptospiral infection *in vivo* by protecting the mice from lethality and downregulating the expression of proinflammatory cytokine and other inflammatory mediators. A) Quantification of mRNA abundance of inflammatory cytokines including TNF-α, IL-1β, IL-4, and IL-10 in leptospires treated mice with or without Ibudilase injection. **B)** Kaplan Meier survival plot for mice treated with PBS, MIF inhibitor, LD50 of leptospires, LD50 pretreated MIF inhibitor. n = 3 experiments. *P<0.05; **P<0.01; ***P<0.001.

9.2.8. Hemocompatibility of Ibudilast

As *in vitro* evaluation studies revealed that anti-MIF ibudilast has no significant effect on cytotoxicity at an optimal concentration of biological activity, we further performed the hemocompatibility assay for the confirmation of the safety of the MIF inhibitor compound to host cells. This is a colorimetric assay based on the release of hemoglobin from damaged erythrocytes. . According to the International Organization for Standardization/Technical Report 7406, the admissible level of hemolysis of biological materials is 5% (Sathishkumar *et al*., 2015). The investigation of the hemocompatibility profile of MIF inhibitors at the different concentrations on host cells showed that no significant hemolytic activity was noticed even at higher concentrations (≥250μM). The percentage of hemolysis of MIF inhibitor-treated cells was

associated with that of the negative controls. No hemolysis was noticed in negative control whereas 100% hemolysis was noticed in positive control-treated cells. The percentage of hemolysis was represented in **Figure 9.8B**. Thus, the anti-MIF Ibudilast has a perfect hemocompatibility with healthy host cells.

9.2.9. Analysis of cell death effect of Ibudilast

After defining the lower cytotoxicity and RBC lysis of the MIF inhibitor compound, we performed the cell death assay by using a fluorescent dye Propidium Iodide (PI). PI staining is based on the integrity and permeability of the cell plasma membrane. PI staining demonstrated that ≤50µM concentration of MIF inhibitor-treated cells showed no significant cell death, whereas the higher concentration (≥100µM) of MIF inhibitor-treated THP-1 cells showed significant cell death (30%) and at ≥250µM concentration, it showed the 85% of cell death which is nearly equal to the number of PI-positive cells of positive control, tBHP treated cells. The number of PI-positive at different concentrations of Ibudilast treated cells, negative and positive control cells were represented in **(Figure 9.9)**

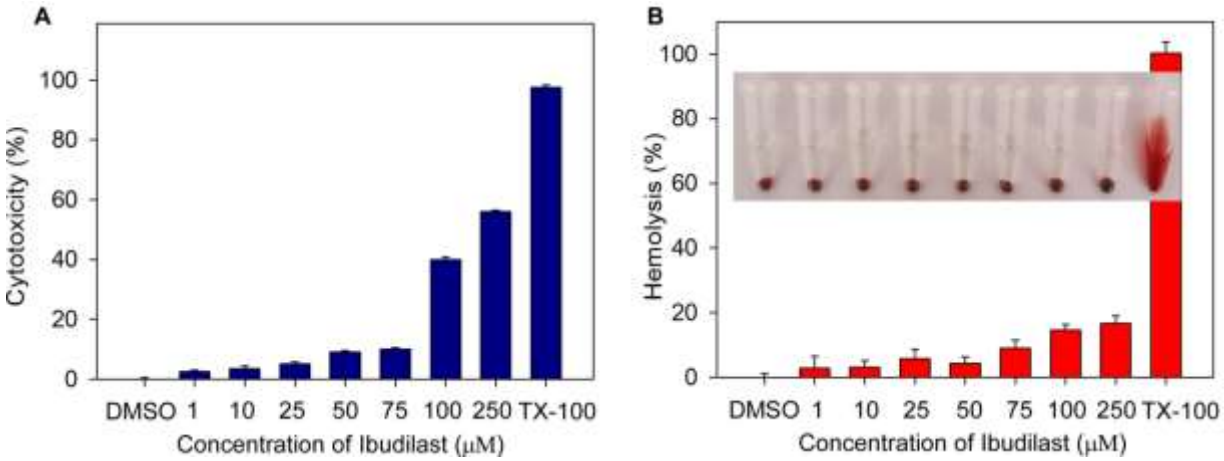

Figure 9.8. *In vitro* cytotoxicity assay and hemocompatibility analysis of Ibudilast. A) Dose-dependent cytotoxic effect of Ibudilast (1 - 250µg) on THP-1 cells. Significant cytotoxic effects were noticed only at higher concentration (≥100 µg)of ibudilast. B) Non- significant lysis of

erythrocytes was observed when compared with the positive control, TX-100. DMSO - Dimethyl Sulfoxide, TX-100 - Triton-X-100.

9.3. Discussion

Studies revealed that the pleiotropic, multi-functional pro-inflammatory protein MIF occupies the major role in the inflammatory pathway, by which MIF has been strongly associated with several inflammatory diseases, autoimmune disorders, and cancer pathogenesis. Therefore, it is not surprising that attempts were initiated to develop MIF-directed therapeutic strategies. Currently, neutralizing antibodies and small molecule inhibitors which target the tautomerase enzymatic active site of MIF is the major MIF-based therapeutic approaches (Trivedi-Parmar & Jorgensen, 2018). Notably, MIF acts as both a cytokine and enzyme that functions as keto-enol tautomerase. Most studies speculated that MIF's enzymatic activity is responsible for the multiple biological activities in both physiological and pathological conditions (Suzuki *et al*., 1997). Due to its beneficial effects such as low cost of production, lack of immunogenic reactions, and the probability of oral administration, small molecule inhibitors gained great interest (Kok *et al*., 2018). The extensively studied, most frequently used reference inhibitor classes of MIF tautomerase activity are isoxazolines inhibitors including ISO-1, ISO-66, and CPSI-1306 (Lubetsky *et al*., 2002; Kithcart *et al*., 2010; Ioannou *et al*., 2014), 1,2,3-triazoles inhibitors as MIF inhibitors including Jorgensen-3g, Jorgensen-3h and Dziedzic-3bb (Jorgensen *et al*., 2010), covalent MIF inhibitors including phenyl pyrimidine (4-IPP) and isothiocyanates, which irreversibly inhibit the tautomerase and biological activity of MIF. Several reversible inhibitors include inhibitors with benzoxazinone scaffold (Alissa-5), allosteric inhibitor (p425), and pyrimidine scaffolds (K664-1) (Bai *et al*., 2012). In this study, we selected the essential tautomerase inhibition classes of chemical compounds (Isoxazoline, plant derived isoflavone,

and quinone, phenyl pyrimidine, quercetin, phenyl pyruvate, cinnamates, and acetylic compound) and 7 existing drugs including ebselen, iguratimod, ibudilast, isoniazid, rifapentine,

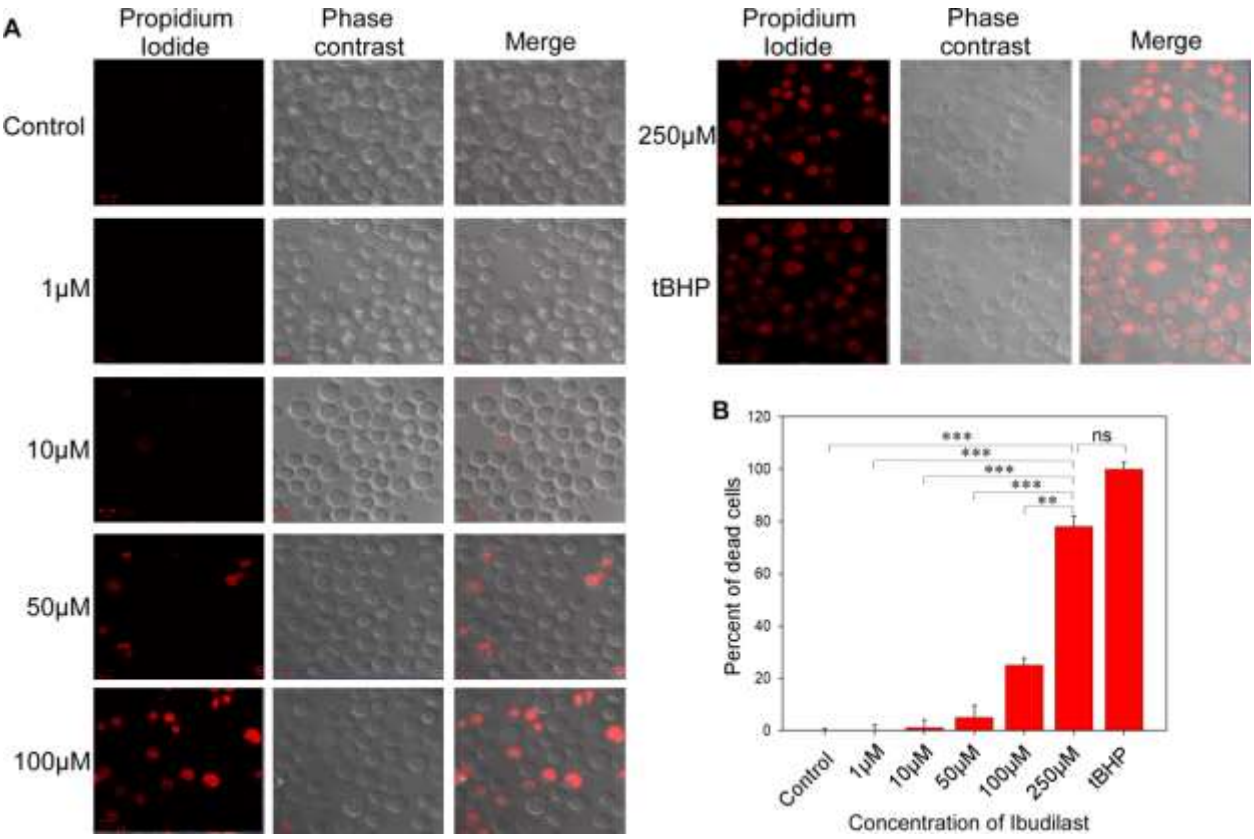

Figure 9.9. *In vitro* cell death assay by PI staining. Analysis of cell death of monocytic cells, THP-1 treated (12h) with various concentrations of Ibudilast (1, 10, 50, 100, and 250 μM). tBHP - *tert*-butyl hydroperoxide (Positive control) treated cells were recognized as controls. n = 3 experiments. **P<0.01; ***P<0.001.

chlorflavonin and isoniazid (InhA) inhibitor which have anti-inflammatory and anti-microbial properties for repurposing old drugs as novel MIF antagonists.

Previous studies evaluated the mechanism of the role of small molecule MIF inhibitors, which includes i) direct binding interaction between the inhibitor and MIF's enzymatic active site, ii) covalent modification in the residues of the enzymatic active site, iii) structural disruption of MIF's active site, iv) allosteric inhibition, and v) indirect inhibition by monomer stabilization to inhibit trimer formation (Ouertatani-Sakouhi *et al*., 2010). Currently, repurposing of old drugs is

studied interestingly, because these are available with known safety clarification and pharmacokinetics. Drugs including histamine, metaraminol, nebivolol, ibudilast, iguratimod, and ebselen were studied (Yang *et al*., 2022). Ibudilast is a non-selective phosphodiesterase inhibitor, used to treat chronic asthma it has a strong anti-inflammatory activity, suitable pharmacokinetics, and high biocompatibility (Rolan *et al*., 2009). Iguratimod, a methanesulfonanilide acts as a potent small molecule drug for rheumatoid arthritis. It downregulates the expression of several inflammatory factors (interleukin-1, -6 and -8 and TNF, NF-κB, cyclooxygenase-2), inhibits the B cell activity to block the secretion of immunoglobulins and autoantibodies, indeed it inhibits the cell-mediated immunity of T lymphocytes (Jiang *et al*., 2020). Ebselen is an organoselenium compound, which reacts with a variety of protein thiol groups leading to the pleiotropic effects and it exhibits anti-viral and anti-inflammatory activity. Recently, researchers have suggested this compound serve as a primary drug candidate for COVID-19 (Sies and Parnham, 2020).

For the first time, our study attempted to develop the MIF-directed therapeutic strategy for the treatment of leptospirosis. The observations from this study elucidated that ibudilast plays a key role in the inhibition of MIF tautomerase activity and significantly reduced the excessive inflammatory responses during leptospiral infection.

9.4. Conclusion

A potential small molecule MIF inhibitor was identified by *in vitro* screening of chemical compounds to assess the MIF tautomerase inhibition. *In vitro* and *in vivo* evaluation of MIF ibudilast confirmed that it has the potential to interfere with the MIF mediated inflammatory processes by decreasing the expression of inflammatory cytokines, cell death, cytotoxicity, and ROS production. Ibudilast protects the mice model from leptospires mediated histopathologies, inflammatory responses, and lethality. Therefore, the results demonstrated herein strongly

suggested that ibudilast will be a promising MIF inhibitor to serve as a repurposing drug against MIF mediated inflammation in leptospirosis.

Summary and conclusion

10. Summary and Conclusion

One of the notable features of the present study is that a significantly higher expression level of circulating MIF was observed in laboratory-confirmed leptospirosis cases than in other febrile cases and healthy control. As per the statement of serological analysis regarding MIF expression, laboratory-confirmed leptospirosis cases with different clinical conditions like febrile illness (8.75ng/mL), pulmonary hemorrhage (14.25ng/mL), Weils' syndrome (8.7ng/mL) and renal failure(11.4ng/mL) has a significant level of MIF concentration and pulmonary hemorrhages has remarkably higher MIF level among them. The results indicated that the level of serum MIF was elevated in human leptospirosis, and it will be possible to use MIF as a marker to improve disease monitoring and management.

Leptospiral LPS triggers the upregulation of MIF mRNA and protein expression in the surrogate model in a time-dependent manner. TLR4 is a major receptor for LPS for further sequential events in cells. Leptospiral LPS induces the SOCE protein expression sequentially (IP3R/Orai1/Stim1) to increase intracellular Ca^{2+} $[Ca^{2+}]_i$ level. LPS stimulated THP-1 cells have significantly increased $[Ca^{2+}]_i$ in a time-dependent manner, which was measured by Ca^{2+} fluorescence. ORAI1 knockdown downregulated the MIF expression evidencing intracellular Ca^{2+}-dependent MIF expression in LPS- stimulated THP-1 cells. Thus, Increased $[Ca^{2+}]_I$ drive the MIF expression. LPS-induced Ca^{2+} influx is also associated with the expression of transcription factor CREB and some inflammatory pathway proteins including, P38 and p-P38. CREB is a calcium-dependent transcription factor having a binding site in the CRE^p region of the MIF gene promoter. ChIP assay illustrated the effective binding of pCREB and MIF gene promoters by the amplification of immunoprecipitated DNA. The data from the luciferase assay indicated that CREB is a positive regulator of the expression of the MIF gene.

In vitro MIF knockdown model was prepared to analyze the underlying mechanism of MIF-regulated leptospiral pathogenesis. Our present investigation clearly indicates that leptospiral LPS stimulates the production of ROS, activation, and translocation of NFAT, the release of TNF-α, and significantly increased expression of ICAM-1 that was quenched by MIF knockdown. These findings develop the mechanism of leptospiral LPS enhanced pathogenesis by using the knowledge of previous studies. Leptospiral LPS causes cell death by inducing cell death machinery by targeting mitochondria. Our reports depict that cell death was promoted by ROS induced significant mitochondrial membrane depolarization that triggers the subsequent events including cytochrome c release to cytosol. These effects are attenuated by MIF knockdown, providing evidence that MIF plays a critical role in leptospiral LPS-induced pathogenesis.

The LD50 of *L. interrogans* serovar Autumnalis strain N2 was calculated as 100 leptospires in mice. As ISO-1 is a MIF antagonist, ISO-1 treatment in leptospires injected mice enhanced the clearance of leptospiral loads from the lung and protects the mice from clinical manifestations of leptospiral pathogenesis and death by increasing the survival rate when compared with leptospires alone injected mice. A median lethal dose of leptospiral infection causes severe lung injury in mice in a dose-dependent manner with the significant histological scoring (Score 9) of lung tissues. The histological alterations including immune cell infiltration, hemorrhage, and congestion of alveolar septa were predominant in $\geq 10^3$ leptospires infected mice. The proteins involved in inflammatory pathways (MIF, TLR-4, ICAM-1, VCAM, pCREB, CREB, P38, p-P38, P44/42, p-P44/42) and calcium-dependent CREB and pCREB are significantly expressed in leptospires injected mice in a dose-dependent manner. MIF inhibition enhanced the considerable reduction in histological scoring and inflammatory protein expression in infected lung tissues.

The increased TNF- α and IL-4 expression and decreased IL-10 expression were observed in infected lung tissues, which was attenuated by the loss of MIF.

MIF -173G>C SNP had a significant difference in genotype frequency among groups. GC genotype was more frequently observed in leptospirosis cases than in controls while GG genotype frequency was highly reported in healthy controls. No GG and CC genotype was found in severe leptospirosis and healthy control subjects, respectively. The distribution of the -173*C allele increases the risk of leptospirosis. The relative mRNA expression and serum MIF level of CC and GC carriers were significantly higher with respect to GG genotype carriers. The carriers of the MIF -173*C allele have significantly higher serum MIF levels than MIF -173G alleles. high MIF expression genotype GC and CC carriers had 10-fold and 11-fold upregulated mRNA expression of TNF-α respectively, whereas 9-fold expression of IL-1β and 11-fold expression of IL-4 when compared to GG carriers. Meanwhile, the anti-inflammatory cytokine IL-10 expression was significantly ($p<0.05$) downregulated in GC and CC genotype carriers. Thus, MIF -173 G/C gene polymorphism will be a suitable severity predictor of leptospirosis.

In vitro screening of 10 essential tautomerase inhibition classes of chemical compounds and 7 old anti-inflammatory drugs, our study identified the potential MIF inhibitor with high biocompatibility. As per the observation of screening experiments and cytotoxicity assay, ibudilast showed strong inhibitory effects on MIF enzymatic activity. 10μM of ibudilast showed the attenuated effect on leptospiral LPS mediated expression of inflammatory cytokines and other mediators such as ICAM-1, p-P38, and p-P44/42 MAPK, ROS production, $\Delta\Psi_m$ loss, and cell death in THP-1 cells. In BALB/c mice, the selected MIF inhibitor significantly reduced the leptospires mediated histopathological changes including alveolar haemorrhage, immune cell infiltration, and alveolar congestion, and also protects the mice from lethality. Thus, the non-

selective phosphodiesterase inhibitor, Ibudilast will be helpful to develop the MIF-directed therapeutic strategy for the treatment of leptospirosis.

Thus, in conclusion, serum MIF may be a promising early diagnostic marker for leptospirosis. As MIF plays a key role in the pathogenesis of leptospirosis, we suggested the MIF targeting therapeutic approaches will be beneficial to leptospirosis patients, especially with organ involvement, and also MIF -173G>C SNP will be a potent severity predictor of leptospirosis. We further suggested that the asthmatic drug, ibudilast will be a lead molecule of MIF-directed therapeutic strategy for the treatment of leptospirosis.

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Assessment of Serum Macrophage Migration Inhibitory Factor (MIF) as an Early Diagnostic Marker of Leptospirosis

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The search for valuable early diagnostic markers for leptospirosis is ongoing. The aim of the present study was to evaluate the diagnostic value of macrophage migration inhibitory factor (MIF) for leptospirosis. MIF is an immunoregulatory cytokine secreted by a variety of cell types involved in immune response and the pathogenesis of various diseases. It was previously described as a severity predictor of diseases. Samples of 142 leptospirosis cases, 101 other febrile cases, and 57 healthy controls were studied. The prevalence of leptospirosis was 47.3%. Autumnalis, Australis, and Canicola were the highly prevalent leptospiral serovars with a microscopic agglutination test (MAT) titer in the range 1:80–1:2,560. Enzyme-linked immunosorbent assay (ELISA) of MIF was carried out to measure the serum MIF levels. We found that the serum MIF levels [median, (interquartile range)] were significantly ($p < 0.001$) elevated in different clinical forms of leptospirosis, such as febrile illness [7.5 ng/ml (5.32–8.97)], pulmonary hemorrhage [13.2 ng/ml (11.77–16.72)], Weil's syndrome [8.8 ng/ml (7.25–9.95)], and renal failure [8.6 ng/ml (7.18–10.5)], than in healthy controls [0.65n g/ml (0.5–1.1)]. Serum MIF had sensitivity, specificity, positive predictive value, and negative predictive value of 100%, >90%, >90%, and 100%, respectively. Receiver operating characteristic (ROC) analysis revealed that the serum MIF levels between leptospirosis cases and control subjects had an area under the curve (AUC) value of >0.9 ($p < 0.0001$). In leptospirosis patients, elevation of serum MIF was significantly ($p < 0.001$) higher in severe cases with organ dysfunction [10 ng/ml (7.8–14.5)] than that in mild febrile cases [7.5 ng/ml (5.32–8.97)], with the difference of 2.5 indicating that serum MIF acts as a predictor of leptospirosis severity. Pearson's correlation test demonstrated that the serum MIF level was strongly correlated ($r = 0.75$, $p < 0.0001$) with disease progression. The median lethal dose (LD_{50}) of leptospiral lipopolysaccharide (LPS) in BALB/c mice was determined to be 20 mg/kg, which gave rise to endotoxemia. Leptospiral LPS triggered the upregulation of MIF expression at 24 h

post-infection, which reached the peak level at 24 h post-treatment in THP-1 cells and showed elevated MIF expressions in different tissues of BALB/c mice at the early stage of infection. Taken together, MIF is an early-phase cytokine that could serve as a rapid diagnostic marker for leptospirosis.

Keywords: leptospirosis, macrophage migration inhibitory factor, diagnostic marker, MIF ELISA, lipopolysaccharide

INTRODUCTION

Leptospirosis is a spirochaetal zoonotic disease caused by pathogenic leptospires infecting both humans and animals. It has a spectrum of clinical presentations ranging from self-limiting mild, nonspecific flu-like illness, to severe fatal conditions (Haake and Levett, 2015). The mortality and morbidity remain significant, and every year, 1.03 million people are affected worldwide (Costa et al., 2015). The signs and symptoms of leptospirosis simulate those of well-known diseases such as typhoid, dengue, malaria, and acute hepatitis (Safa et al., 2017), leading to underdiagnosis and inadequate timely treatment (Izurieta et al., 2008). The two major limitations that complicate leptospirosis management are the lack of knowledge on the importance of disease considerations and the lack of early and accurate diagnostic tools. Hence, frequent estimation of the disease prevalence in tropical regions and the development of early diagnostic markers are urgently needed.

Currently, leptospirosis represents a major challenge in the healthcare system. It has been reported throughout the world, especially in tropical and subtropical regions. In India, leptospirosis is endemic in seven states, including Tamil Nadu, Kerala, Karnataka, Maharashtra, and Gujarat, and in one union territory, Andaman and Nicobar Islands, especially in North and South Andaman (Loganathan and Shivakumar, 2008). Natural catastrophes such as flooding and cyclones can increase human exposure to leptospires, which leads to its outbreak (Kobayashi, 2005). During the post-monsoon, the outbreak of leptospirosis occurs almost every year in Andaman Islands, especially in regions where people engage in agricultural activities (Sehgal et al., 1995; Vijayachari et al., 2004; Sugunan et al., 2009). In Kerala, more than 1,000 leptospirosis cases are reported every year, and it has the greatest mortality rate compared to other contagious diseases. Indeed, a noticeable outbreak in Kerala has been recently reported in 2018 (James et al., 2018). Epidemics of leptospirosis are often reported in urban areas, such as Chennai and Mumbai (Bharadwaj et al., 2002; Loganathan and Shivakumar, 2008; Loganathan et al., 2012).

Although it has been reported for decades, the problem of leptospirosis is not well documented in developing countries. Frequent estimation of the disease prevalence and the incidence rates in high-risk regions is required, serving as an important public health tool to prevent and control the disease outbreak (Ward, 2013). As leptospirosis is still underdiagnosed and underreported in Tamil Nadu, our research team has frequently investigated and reported the prevalence of leptospirosis in Tiruchirappalli (Vedhagiri et al., 2013; Prabhakaran et al., 2014; Vanithamani et al., 2015; Raja et al., 2016; Kanagavel et al., 2017), where 70% of the population engage in agriculture and allied activities. The preliminary part of the present study was designed to evaluate the disease burden in Tiruchirappalli from June 2017 to February 2018.

Another crucial complication with leptospirosis is the limitation of a rapid diagnosis (Rajapakse et al., 2015). The standard serological assays are dependent on circulating antibodies and thereby fail to diagnose the early phase of leptospirosis (Toyokawa et al., 2011). Misdiagnosis in the early phase of the disease would lead to the life-threatening severe form of leptospirosis with multi-organ involvement, including renal failure, liver dysfunction, pulmonary hemorrhage, and meningitis, which bring about dramatically increased mortality rates (Cagliero et al., 2018). Previous investigations suggested that the development of rapid diagnostic markers and the initiation of antibiotic therapy can achieve successful treatment of leptospirosis (Toyokawa et al., 2011). Recent research on leptospirosis has mainly focused on the identification of novel biomarkers with high predictive value. Previous studies suggested that host immunological factors can serve as diagnostic markers and are deemed to play critical roles in the progression into severe leptospirosis (Zuerner, 2015). Several host mediators have been investigated as potential biomarkers of leptospirosis, such as human serum (mannose binding lectin, MBL) (Miranda et al., 2009), interleukin 6 (IL-6), IL8, IL-10, soluble suppression of tumorigenicity 2 receptor (Wagenaar et al., 2009a), pentraxin 3, and copeptin (Wagenaar et al., 2009b). In this study, we propose the consideration of serum macrophage migration inhibitory factor (MIF) for the diagnosis of leptospirosis because the expression of MIF is significantly elevated at the early stage of disease induction.

MIF is a pro-inflammatory cytokine that acts as a potential regulator of host response to infection (Calandra and Roger, 2003). MIF is an approx. 12.5-kDa highly conserved secreted protein (Yang et al., 2017) with pro-inflammatory and immunomodulatory activities. It has been involved in several immunological processes such as leukocyte recruitment, inflammation, immune response, cell proliferation, tumorigenesis, and counter-regulation of glucocorticoids (Chen et al., 2017). Growing evidence supports the correlation between serum MIF concentration and the pathogenesis of several inflammatory diseases (Kithcart et al., 2010). More recently, emphasis has been on the use of serum MIF as an early diagnostic marker for diseases to achieve early diagnosis and improve disease management. Previous investigations showed that elevated cytokine production plays a predominant role in the development of severe leptospirosis (Cagliero et al., 2018). In this case–control study, we determined the disease prevalence

and proposed the use of serum MIF as a potential early diagnostic tool to successfully combat the outbreak of leptospirosis in developing countries.

MATERIALS AND METHODS

Study Design and Study Site

A case–control study was conducted to estimate the prevalence of leptospirosis in Tiruchirappalli, to determine the serum levels of MIF in leptospirosis patients compared with other febrile cases and healthy control subjects, and to assess the leptospiral lipopolysaccharide (LPS)-mediated MIF upregulation in *in vitro* and *in vivo* experimental models. This study was carried out from June 2017 to February 2018 by active hospital-based surveillance at the Annal Gandhi Memorial General Hospital, Tiruchirappalli, Tamil Nadu, India. The temperature in the study area ranges from 36°C to 41°C, and its geographical position is 10°48′18″ N latitude and 78°41′08″ E longitude.

Study Population and Case Definition

A total of 300 study subjects were recruited to participate in the study. In total, 243 blood samples from clinically suspected cases with clinical manifestations such as fever, myalgia, body ache, arthritis, icterus, rigors, breathlessness, abdominal pain, conjunctival suffusion, subconjunctival hemorrhages, and jaundice with acute renal failure were collected for the diagnosis of leptospirosis. The samples were collected before any treatment was given to the patients. A total of 57 seronegative healthy controls recruited from the general population in the same geographical area matched for age (± 5 years) and sex were included as controls. Healthy control subjects who had fever in the previous 2 weeks were excluded from the study. The obtained serum samples were divided into aliquots and stored at −80°C until the assay was performed.

Live Antigens and Microscopic Agglutination Test

For serological evidence of leptospirosis in the study population, microscopic agglutination test (MAT) was performed using a panel of 12 live leptospiral serovars. Leptospiral cultures were maintained by regular sub-culturing in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium supplemented with bovine serum albumin and Tween-80 at the Medical Microbiology Laboratory, Bharathidasan University, Tiruchirappalli. The following serogroups were used as live antigens: Australis (serovar Australis, strain Ballico), Autumnalis (serovar Autumnalis, strain Akiyami A), Ballum (serovar Ballum, strain Mus 127), Bataviae (serovar Bataviae, strain Swart), Canicola (serovar Canicola, strain Hond Utrecht IV), Icterohaemorrhagiae (serovar Icterohaemorrhagiae, strain RGA), Grippotyphosa (serovar Grippotyphosa, strain Moskva V), Hebdomadis (serovar Hebdomadis, strain Hebdomadis), Javanica (serovar Poi, strain Poi), Pomona (serovar Pomona, strain Pomona), Pyrogenes (serovar Pyrogenes, strain Salinem), and Sejroe (serovar Hardjo, strain Hardjoprajitno). Seven-day-old live leptospiral culture of 1×10^8 organisms/ml was used as the antigen. Double dilution of serum was performed serially starting from 1:20 and incubated with live antigens for agglutination. A titer of ≥1:160 and agglutination of ≥50% were considered as positive for MAT. Phosphate-buffered saline (PBS) was used as a diluent in the assay.

IgM Enzyme-Linked Immunosorbent Assay

Immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) was performed to further confirm leptospiral infection in suspected cases by assessing the early immune response of cases to leptospirosis. Heat-extracted leptospiral antigens were prepared as described earlier (Kanagavel et al., 2017). Of the leptospiral antigens, 0.2 μg was coated on 96-well microtiter plates at appropriate wells using carbonate coating buffer (pH 9.6) and then stored at 4°C for 12 h. Each well was washed three times with PBST (PBS + 0.1% Tween-20) for 10 min each. About 3% blocking solution (non-fat milk) was added to each well and incubated at 37°C for 1 h. Each well was washed, as previously mentioned. The test sera were added into appropriate wells at a dilution of 1:100 and incubated at 37°C for 1 h. After washing the wells, the bound IgM antibody was detected by adding peroxide-conjugated anti-human IgM antibody (1:1,000) and incubated at 37°C for 1 h, followed by developing with *o*-phenylenediamine dihydrochloride (OPD). Fifty microliters of 1 N H_2SO_4 was added to stop the reaction, and then the optical density was measured at 490 nm using a microtiter plate reader.

MIF Immunoassay

Quantitative measurement of human MIF in patient sera was carried out using Human MIF ELISA Kit (Sigma-Aldrich, St. Louis, Mo, USA). All procedures were in accordance with the manufacturer's instructions. In brief, all reagents and samples were allowed to reach 18–25°C before use. All samples and standards were performed in triplicate. Of the samples, 100 μl was added into microtiter wells and incubated for 3 h at room temperature with gentle shaking. Then, the solution was discarded and the wells washed four times with 1× wash buffer. Subsequently, 100 μl of 1× biotinylated detection antibody was added to each well and incubated for 1 h at room temperature with gentle shaking. Following washing, 100 μl of horseradish peroxidase (HRP)–streptavidin solution was added to each well and incubated for 45 min at room temperature with gentle shaking. The wells were again washed and 100 μl of ELISA colorimetric 3,3′,5,5′-tetramethylbenzidine (TMB) reagent was added to each well and incubated for 30 min at room temperature in the dark. Fifty microliters of the stop reagent was added to each well and the plates immediately read at 450 nm. The mean absorbance values for each set of standards, controls, and samples were calculated and the average zero standard optical densities were subtracted for background correction. The standard curve was plotted using SigmaPlot 11.0 software, with the standard concentration on the *x*-axis and absorbance on the *y*-axis to quantify the MIF. Serum MIF levels were expressed as nanograms per milliliter.

Mouse-Adapted Challenge Strain

Mouse-adapted challenge strains (MACS) were prepared from their corresponding parent strains (reference laboratory strains/isolates) by passaging them in immunocompromised BALB/c mice (300 mg/kg body weight of cyclophosphamide treatment)

~15 times (Adler and Faine, 1976; Adler and Faine, 1977). The cultures used in all the experiment in the current study were *Leptospira interrogans* serovar Autumnalis strain N2 (human isolate) MACS passaged *in vitro* less than three times (Kanagavel et al., 2017).

Extraction of LPS From MACS

L. interrogans serovar Autumnalis strain N2 MACS were grown in liquid EMJH medium at 30°C and collected at a density of ~5 × 10^8 leptospires/ml. Leptospiral LPS was extracted with the standard hot phenol–water method (Westphal and Jann, 1965). The phenol phase was purified by dialysis. The extracted LPS were quantified using the phenol/sulfuric acid method (Vanithamani et al., 2015).

Cell Culture and LPS Induction *In Vitro*

THP-1 cells were purchased from the National Centre for Cell Science, Pune, and cultured in RPMI 1640 medium with 10% fetal bovine serum at 37°C and 5% CO_2. The cells were pelleted and washed three times with serum-free culture medium. Approximately 5 × 10^5 cells per well were added into six-well plates and then treated with LPS (1 μg/ml) at different time intervals (0, 5, 10, 20, and 30min and 1, 2, 3, 6, 12, 24, 48, and 60 h). All treatments were performed in triplicate. Treated cells were pelleted for further analysis.

BALB/c Mice and Determination of LPS LD_{50}

An inbred strain of BALB/c mice (weighing about 20 ± 2 g) was used throughout the study. To accustom the mice, they were housed under ambient room temperature (25 ± 2°C), with a 12-h light/dark cycle, and given standard feed and water *ad libitum* over a period of 10 days before the start of the experiments. Mice 4–6 weeks old were separated into five groups. Five mice (immunocompromised) in each group were challenged (intraperitoneally) with different doses (5, 10, 20, and 30 mg/kg) of *L. interrogans* serovar Autumnalis strain N2 for the determination of the survivability of the BALB/c mouse model. Mice injected with PBS were considered as untreated control. Survival over 4 days was frequently evaluated and the LD_{50} of leptospiral LPS determined.

Detection of Endotoxemia in the Mouse Model

Dot blot assay and cytokine profiling were performed to examine endotoxemia in LD_{50} LPS-injected mice. Blood sample was collected from the control (PBS-injected) and LPS-injected mice and the plasma separated for analysis. Two miroliters of plasma samples and 5 μg purified LPS were loaded into a 0.2-μm nitrocellulose membrane, probed with patient sera (1:100), and incubated for 1 h at room temperature. Anti-human IgM (ALP-conjugated) was added to the membrane and incubated for 1 h. The membrane was washed for 30 min with PBST after every incubation step, developed with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA), and documented in Fusion Solo™ Personal Blot and Gel Imaging System (Vilber Lourmat, Paris, France). Dot intensity was measured densitometrically and expressed in arbitrary units.

Quantitative real-time PCR (qRT-PCR) was carried out to measure the expressions of the inflammatory cytokines [tumor necrosis factor alpha (TNF-α), IL-1β, IL-4, and IL-10] of mice injected with LD_{50} LPS. Monolayer cells from the plasma were collected and RNA was isolated with the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). Complementary DNA (cDNA) was synthesized and qRT-PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The primers used in this study are listed in **Supplementary Table S1**.

LPS Induction *In Vivo*

The mice to be injected with leptospiral LPS were primarily immunocompromised with cyclophosphamide (300 mg/kg body weight). The mice were subjected to LPS exposure by intraperitoneal injection. The untreated control group was maintained by injecting with PBS alone. The dosage of LPS was 20 mg/kg body weight. Infected mice were monitored every 12 h for clinical outcomes and survival up to 8 days. Dead and moribund animals were euthanized for harvesting the tissues. Vital organs including the heart, lungs, kidney, spleen, and liver and lymphoid organs such as the thymus, bone marrow, spleen, and lymph node were collected and stored in liquid nitrogen for MIF profiling experiments.

Protein Extraction and Western Blotting

The collected THP-1 cells and homogenized tissues were washed three times with ice-cold PBS. Cell lysates were prepared using ice-cold RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, and 1% NP-40) (Thermo Fisher Scientific, Waltham, MA, USA) and stored at −20°C. The extracted protein was quantified using the bicinchoninic acid (BCA) method. Thirty micrograms of protein per well was loaded onto 12% polyacrylamide gel and electrophoresed at 60 V in Mini-PROTEAN Tetra System (Bio-Rad, Hercules, CA, USA). The separated proteins were transferred into a nitrocellulose membrane (pore size, 0.2 μm) electrophoretically at 12 V for 1 h using a V20 semi-dry blotter (Scie-Plas, Cambridge, UK). Subsequently, the membranes were blocked with a blotting grade blocking solution (5%, *w*/*v*) at room temperature for 1 h, washed three times for 10 min each with 1× TBST (TBS + 0.1% Tween-20), and incubated with rabbit anti-MIF antibody (1:1,000; Invitrogen, Carlsbad, CA, USA) at 4°C for 12 h. Bound antibodies were detected by incubating with HRP-conjugated anti-rabbit antibody (1:5,000; Sigma-Aldrich, St. Louis, MO, USA). Bands were developed with the West Pico Signal Chemiluminescence developing kit (Thermo Fisher Scientific, Waltham, MA, USA) and documented in Fusion Solo™ Personal Blot and Gel Imaging System (Vilber Lourmat, Paris, France). Band intensities were calculated using ImageJ software, and the data were normalized to the loading control.

Quantitative Real-Time PCR Analysis

RNA was extracted from the harvested tissues and THP-1 cells using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA, USA). The concentration and the purity of mRNA were determined using the BioPhotometer Plus system (Eppendorf, Hamburg, Germany). cDNA was synthesized

using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). qRT-PCR was performed using a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) The primers used in this study are listed in **Supplementary Table S1**. The qRT-PCR using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) and primers was carried out in a 10-µl reaction volume (20 ng cDNA, 5 µl Master Mix, and 0.2 µM of each primer). GAPDH was used as the loading control.

Statistical Analysis

Data from triplicate experiments were quantified and expressed as the mean ± SD (n = 3). Serum MIF levels with outlier points were expressed as median (interquartile range, IQR). Data were computed either with GraphPad Prism version 9.2.0 or SigmaPlot 11.0 software. Receiver operating characteristic (ROC) analyses were performed to estimate the sensitivity and specificity of MIF as a diagnostic marker for leptospirosis. Pearson's correlation coefficient test was performed to assess the correlation of serum MIF with disease duration and patient age. A two-tailed paired Student's t-test or the Mann–Whitney U test was performed to analyze differences between the study groups. Kaplan–Meier plots were generated using GraphPad Prism version 7.0 to quantify survivability. A p-value ≤0.05 was considered significant.

Ethics Statement

The studies involving human participants (for the collection of blood samples) were reviewed and approved by the Institutional Ethical Committee (no. DM/2014/101/51) of Bharathidasan University. Informed consent was obtained from both patients and healthy controls prior to the sample collection; in the case of minor study participants, their surrogates signed an informed consent form. The animal experimental protocols were approved by the Institutional Animal Ethical Committee (BDU/IAEC/P30/2018), Bharathidasan University.

RESULTS

Seroprevalence of Leptospirosis

The seroprevalence of leptospirosis was determined with the MAT assay with the panel of 12 leptospiral serovars as an antigen. Out of the 300 serum samples tested for antibodies against pathogenic *Leptospira*, a total of 142 cases tested positive at agglutination titer of ≥1:80, giving an overall seroprevalence of 47.3%. Seropositivity was uniformly distributed in both genders and in all age groups. The demographic characteristics of the clinical subjects included in the study are shown in **Table 1**.

Of the 12 leptospiral serovars tested, six were detected among the samples from the study subjects. The prevalent infecting serovars were Autumnalis (50.7%), followed by Australis (21.2%), Canicola (16.2%), Icterohaemorrhagiae (15.6%), Grippotyphosa (3.5%), and Ballum (2.9%). MAT titers were reported to be in the range between 1:80 and 1:2,560. The MAT-positive titers with the respective serovars are represented in **Table 2**. Among the 142 patients who tested positive for MAT, 9 (6.3%) cases were negative for IgM ELISA.

Elevated Serum MIF in Leptospirosis Cases

To identify whether MIF protein was differentially expressed between leptospirosis patients and healthy control subjects, we used the sera of the study subjects to assess circulating MIF concentrations with MIF ELISA. The cutoff point for a positive MIF level was 0.008 ng/ml. As shown in **Figure 1A**, it was found that the serum MIF levels were significantly ($p < 0.001$) elevated in different clinical conditions of patients with leptospirosis, including febrile illness (median = 7.5 ng/ml, IQR = 5.32–8.97), pulmonary hemorrhage (median = 13.2 ng/ml, IQR = 11.77–16.72), Weil's syndrome (median = 8.8 ng/ml, IQR = 7.25–9.95), and renal failure (median = 8.65 ng/ml, IQR = 7.18–10.5), compared to those in healthy controls (median = 0.65 ng/ml, IQR = 0.5–1.1) and other febrile cases such as typhoid (median = 1.32 ng/ml, IQR = 0.57–1.9), malaria (median = 1.1 ng/ml, IQR = 0.57–1.35), dengue (median = 2.2 ng/ml, IQR = 1.58–2.8), hepatitis (median = 1.32 ng/ml, IQR = 0.62–1.56), syphilis (median = 1.9 ng/ml, IQR = 1.46–2.2), shigellosis (median = 1.31 ng/ml, IQR = 0.67–1.5), and enteritis (median = 1.2 ng/ml, IQR = 0.8–1.85). Patients with pulmonary hemorrhage have remarkably higher MIF levels than with other clinical manifestations. The MIF levels of all study subjects are presented in **Table 3**.

ROC Curve Analysis of MIF as a Candidate Biomarker

To estimate the diagnostic value of serum MIF as a biomarker for leptospirosis, ROC curve analysis was performed. ROC analysis revealed that the serum MIF levels discriminated significantly between leptospirosis cases and healthy control subjects. The area under the curve (AUC) of the different clinical

TABLE 1 | Demographic characteristics of the study subjects.

S. no.	Variables		Confirmed leptospirosis cases	Other febrile cases	Heathy control subjects
1	Age (years)	Range	6–75	5–75	8–74
		Mean ± SD	34.2 ± 17.7	28 ± 16.8	35.8 ± 18.0
2	Sex (%)	Female	48	43.5	54.4
		Male	52	56.5	45.6
3	Duration of disease (days)	Range	7–31	2–41	–
		Mean ± SD	19.8 ± 7	11.9 ± 7.5	–
4	Serology	MAT titers (range)	1:80–1:2,560	–	–
		IgM ELISA titer	1:100	–	–

MAT, microscopic agglutination test; IgM, immunoglobulin M.

TABLE 2 | Serovar distribution and MAT titers in the study subjects.

Serovar	Frequency, *n* (%)	Median MAT titers	1:80 (%)	1:160 (%)	1:320 (%)	1:640 (%)	1:1280 (%)	1:2560 (%)
Autumnalis	72 (50.7)	1:640	12.5	13.8	16.6	33.3	16.6	6.9
Australis	30 (21.1)	1:640	16.6	16.6	13.3	26.6	23.3	3.3
Canicola	23 (16.2)	1:320	17.3	17.3	26	26	8.6	4.3
Icterohaemorrhagiae	8 (15.6)	1:640	12.5	12.5	0	50	12.5	12.5
Grippotyphosa	5 (3.5)	1:320	20	20	40	20	20	0
Ballum	4 (2.9)	1:160	25	25	50	0	0	0

MAT, microscopic agglutination test.

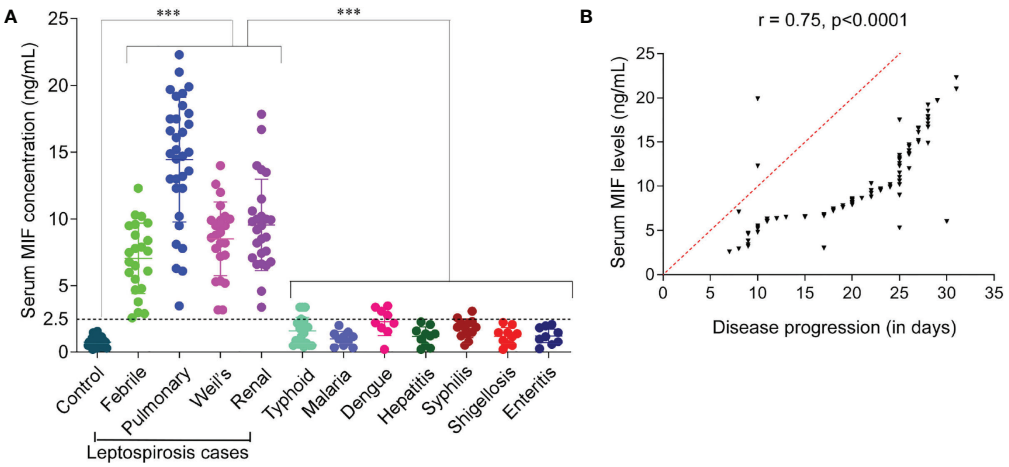

FIGURE 1 | Serum macrophage migration inhibitory factor (MIF) profiling and analysis of its diagnostic value for the early diagnosis of leptospirosis. **(A)** Profiling of MIF in sera of patients with leptospirosis with different clinical manifestations, other febrile illnesses, and healthy controls. Study groups are indicated on the *x*-axis and the MIF concentration on the *y*-axis. *Dotted line* represents the cutoff with the absolute values on the *left*. $n = 3$ experiments. ***$p < 0.001$. **(B)** Pearson's correlation coefficients between serum MIF levels and disease progression. This analysis showed that serum MIF was positively correlated with disease progression, which indicates the contribution of serum MIF to disease severity ($r = 0.75$, $p < 0.0001$), acting as a severity predictor.

manifestations of leptospirosis was >0.9 ($p < 0.0001$) (**Supplementary Figures S1A–D**). An AUC value >0.9 was considered as an outstanding quality of discrimination between groups. The optimal cutoff value for MIF was predicted as 2.5 ng/ml by stressing the higher sensitivity. The sensitivity and the specificity of serum MIF profiling for leptospirosis cases were found to be 100% and >90%, respectively, in different clinical forms of leptospirosis. The positive predictive value (PPV) and the negative predictive value (NPV) were >90% and 100%, respectively. The sensitivity/specificity values and the PPV/NPV for the different clinical manifestations of leptospirosis are shown in **Table 4**. Our results demonstrated that the levels of serum MIF were significantly upregulated in leptospirosis patients compared to those in healthy control subjects and that it will be possible to use MIF as a biomarker to improve disease monitoring and management.

Serum MIF Level Correlates With Leptospirosis Disease Progression and Severity

The difference in the serum MIF levels between leptospirosis cases with febrile illness (mild, non-hospitalized) and those with organ dysfunction (severe, hospitalized) was significantly high (2.5, $p < 0.001$). Our results revealed that the serum MIF levels were more significantly elevated in patients with severe than in those with mild leptospirosis. Here, we analyzed whether there was any association between serum MIF and leptospirosis disease progression. We evaluated the serum MIF levels associated with disease progression using Pearson's correlation test and found a significantly high positive correlation ($r = 0.75$, $p < 0.0001$). A correlation coefficient index (r) of ≥0.7 was considered as a high positive correlation. There was no significant correlation found in the serum MIF levels of study subjects in respect to age ($r = 0.006$). An r value of <0.3 was considered as negligible correlation between variables. The correlation of elevated serum MIF with the disease progression of leptospirosis is represented in **Figure 1B**. The analysis suggested that serum MIF may serve as a severity predictor of human leptospirosis.

Determination of LPS LD_{50} and Endotoxemia in BALB/c Mice

For the determination of LD_{50}, LPS-challenged mice were frequently evaluated for disease progression and moribund state. The number of deceased mice was recorded to plot the Kaplan–Meier survival graph. The LD_{50} for leptospiral LPS was found to be 20 mg/kg, which caused the death of about 50% of the mice. Mice

TABLE 3 | Serum macrophage migration inhibitory factor (MIF) levels in patients and healthy controls.

Study subjects (*n*)	Serum MIF level (ng/ml)		
	Range	Median (interquartile range)	*p*-value
Laboratory-confirmed leptospirosis cases (142)			
Febrile illness (41)	2.6–12.3	7.5 (5.32–8.97)	<0.001
Pulmonary hemorrhage (37)	3.5–22.3	13.2 (11.77–16.72)	<0.001
Weil's syndrome (35)	3.2–14	8.8 (7.25–9.95)	<0.001
Renal syndrome (29)	3.4–17.85	8.65 (7.18–10.5)	<0.001
Other febrile cases (101)			
Typhoid (40)	0.4–3.4	1.62 (0.57–1.9)	1
Malaria (10)	0.32–2.03	1.1 (0.57–1.35)	1
Dengue (9)	0.2–3.5	2.23 (1.58–2.8)	1
Hepatitis (10)	0.2–2.3	1.32 (0.62–1.56)	1
Syphilis (13)	0.52–3.1	1.9 (1.46–2.2)	1
Shigellosis (10)	0.2–2.25	1.31 (0.67–1.5)	1
Enteritis (9)	0.25–2.1	1.2 (0.8–1.85)	1
Healthy controls (57)	0.2–1.6	0.65 (0.5–1.1)	–

TABLE 4 | Receiver operating characteristic (ROC) analysis of macrophage migration inhibitory factor (MIF) as an early diagnostic marker for leptospirosis.

Clinical parameters	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC ± SE	*p*-value
Febrile illness	100	91.7	91.54	100	0.9910 ± 0.006	<0.0001
Pulmonary hemorrhage	100	99	98.9	100	0.9999 ± 0.0003	<0.0001
Weil's syndrome	100	95.4	95.12	100	0.9960 ± 0.003	<0.0001
Renal syndrome	100	95.4	95.12	100	0.9989 ± 0.001	<0.0001

PPV, positive predictive value; NPV, negative predictive value; AUC, area under the curve.

challenged with 5, 10, and 30 mg/kg of LPS exhibited 80%, 80%, and 20% of survival, respectively (**Figure 2A**). Thus, we used 20 mg/kg of leptospiral LPS for subsequent experiments.

The dot blot immunoassay detected the significant occurrence of LPS in the blood stream of infected mice. This confirmed that the intraperitoneal injection of LD_{50} LPS induced endotoxemia in the mouse model (**Figures 2B, C**). Endotoxemic inflammation after injection of LD_{50} LPS was characterized by analysis of the cytokine expressions. The qRT-PCR results showed a significant (15-fold) increase of the expressions of TNF-α and IL-1β and a threefold increase of the expression of IL-4 (**Figure 2D**). The high expressions of pro-inflammatory

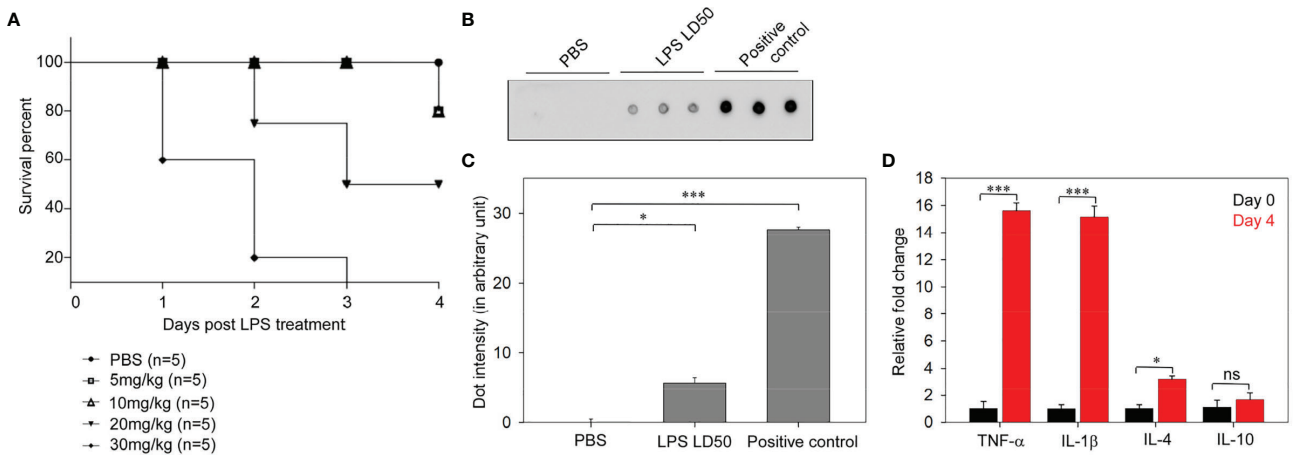

FIGURE 2 | Determination of lipopolysaccharide (LPS) median lethal dose (LD_{50}) of *Leptospira interrogans* serovar Autumnalis strain N2 in BALB/c mice. **(A)** Representative Kaplan–Meier plot of LD_{50} determinations showing the survivability at different doses (5, 10, 20, and 30 mg/kg) of leptospiral LPS-injected mice. **(B)** Dot blot assay of the LD_{50} (10 mg/kg) of leptospiral LPS causing endotoxemia in infected mice. Plasma samples of mice injected with PBS and LD_{50} LPS were assayed. Purified LPS was loaded as a positive control. **(C)** Representative graph of the dot intensity of samples. **(D)** Analysis of the expressions of inflammatory cytokines in the leptospiral LPS-injected mouse model by qRT-PCR. $n = 3$ experiments. $^{*}p < 0.05$; $^{***}p < 0.001$, ns, no significant.

cytokines in LPS-infected mice revealed endotoxemic inflammation. Therefore, the administration of leptospiral LD_{50} LPS induced endotoxemic inflammation in BALB/c mice.

MIF Profiling *In Vitro* and *In Vivo*

To demonstrate the MIF expression profile during the early phase of leptospiral infection, we have experimentally induced leptospiral infection in *in vitro* and *in vivo* models. Cell lysates prepared from THP-1 cells treated with leptospiral LPS (1 µg/ml) were assayed using Western blotting to determine the intracellular MIF profile (**Figures 3A, D**). MIF expression was at the baseline in untreated cells. Leptospiral LPS significantly ($p < 0.001$) increased the expression level of MIF in a time-dependent manner. The expression of MIF in THP-1 cells was markedly increased at 30 min post-treatment, which gradually upregulated and reached the peak level at 24 h post-treatment and then significantly diminished at 60 h.

To assess the elevated MIF expression in lymphoid organs and other vital organs during leptospiral pathology, tissue samples were harvested from leptospiral LPS-administered BALB/c mice and were assayed with Western blotting. The results showed significantly ($p < 0.01$) upregulated MIF expression in vital organs post-LPS injection, particularly in the lung, spleen, and heart compared to other organs (**Figures 3B, E**). As MIF is an immune mediator, we investigated the regulation of its expression in both primary and secondary lymphoid organs, including the thymus,

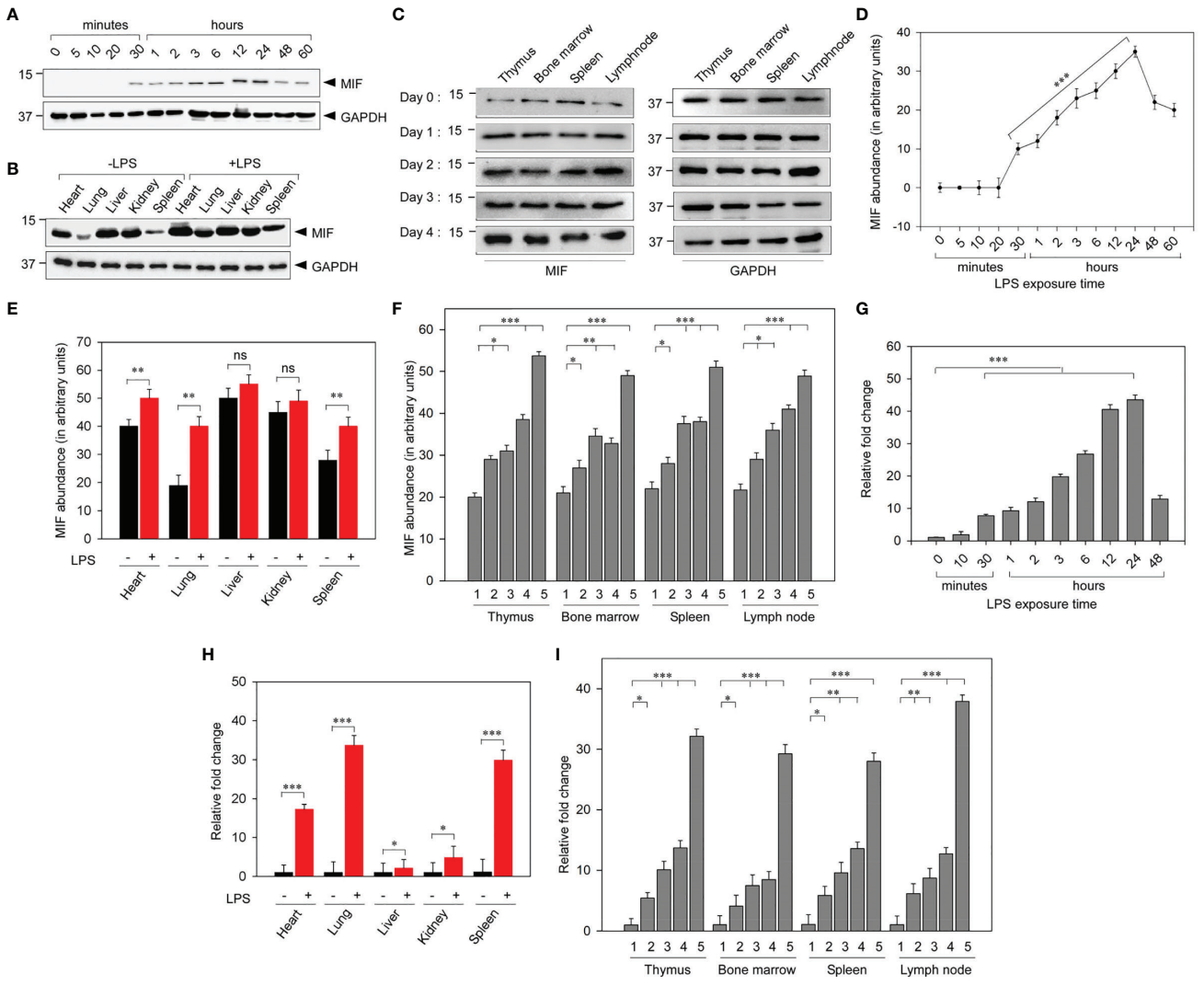

FIGURE 3 | Analysis of the macrophage migration inhibitory factor (MIF) profile in leptospiral lipopolysaccharide (LPS)-induced experimental models. **(A)** Representative Western blot analysis of the increased expression of MIF in LPS-treated THP-1 cells in a time-dependent manner. **(B)** Representative Western blot analysis of the significantly upregulated expression of MIF in the lungs and spleen among the vital organs of LPS-induced BALB/c mice. **(C)** Representative Western blot analysis of the progressive MIF upregulation in primary and secondary lymphoid organs at different time intervals. **(D–F)** Quantification of MIF abundance by densitometric measurement from **(A–C)**, respectively. **(G–I)** Analyses of leptospiral LPS-stimulated MIF mRNA expressions in THP-1 cells **(G)** and in vital organs **(H)** and lymphoid organs **(I)** of BALB/c mice by qRT-PCR. *1*, day 0; *2*, day 1; *3*, day 2; *4*, day 3; *5*, day 4. $n = 3$ experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. ns, no significant.

bone marrow, spleen, and lymph node, on days 0, 1, 2, 3, and 4 post-LPS treatment. The expression of MIF was significantly increased in all lymphoid organs on days 1, 2, 3, and 4 post-LPS treatment when compared with day 0. In untreated control mice, differential MIF expression was observed in vital organs, whereas no significant difference in its expression was noticed between lymphoid organs (**Figures 3C, F**).

Furthermore, RT-PCR analysis confirmed the LPS-mediated upregulated *MIF* gene expression by measuring the increased MIF mRNA levels. Leptospiral LPS gradually upregulated the *MIF* gene expression in a time-dependent manner in LPS-treated THP-1 cells (**Figure 3G**). As *in the vitro* MIF protein profile, the *MIF* gene expression was remarkably increased in the lung, spleen, and heart of LPS-injected mice (**Figure 3H**). *MIF* gene expression analysis on lymphoid organs at different time intervals revealed that the mRNA expression was gradually increased in all lymphoid organs up to day 3 and drastically increased on day 4. No significant morphological changes were observed in the lymphoid organs of mice injected with LD_{50} LPS (**Figure 3I**). These results confirmed that MIF is an early-phase secreted cytokine and that its use as a biomarker will be a promising early diagnostic tool for leptospirosis. Taken together, the mRNA and protein expressions of MIF were significantly increased as the duration of infection increased, with MIF possibly driving the pathogenesis of leptospirosis.

DISCUSSION

We began this study with the determination of leptospirosis prevalence in Tiruchirappalli district. It was found that evaluation of the serum MIF levels of patients has diagnostic implications for leptospirosis. To the best of our knowledge, our study revealed, for the first time, that MIF-specific ELISA may be a promising biomarker for the early diagnosis of leptospirosis. In this study, we also explored the MIF protein expression profile in leptospiral LPS-induced *in vitro* and *in vivo* models to confirm MIF as an early-phase secreted cytokine. We are aware that our research may have a limitation, which is the period of clinical investigation from 2017 to 2018. Therefore, the seroprevalence of leptospirosis may not match those of current investigations.

Leptospirosis is a neglected tropical zoonotic disease with a high disease burden and high mortality rates in developing countries due to the limited knowledge of physicians on leptospirosis, difficulty of obtaining early diagnosis, and the delayed initiation of effective treatments. The primary step of disease management is to attain knowledge regarding the disease prevalence in the area and develop an early diagnostic tool. Here, we reported the significant percentage of disease burden and the prevalent serovars, which included Autumnalis, Australis, Canicola, Icterohaemorrhagiae, Grippotyphosa, and Ballum. This report addressed the importance of disease consideration in tropical regions and increased the research base of leptospirosis to accomplish complete documentation of the disease. Our study also suggested that the prevalent serovars should be included in the panel of MAT antigens in clinical laboratories of the study area in order to minimize false-negative results.

Currently, various serological tests are available for the diagnosis of leptospirosis, such as MAT, ELISA, macroscopic agglutination test, microcapsule agglutination, and the dipstick assay (Vanithamani et al., 2015). Despite being the gold standard reference for leptospirosis, serogroup-specific MAT assay is complex to perform and has shown technical limitations and low sensitivity in patients with the early phase of the disease. Indeed, MAT exhibited sensitivity values of 41%, 82%, and 96% in the first, second to the fourth, and after the fourth week of the onset of disease, respectively (Musso and La Scola, 2013). During an outbreak, these complex methods are not suitable for evaluating a large quantity of samples. A novel diagnostic biomarker that has high sensitivity and specificity and is affordable is urgently required in regions of developing countries with high leptospirosis prevalence in order to combat frequent disease outbreaks. The available diagnostic assays are dependent on the activation of the adaptive immune response, whereas the IgM antibody is detectable only at 5–7 days post-infection, which leads to delayed antibiotic therapy for patients. Leptospirosis patients begin to worsen if they are not properly treated within 2–3 days (Kobayashi, 2005; Musso and La Scola, 2013). A misdiagnosed and untreated early phase of leptospirosis causes cytokine storm and tissue damage, which lead to the development of severe leptospirosis with multi-organ dysfunction (Cagliero et al., 2018). However, the currently evaluated diagnostic marker (MIF) is an early expressed inflammatory cytokine, which may serve as a promising early diagnostic marker for leptospirosis. MIF ELISA exhibited sensitivity of 100% and specificity of more than 90% for leptospirosis.

Previous studies reported a wide range of cytokines secreted by the host immune system during leptospirosis. Especially, TNF-α and the interleukins IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10 were elevated in severe leptospirosis cases, whereas TNF-α, IL-6, IL-8, IL-10, interferon-γ, and soluble TNF receptor 1 were elevated in high fatality cases (Senavirathna et al., 2020). The hemolysin of *L. interrogans* acts as a pro-inflammatory stimulator that triggers the production of cytokines by the Toll-like receptor 2- and 4-dependent JNK (c-Jun N-terminal kinase) and NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cell) pathways (Wang et al., 2012). Patients' immune response to leptospirosis, especially the cytokine production, causes the variations of disease outcomes. As inflammatory cytokines are secreted during the early stages of infection, the implication of these cytokines as diagnostic biomarkers facilitates the detection of the acute phase of the disease.

In recent decades, researchers have focused on the contribution of MIF in inflammatory diseases. MIF is an immunoregulatory/inflammatory cytokine that is differentially expressed between patients and healthy controls. Normally, MIF circulates at a concentration of 2 ng/ml in human blood plasma. During infection, the MIF level is drastically elevated, which makes it possible to function as a biomarker for specific diseases (Grieb et al., 2010). The combined determination of MIF and other biomarkers upgrades the detection of fatal outcomes (Grieb et al., 2010). Therefore, MIF was considered in this study to evaluate its diagnostic prospects. In general, the major problem in leptospirosis

is its diagnosis, which is still mostly misleading due to the febrile-related symptoms of typhoid, dengue, malaria, hepatitis, enteritis, and shigellosis (Gasem et al., 2020; Md-Lasim et al., 2021). In this study, our observations clearly stated that MIF ELISA has high sensitivity (100%) and specificity (>90%) for the diagnosis and distinction of leptospirosis cases from other febrile cases. A significantly different cutoff point of the serum MIF level ($p < 0.001$) was detected in leptospirosis patients when compared with other cases, which greatly suggested that MIF is a potential early diagnostic marker for leptospirosis. AUC values of 0.5, 0.7–0.8, 0.8–0.9, and >0.9 were considered as showing non-acceptable, acceptable, excellent, and outstanding discriminating ability to diagnose patients with and without the disease (Mandrekar, 2010). MIF had an AUC value of >0.9 for the different outcomes of leptospirosis. According to the ROC analysis, MIF is strongly suggested as an efficient biomarker for rapid diagnostic purposes.

Pearson's correlation test demonstrated that the serum MIF levels were strongly correlated ($r = 0.75$) with disease duration, directly indicating that MIF might be deeply involved in the pathology of leptospirosis. However, no significant correlation was found with respect to age, which is consistent with previous studies on autoimmune and inflammatory diseases. The degree of infection, microbial count, and host immune responses showed more potential as factors than age, which may have obscured the impact of age on the levels of serum MIF (Mizue et al., 2000; Sreih et al., 2011). The correlation coefficient index (r) values of 0.0–0.3, ≥0.3, ≥0.5, ≥0.7, and 0.9–1.0 referred to negligible, low, moderate, high, and very high positive correlations, respectively (Mukaka, 2012). Therefore, MIF not only showed high diagnostic values but was also associated with disease severity. As there is still no remarkable severity predictor for leptospirosis, it is assumed that our significant range of elevated serum MIF may serve as a potential predictor of severe leptospirosis. As severe leptospirosis with multi-organ dysfunction may be fatal, the early prediction of the disease severity and the development of appropriate treatments are needed. Furthermore, certain cases such as severe leptospirosis patients with acute renal failure have not shown clinical response to most treatments. Previous investigations suggested that convalescent plasma therapy may be very useful for the treatment of these cases due to its fast recovery and ability to reduce the bacterial load (Tse et al., 2002). Thus, a therapeutic strategy applicable for the treatment of both mild and severe leptospirosis cases is also urgently needed.

Existing research works have addressed MIF as being implicated in the pathogenesis of various inflammatory and autoimmune diseases, including sepsis (Bozza et al., 2004), rheumatoid arthritis (Kim et al., 2011), diabetes (Sanchez-Zamora et al., 2010), solid tumors and cancer (Nobre et al., 2017), acute respiratory distress syndrome (Donnelly et al., 1997), hepatic inflammatory diseases (Marin et al., 2017), multiple sclerosis (Benedek et al., 2017), systemic lupus erythematosus (Tu et al., 2019), psoriasis (Bezdek et al., 2018), and dengue (Chuang et al., 2015). Leptospiral LPS is a serovar-specific major immunodominant antigen that initiates the pathogenesis of leptospirosis (Vanithamani et al., 2015). The present study determined the LD_{50} (20 mg/kg) of *L. interrogans* serovar Autumnalis strain N2 for the development of the experimental animal model of leptospirosis to analyze the expression profile of MIF during the disease. Endotoxemia and upregulation of pro-inflammatory cytokines were reported in leptospiral LPS-challenged mice. The intraperitoneally injected LPS can cross the gastrointestinal barrier to enter the blood stream. Circulating LPS then bound to the LPS-binding protein and complexed with the CD14 receptor, which can stimulate the pro-inflammatory cytokines (André et al., 2019). Monocytes and macrophages rapidly secreted the MIF protein after exposure to microbial products, especially LPS (Bernhagen et al., 1993; Calandra and Roger, 2003). LPS and TNF stimulation regulated MIF/CD74 signaling to promote B-cell proliferation and inflammation (Klasen et al., 2018) and regulated the expression of TLR4 in fibroblasts (Xi et al., 2016). The immunopathological mechanism underlying MIF expression and its role are not well understood. MIF is an acute-phase secretory protein that appears in the blood at 2–8 h of infection (Calandra et al., 1994).

Our study is the first to investigate the MIF profile in leptospiral LPS-treated *in vitro* and *in vivo* models. THP-1 cells and mice with experimentally induced leptospirosis showed significantly upregulated MIF expression at the early phase of leptospirosis. MIF profiling in untreated control mice showed differential MIF expression in the heart, lung, liver, kidney, and spleen, among which the kidney and liver showed higher MIF expressions. However, our results for the spleen, which is a secondary lymphoid organ, revealed that it had a low MIF expression in normal mice. MIF expression is regulated by several immune mediators, particularly the pro-inflammatory cytokine TNF-α, which enhances the promoter activity of MIF by activating the nuclear transcription factor NF-κB (Cao et al., 2006). Spleen is a notable organ that showed very low TNF-α expression, but the liver and kidney cells exhibited predominant expressions (Hunt et al., 1992). MIF has a wide tissue distribution; specifically, the organs involved in stress response have high levels of MIF expression (Calandra and Roger, 2003).

Upon infection, the expression of MIF was significantly increased in the spleen, lung, and heart. In general, during infection, matured lymphocytes (one of the predominant sources of MIF expression) migrate into the spleen to fight antigens. Thereby, the spleen showed a significantly higher MIF expression during leptospiral infection in mouse models. Alveolar macrophages and the immune cells in mucosa-associated lymphoid tissue (MALT) could be responsible for the elevated MIF expression during infection. The infiltration of immune cells in tissues may play a key role in the differential MIF abundance in different tissues. Besides, we found that the expression of MIF was dramatically increased in all lymphoid organs in response to the duration of infection. As immune cells are the primary sources of MIF secretion during infection, the formation and activation of lymphocytes, macrophages, and other immune cells in primary and secondary lymphoid organs play a key role in MIF expression (Calandra and Roger, 2003). In the present study, the early expression of MIF and its time-

dependent increase upon infection suggested a role of this cytokine in disease pathogenesis and progression. Thus far, only very few studies have investigated the mechanism of the contribution of MIF in the pathology of inflammatory diseases. Further studies are required to better understand its role in different inflammatory diseases and in the development of therapeutic regimens.

CONCLUSION

Our data provided the first evidence of differential MIF levels in sera of leptospirosis patients, other febrile cases, and healthy control subjects. According to our results, MIF is an early-phase cytokine biomarker that provides positive results in the early phase as possible and exhibits high sensitivity and specificity. Thereby MIF meets the desired attributes of an ideal biomarker. MIF profiling in leptospirosis patients also suggested that an elevated concentration of serum MIF is a superior indicator for predicting severe leptospirosis. *In vitro* and *in vivo* MIF profiling determined the timeline of early MIF gene expression and its differential expression in different organs during leptospiral infection. Thus, the present findings provide the framework for further studies for understanding the role of MIF in leptospirosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Ethical Committee (no. DM/2014/101/51) of Bharathidasan University. The patients/participants provided written informed consent to participate in this study. The animal study was reviewed and approved by the Institutional Animal Ethical Committee (BDU/IAEC/P30/2018), Bharathidasan University.

AUTHOR CONTRIBUTIONS

KS: Conceptualization, Methodology, Software, Writing-Original draft preparation. CM: Conceptualization, Data curation, Methodology, Writing- Original draft preparation. GM: Data curation, Methodology. AH: Visualization, Investigation, Software, Data curation, Methodology, Reviewing and Funding acquisition. AA: Supervision, Reviewing, Editing, Funding acquisition. KN: Conceptualization, Validation, Funding acquisition, Project administration, Writing, Reviewing and Editing.

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SUPPLEMENTARY MATERIAL

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Macrophage migration inhibitory factor gene promoter polymorphism (−173G/C SNP) determines host susceptibility and severity of leptospirosis

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ABSTRACT

The biological mechanisms that are associated with the severity of leptospirosis are far from complete. The aim of the present study was to investigate whether the macrophage migration inhibitory factor (MIF) gene promoter polymorphisms determine susceptibility to and severity of human leptospirosis. MIF is a potent pro-inflammatory cytokine, which has been reported to correlate with the risk of inflammatory disease onset and severity. In the present study, MIF 173G/C single nucleotide polymorphism (SNP) was analyzed by PCR-RFLP (Restriction fragment length polymorphism). A statistically significant increase of MIF -173*C allele related genotypes was observed in leptospirosis patients when compared with healthy control subjects. Genotypes GC (OR: 28.4; 95% CI: 10.9–73.6; $p < 0.001$) and CC (OR: 40; 95% CI: 2.3–686.5; $p < 0.001$) of −173 G/C MIF polymorphism was associated with susceptibility and severity of leptospirosis respectively. In leptospirosis cases, 69.8% of leptospirosis patients were GC genotype carriers while 19.8% and 10.4% cases were CC and GG carriers; in severe leptospirosis, 68% cases were CC carriers and 32% were GC carriers; and in healthy controls, 92.5% subjects were GG carriers and 7.5% were GC carriers. MIF -173*C allele was (OR: 15; 95% CI: 6.1–36.8; $p < 0.001$) significantly associated with the risk of leptospirosis than −173*G allele (OR: 0.06; 95% CI: 0.02–0.16; $p < 0.001$). The relationship of −173G/C MIF polymorphism with mRNA and serum level of MIF and inflammatory cytokine expression was analyzed by quantitative real-time PCR and MIF ELISA. MIF mRNA expression was significantly increased in carriers of MIF -173*C allele associated genotypes, GC and CC. A substantial increase of serum MIF (Mean ± SD) was found in risk genotypes GC (5.81 ± 0.61 ng/mL) and CC (10.12 ± 0.23 ng/mL) carrying leptospirosis patients than GG genotype (0.86 ± 0.3 ng/mL) carrying healthy controls. Pearson correlation test showed a significant positive correlation between elevated serum MIF and −173*C allele ($r = 0.99$, $p < 0.001$). High MIF expression genotypes GC and CC upregulated the mRNA expression of TNF-α, IL-1β and IL-4 whereas downregulated the IL-10 expression. Thus, MIF -173 G/C SNP genotype GC carriers have highly susceptible to leptospirosis and the leptospirosis patients with CC genotype had an increased risk of developing a severe form of the disease. The observations of this study conclude that MIF -173G/C polymorphism is associated with leptospirosis susceptibility and severity and also could be a promising severity predictor of leptospirosis.

1. Introduction

Leptospirosis is the most neglected, re-emerging tropical zoonotic disease affecting both humans and animals especially in developing countries [1]. The estimated global burden of leptospirosis is an average of 1.03 million annually and 58900 deaths were reported [2]. About 10% of leptospirosis patients are complicated with multiorgan failure including hepatic dysfunction, acute renal failure, pulmonary involvement, circulating system dysfunction, acute pancreatitis and rhabdomyolysis [3]. An unresolved mystery of leptospirosis is the heterogeneity of disease outcomes. Leptospiral infection in some cases results in severe leptospirosis with multi-organ involvement and high mortality while others have mild subclinical manifestations. The factors driving the disease progression towards severe leptospirosis with fatal outcome remains unclear. Therefore, studies on human genetics associated susceptibility and severity of infectious diseases are essential for advancing our knowledge of disease pathogenesis, stratification of risk and the development of specific drug targets [4].

There is some evidence hypothesized that pathogen and host related factors are significantly related to this heterogeneity. The factors

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including i) highly prevalent pathogenic strain (*Leptospira interrogans* serovar Icterohemorrhagiae) and the ii) leptospiral load - $>10^3$ leptospires/mL at pre-treatment [5], iii) host immunity - the pre-existing antibodies protect the patients from severe outcomes [6], iv) chronic alcoholism [5], and v) other host clinical profiles during leptospiral infection such as septic shock, hypertension, hypotension, oligoanuria, abnormal chest auscultation, leukocytosis, increased lactate, lipase, amylase, creatinine, IL-6, IL-8, IL-10 levels, increased aspartate aminotransferase/alanine aminotransferase (AST/ALT) ratio, decreased IL-10/TNF-α ratio, low platelet counts, low hematocrit and abnormal coagulation and fibrinolytic pathway activation [7–12] were all believed to play a key role in developing severe leptospirosis. In addition, many studies reported that host genetic factors are the major determinants that influence the disease severity [13]. The identification of a reliable parameter would be helpful to physicians for the purpose of triage.

Recent studies indicated that MIF gene polymorphisms play a key role in disease susceptibility and severity. Human MIF is encoded by a single gene located on chromosome 22q11.23, 0.8 kb long. It consists of 3 exons and 2 introns. The gene product of MIF is composed of 115 amino acids with a molecular weight of 12.5 kDa. MIF is a proinflammatory and immunoregulatory cytokine shown to be associated with many diseases [14]. In the human MIF gene, five single nucleotide polymorphisms (SNPs) at the location of −173G/C (rs755622), +254 (rs2096525), +656 (rs2070766), 3.8 kb 3′ of the translation termination codon (rs1007888) and −794 $CATT_{5\text{-}8}$ microsatellite were reported [15]. Among these genetic polymorphisms, −173G/C (SNP located at −173 (G to C)) and −794 $CATT_{5\text{-}8}$ (short tandem repeat polymorphism located at the nucleotide position, −794) at the MIF gene promoter are functionally significant and closely related to the severity of numerous diseases including tuberculosis [14], autoimmune disease [16], cancer [17], gestational diabetes mellitus [18], rheumatoid arthritis [19], coronary artery disease [15], vitiligo [20], pneumococcal meningitis [21] etc. Genetic polymorphism of MIF correlates with circulating MIF concentration by influencing transcriptional activity of MIF. Elevated circulatory MIF concentration has been reported in several inflammatory and autoimmune diseases [22]. The longer CATT repeats (>5 CATT variants) and −173*C are considered as high expression MIF genotypes whereas shorter CATT repeats (5-CATT variants) and −173*G are the low expression MIF genotypes [23].

Genotyping of MIF gene polymorphisms separate the patients into high and low MIF expressers, which are directly proportional to high and low risk of disease severity, therefore physicians can provide the early and appropriate treatment to high-risk patients to decrease the disease severity and mortality in an outbreak situation [5]. Based on this knowledge, we decided to examine the association between MIF gene promoter polymorphism and leptospirosis susceptibility and severity in the study population. We report herein the correlation of high MIF expression genotypes (GC and CC) with susceptibility and severity of leptospirosis and low MIF expression genotype (GG) has no effect on disease onset and progression.

2. Materials and methods

2.1. Study design

A case control study was conducted in the period of August 2019 to January 2020 at Mahatma Gandhi memorial government hospital, Tiruchirappalli, Tamil Nadu, India. This study was performed to examine the role of host MIF gene polymorphisms in the determination of disease susceptibility and severity in patients with leptospirosis. The genotypes of MIF polymorphisms were compared in leptospirosis and healthy control subjects.

2.2. Study subjects

This study was comprised of two groups, the first group consisted of 86 patients with confirmed serology of leptospirosis and for the second group, 80 healthy individuals with no symptoms or history of leptospirosis were consecutively recruited for this study. Leptospirosis confirmed cases constitute two subgroups, a) Hospitalized patients with a severe form of leptospirosis (n = 22) and b) Non-hospitalized patients with mild leptospirosis (n = 64). Microscopic agglutination test (MAT) and IgM enzyme-linked immunosorbent assay (ELISA) were performed to confirm leptospirosis [24]. Any healthy control subjects who had a fever within the previous two weeks were excluded from the study.

2.3. Ethical considerations

The study was ethically approved by the Institutional Ethical Committee (No: DM/2014/101/51) at the Bharathidasan University (Tiruchirappalli, Tamil Nadu, India). Informed written consents were obtained from all study subjects after explaining the purpose of this study, in the case of minor study subjects; their surrogates signed an informed consent form.

2.4. Genomic DNA extraction

The peripheral blood was collected from all patients and control subjects in vacutainer tubes containing ethylene diamine tetra acetic acid (EDTA). The genomic DNA was extracted by DNeasy blood and tissue kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. The extracted DNA was stored in TE buffer and frozen at −20 °C until use and the quality of DNA was assessed by agarose gel electrophoresis.

2.5. Genotyping of MIF gene promoter −173G/C SNP

Polymerase chain reaction- Restriction fragment length polymorphism (PCR-RFLP) was performed to analyze the functional SNP (-173G/C) in the promoter region of MIF. The thermal cycling reaction conditions included 1 cycle of 95 °C for 10 min, 35 cycles of 95 °C for 45 s, 62 °C for 45 s, 72 °C for 1 min, 1 cycle of 72 °C for 7 min. The resulting amplified product was further visualized by 1.5% agarose gel. The amplified PCR product was digested with FastDigest *Alu*I restriction endonuclease enzyme (Thermo Scientific, USA) at 37 °C for 1 h and the digested fragments were separated onto 6% polyacrylamide gel and stained with Ethidium bromide. The percentage of allele and genotype frequencies was calculated. The genotype GG, CC and GC was characterized by 2 bands of 268 and 98bp in size, 3 bands of 205,98 and 63 bp in size and 4 bands of 268, 205,98 and 63bp sized fragments respectively.

2.6. Quantitative real-time PCR analysis

RNA was extracted from peripheral blood leukocytes and the cDNA was synthesized by using the RNeasy mini kit (Qiagen, Valencia, CA) and iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) respectively. Quantitative Real-time PCR (qRT-PCR) was performed using a CFX96 Touch™ Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). The primers used in this study are listed in Table 1. The qRT-PCR using SYBR Green PCR Master Mix (Bio-Rad, Hercules, CA, USA) and primers was carried out in a 10 μL reaction volume (20 ng cDNA, 5 μL Master Mix, 0.2 μM of each primer). GAPDH was used as an internal control for normalization.

2.7. MIF ELISA

The peripheral blood was collected from all cases and controls in clot activator tubes and the serum was separated and then stored at −20 °C

Table 1
List of primers used in this study.

Gene	Primer sequence
Human MIF -173 G/C SNP	FP: 5′-CAGTGCGTGTCGTGGAGT-3′ RP: 5′-GGGTGAGAACTGAATTCCA-3′
Human MIF	FP: 5′-CGCAGAACCGCTCCTACAG-3′ RP: 5′-GGAGTTGTTCCAGCCCACAT-3′
Human TNF-α	FP: 5′-AGAGGGAAGAGTTCCCCAGGAC-3′ RP: 5′-TGAGTCGGTCACCCTTCTCCAG-3′
Human IL-1β	FP: 5′-CCAGCTACGAATCTCGGACCACC-3′ RP: 5′-TTAGGAAGACACAAATTGCATGGTGAAGTCAGT-3′
Human IL-4	FP: 5′-CTGCAAATCGACACCTATTA-3′ RP: 5′-GATCGTCTTTAGCCTTTC-3′
Human IL-10	FP: 5′-ATGCCCCAAGCTGAGAACCAAGACCCA-3′ RP: 5′ TCTCAAGGGGCTGGGTCAGCTATCCCA-3′
Human GAPDH	FP: 5′-AACGACCCCTTCATTGAC-3′ RP: 5′-TCCACGACATACTCAGCA -3′

until use. MIF ELISA was performed by Human MIF ELISA kit (Sigma-Aldrich, St. Louis, Mo, USA) according to the manufacturer's instructions to measure the serum MIF level in leptospirosis patients and healthy control subjects. The experiment was performed in triplicates.

2.8. Statistical analysis

The data was measured with SigmaPlot 11.0 software. Triplicates were quantified and expressed as Mean ± Standard Deviation. The cut-off value was calculated by mean ± 2 (Standard deviation) of controls. Pearson's Chi-Square test was performed to analyze the Hardy-Weinberg equilibrium for MIF gene polymorphisms. The genotype and allele frequencies were determined by direct counting and the difference between the distribution of genotypes and alleles was calculated by Chi-Square test. The measurement of odds ratio and 95% confidence interval and was used to analyze the risk of MIF polymorphism associated leptospirosis. Pearson correlation coefficient analysis was performed to estimate the associations of polymorphism and serum MIF. For all tests, p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1. Baseline features of study subjects

A total of 86 leptospirosis cases and 80 healthy control subjects were evaluated in this study. MAT assay determined the seropositive of leptospirosis with the titre of 1:80 to 1:2560. The highly infecting serovars were Autumnalis (43%), Australis (29%) and Icterohaemorrhagiae (17.5%). The mean age of leptospirosis confirmed hospitalized and non-hospitalized cases and healthy control subjects were 38.6 ± 15.9, 32 ± 16.2 and 33.2 ± 15.8 years old respectively. The gender distribution among leptospirosis cases was 45% female and 55% male. There was no noteworthy difference was found between studied groups in respect to age and sex. The mean difference of disease duration between hospitalized and non-hospitalized patients was found to be 12.4. The clinical and demographic characteristics of study subjects are summarized in Table 2.

3.2. MIF gene polymorphism on leptospirosis susceptibility and severity

In order to determine the influence of MIF gene polymorphism on leptospirosis susceptibility and severity, genotypic and allelic frequencies of MIF polymorphism, −173G/C SNP were analyzed in leptospirosis cases and healthy controls. For both the leptospirosis and healthy control subjects, the distribution of MIF polymorphisms adopted the Hardy-Weinberg equilibrium (P = 1). When we analyzed the distribution of MIF polymorphic genotypes of −173G > C between leptospirosis patients and healthy control groups, we observed that MIF

Table 2
Demographic, clinical and serological characteristics of leptospirosis patients and healthy control subjects.

Variables	Leptospirosis patients		Healthy controls
	Hospitalized patients	Non- Hospitalized patients	
Sex	60	47.4	45
Female (%)	40	52.6	55
Male (%)			
Age	8–53	6–68	7–65
Range (years)	38.6 ± 15.9	32 ± 16.2	33.2 ± 15.8
Mean (years) ± S.D			
Duration of	12–25	4–10	–
disease	19 ± 4.1	6.6 ± 1.5	–
Range (days)			
Mean (days) ± S.D			
Serology	(1:80–1:2560)	(1:80–1:2560)	–
MAT titres Range	1:100	1:100	–
IgM ELISA titre			

-173G > C SNP had a significant difference in genotype frequency among groups.

The distribution of MIF -173G/C genotype variants GG, GC and CC were 0% (OR: 0.12, 95% CI: 0.007–2.32), 32% (OR: 0.09, 95% CI: 0.003–0.29) and 68% (OR: 66.42, 95% CI: 12.5–352.74) among severe leptospirosis cases and were 14% (OR: 0.01, 95% CI: 0.004–0.03), 83% (OR: 59.42, 95% CI: 20.68–170.7) and 3% (OR: 6.44, 95% CI: 0.3–136.57) in mild leptospirosis while 92%, 8% and 0% in healthy control subjects respectively. GC genotype was more frequently observed in leptospirosis cases than controls while GG genotype frequency was highly reported in healthy controls. In addition, the majority of the severe leptospirosis cases have a genotype frequency of CC (68%). No GG and CC genotype was found in severe leptospirosis and healthy control subjects respectively. The frequency of C allele in severe and mild leptospirosis patient groups were 84% (OR: 7.7, 95% CI: 0.42–138) and 45% (OR: 75.37, 95% CI: 25.33–224.25) respectively, which is significantly higher than the healthy control subjects (6%). While the frequency of G allele in severe and mild leptospirosis patient groups were 16% (OR: 0.01, 95% CI: 0.002–0.07) and 55% (OR: 0.15, 95% CI: 0.007–3.29) respectively, which is considerably lower than the healthy control subjects (80%). With regard to the MIF -173 G/C polymorphism, genotype and allele distributions in leptospirosis and controls are shown in Table 3 and Fig. 1A. The representative image of genotype analysis by polyacrylamide gel electrophoresis was presented in Fig. 1B. This result strongly suggests that the distribution of −173*C allele increases the risk of leptospirosis. The overall and stratified analysis revealed a strong positive correlation between leptospirosis risk and MIF -173G/C polymorphism.

3.3. Influence of MIF -173G/C SNP on mRNA and serum MIF levels

To demonstrate the functional impact of MIF -173G/C polymorphism, we analyzed the MIF mRNA expression and serum concentration between different genotype carriers. The quantitative analysis of MIF RNA expression according to −173G/C SNPs showed that the relative mRNA expression of CC and GC carriers was significantly higher with respect to GG genotype carriers ($p < 0.001$) as shown in Fig. 2A. The quantification of serum MIF levels in different allele carriers determined the association between −173G/C SNP of the MIF gene and the production of circulating MIF proteins. The analysis highlighted the significant increase of serum MIF with carriage of CC genotype followed by GC. The mean score of serum MIF was significantly elevated 11-fold in carriers of CC (10.12 ng/mL) and 6-fold in GC (5.81 ng/mL) of leptospirosis patients with respect to GG (0.86 ng/mL) of healthy

Table 3
Distribution of genotype and allele frequency of MIF -173 SNP and association with susceptibility and severity of leptospirosis.

Genotype	Healthy controls n = 80, Frequency (n)	Leptospirosis (n = 86)					
		Severe and hospitalized (n = 22)			Mild and non-hospitalized (n = 64)		
		Frequency (n)	OR (95% CI)	P value	Frequency (n)	OR (95% CI)	P value
GG	0.92 (74)	0 (0)	0.12 (0.007–2.32)	= 0.16	0.14 (9)	0.01 (0.004–0.03)	<0.0001
GC	0.08 (6)	0.32 (7)	0.09 (0.03–0.29)	<0.0001	0.83 (53)	59.42 (20.68–170.7)	<0.0001
CC	0 (0)	0.68 (15)	66.42 (12.5–352.74)	<0.0001	0.03 (2)	6.44 (0.3–136.57)	= 0.23
Allele							
G	0.97 (80)	0.16 (7)	0.01 (0.002–0.07)	<0.0001	0.55 (61)	0.15 (0.007–3.29)	= 0.23
C	0.03 (6)	0.84 (22)	7.7 (0.42–138)	= 0.16	0.45 (55)	75.37 (25.33–224.25)	<0.0001

No deviation from Hardy-Weinberg equilibrium was observed in leptospirosis cases or healthy controls for MIF -173G/C polymorphisms.

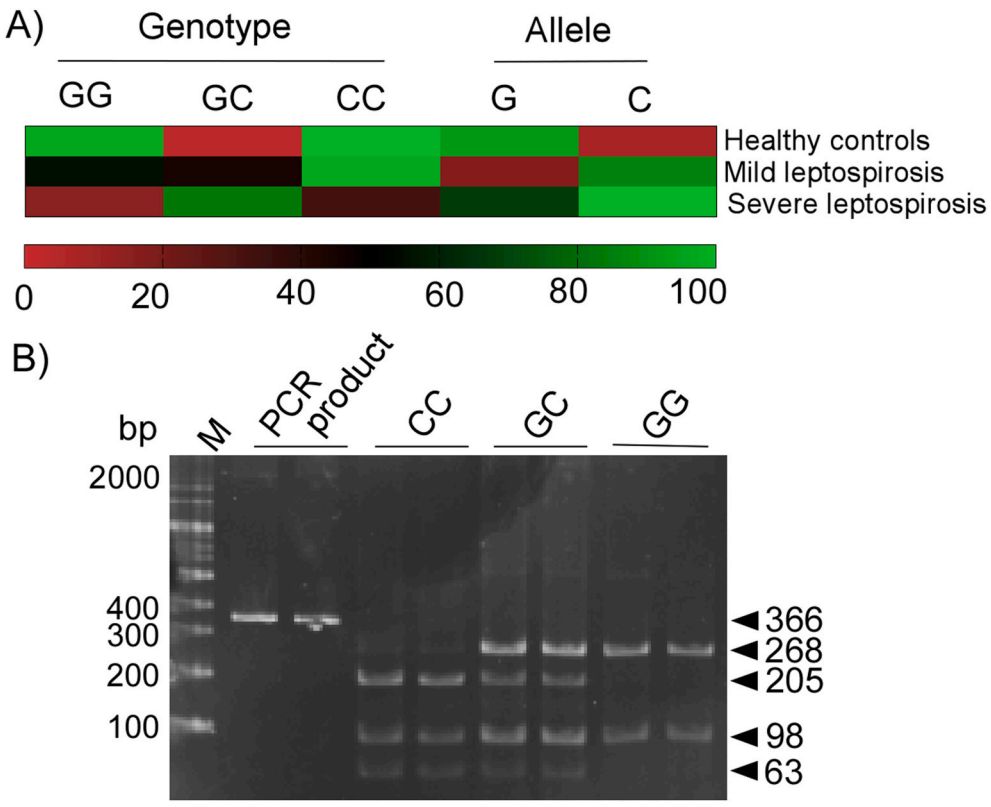

Fig. 1. A) Genotypes (GG, GC and CC) and alleles (G and C) frequency of MIF -173G/C polymorphism were observed in leptospirosis patients and healthy control subjects. Heatmap showed that C allele associated genotypes (GC and CC) were highly observed in leptospirosis cases whereas GG genotype was frequently distributed in healthy controls. B) Representative image of MIF -173 G/C SNP genotype analysis by polyacrylamide gel electrophoresis.

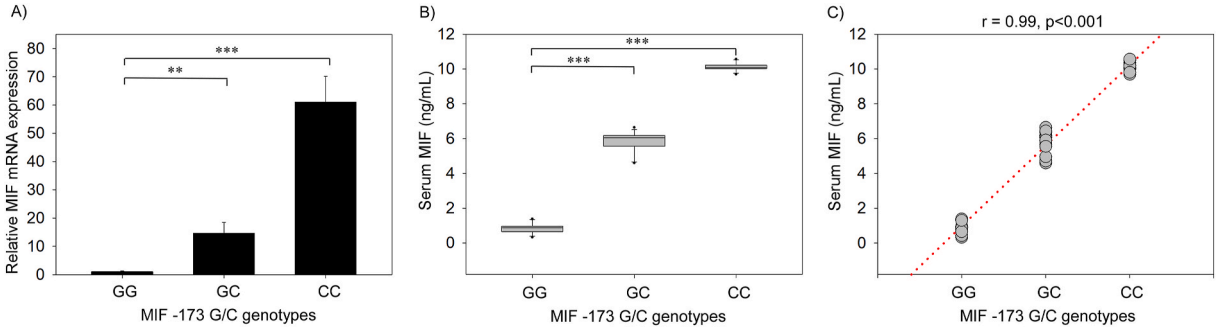

Fig. 2. Association of MIF -173G/C SNP with MIF mRNA expression and serum MIF levels. A) Relative MIF mRNA expression with respect to −173G/C genotypes, GG, GC and CC. B) Box plot showed the serum MIF levels in different −173 G/C genotype carriers. Both MIF mRNA expression and serum MIF levels were significantly higher in −173*C allele associated genotypes GC and CC when compared with GG. C) Pearson correlation coefficient analysis between serum MIF level and MIF gene polymorphism −173G/C SNP. This analysis showed that elevated serum MIF level was positively correlated with −173*C allele associated genotypes GC and CC ($r = 0.99$, $P < 0.001$). n = 3 experiments. **P < 0.01, ***P < 0.001.

controls ($p < 0.001$) (Table 4). It is also important to note that the carriers of MIF -173*C allele have significantly higher serum MIF levels than MIF -173G alleles (7.96 ng/mL vs 3.34 ng/mL, $p < 0.001$) (Fig. 2B). The most remarkable result to emerge from the data is that C allele related genotypes are positively correlated with serum MIF levels upon leptospiral infection ($r = 0.99$, $p < 0.001$) (Fig. 2C).

3.4. MIF -173G/C SNP regulates cytokine profile of leptospirosis patients

Relative mRNA expression of inflammatory cytokines TNF-α, IL-1β and IL-4 and anti-inflammatory cytokine IL-10 with respect to −173G/C genotypes, GG, GC and CC was analyzed by qRT-PCR (Fig. 3). The results revealed that high MIF expression genotype GC and CC carriers had 10-fold and 11-fold upregulated mRNA expression of TNF-α respectively, whereas 9-fold expression of IL-1β and 11-fold expression of IL-4 when compared to GG carriers. Meanwhile, the anti-inflammatory cytokine IL-10 expression was significantly ($p < 0.05$) downregulated in GC and CC genotype carriers. This elevated expression of inflammatory cytokines may drive the disease severity by accelerating other inflammatory responses and organ damage.

4. Discussion

The outcome of leptospirosis infection varies from mild to severe clinical manifestations. The investigations on host genetic factors responsible for the disparate outcomes are of great importance for attaining novel insights into pathogenic mechanisms and developing the potential personalized treatment for high-risk populations. Previous studies revealed that MIF gene promoter polymorphisms are a firm genetic risk factor for the susceptibility and severity of diseases [20,25–28]. In the present investigation, we found MIF gene polymorphism (-173G/C SNP) was significantly associated with the risk of leptospirosis.

Since MIF is a pluripotent immunoregulatory cytokine implicated in disease pathogenesis, MIF gene polymorphism influences the risk and progression of diseases. Two functionally significant MIF gene promoter polymorphisms are $CATT_{5\text{-}8}$ microsatellite and G/C SNP. Previous studies have determined that the longer length CATT repeats of MIF -794 $CATT_{5\text{-}8}$ polymorphism was strongly correlated with disease onset and/or progression [14,21,29–31]. Regarding MIF -173G/C SNP, previous reports showed that −173 GC and CC genotype carriers have an increased risk of developing diseases than GG genotype carriers. This polymorphism was associated with various infectious and autoimmune diseases including cutaneous leishmaniasis [32], pulmonary tuberculosis [33], coronary artery disease [15], psoriasis [34], multiple sclerosis [29] and cancer [35]. In certain cases, the polymorphisms are associated with either susceptibility or severity of the disease. Barton et al. revealed that −173*C alleles were found to be correlated with susceptibility to inflammatory polyarthritis but not associated with disease progression and severity [36]. The host genetic factors have not only determined the outcome of disease but also the outcome of treatment, so the consideration of host genetic or epigenetic signatures is of great importance in disease management [37].

For the first time, we conducted a case-control study to investigate the association of MIF -173G/C gene polymorphism with susceptibility and severity of leptospirosis. The results of the present study are the first demonstration of the relationship between the outcome of leptospirosis and MIF polymorphisms. The association analysis revealed that MIF -173G/C SNP is significantly correlated with leptospirosis susceptibility and severity. The −173*C allele associated genotypes GC and CC are frequently observed in leptospirosis patient groups, while genotype GG was highly observed in healthy controls. The genotype GC was highly distributed in mild leptospirosis patients while CC was observed in severe leptospirosis. Polymorphisms in host MIF gene makeup may lead to variations in leptospirosis susceptibility and severity. During outbreak situations, the typing of MIF -173G/C SNP may be instruct to identify the patients at increased risk in order to provide personalized and/or prophylactic treatments. Thus, the identification of MIF -173G/C variants of leptospirosis patients could be a better severity predictor of leptospirosis.

Previous studies also investigated various cytokine gene polymorphisms in leptospirosis. The research by Fialho et al. revealed that IL-4 -33T/T and IL-4Rα +1902A/G gene polymorphisms are highly distributed in leptospirosis patients [38]. Previous studies found that the genetic variants including IL-1β −511G/G, IL-12RB1 +1196C/G, Cytokine-inducible SH2-containing protein −292T/A and +3415C/G confer the host susceptibility towards leptospirosis [39]. The study by Cédola et al. highlighted that TLR2 Arg753Gln G/A, and TLR1 Ile602Ser G/G genotype carriers are highly susceptible to leptospirosis while TLR2 Arg753Gln G/A genotype carriers are the high-risk population for severe leptospirosis with hepatic insufficiency and jaundice [40].

MIF has been reported to be elevated in the serum of patients with inflammatory or autoimmune diseases. Based on the genetic location, the genetic variants alter the gene expression. The SNPs within the transcriptionally regulatory elements can modify mRNA expression of a gene; SNPs within genes can alter splicing and translation; SNPs within coding sequence can modify the activity of the protein [41]. The G to C allele transition in MIF -173G/C polymorphism can enhance the binding of the transcription factor, activator protein-4 (AP-4) to increase the mRNA and serum level of MIF [42]. Previous reports revealed that −173*G allele and −173*C allele were considered as low and high expression MIF alleles. Regarding MIF mRNA and protein expression, our results are in line with previous investigations [33,43]. −173*C allele associated genotypes GC and CC were associated with transcript and serum level of MIF than GG genotype.

In addition to analyze the correlation between the MIF -173G/C polymorphism and the MIF gene expression, we performed cytokine profiling to evaluate the MIF -173G/C SNP regulated immune responses in leptospirosis patients. The upregulated MIF expression stimulates the production of inflammatory cytokines by downregulates the anti-inflammatory cytokine. Previous investigations revealed that macrophage MIF counter-regulates the anti-inflammatory activity of glucocorticoids and promotes the secretion of pro-inflammatory cytokines, TNFα, IL-1β, IL-6, and IL-8 upon lipopolysaccharide (LPS) stimulation [44]. The elevated circulated levels of inflammatory cytokines cause cell toxicity, tissue damage leads to disease severity [45].

The predominant source of MIF is macrophages which are important effector cells of innate immune system. As macrophages involved in the first line immune defense against infection, the MIF expression was observed earlier in disease onset and also it regulates the responses of macrophage and other immune cells against endotoxin and other pathogenic stimuli. MIF upregulates the cellular expression of TLR4 and promotes the recognition of and response to endotoxin to triggers the inflammatory and immune responses [46,47]. The MIF receptors and their complexes CD74, CXCR2, CXCR4, CXCR7, CD74/CXCR4, CXCR4/CXCR7 and CD74/CXCR2 carried out the regulatory functions of MIF. These functionally important receptors regulate signal transduction pathways associated with TLR, Nuclear factor kappa B ligand (NF-κB), P53, Mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) to promote immune response, production of inflammatory cytokines and chemotaxis etc. In sharp, CD74 and

Table 4
Serum MIF levels in study subjects, according to MIF -173 G/C polymorphism.

Genotype	MIF concentration (Mean ± SD)	p-value
GG	0.86 ± 0.3 ng/mL	<0.001
GC	5.81 ± 0.61 ng/mL	
CC	10.12 ± 0.23 ng/mL	
Allele		
G	3.34 ± 2.56 ng/mL	<0.001
C	7.96 ± 2.23 ng/mL	

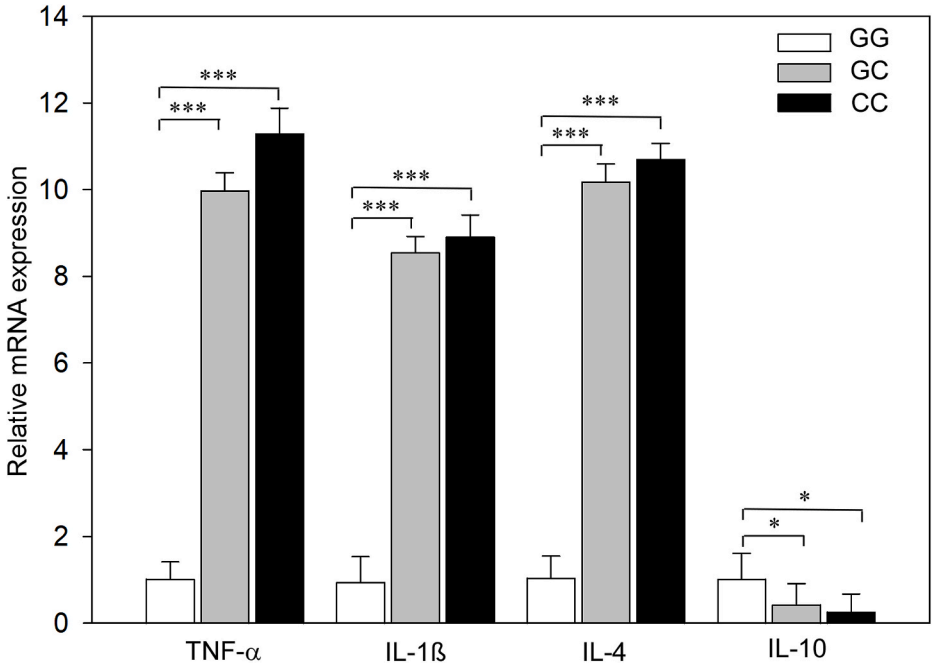

Fig. 3. MIF -173G/C SNP regulated cytokine expression during leptospirosis. Relative mRNA expression of TNF-α, IL-1β, IL-4 and IL-10 with respect to −173G/C genotypes, GG, GC and CC was determined by qRT-PCR analysis. This analysis showed that TNF-α, IL-1β and IL-4 expression was significantly higher in MIF -173 GC and CC genotype carriers when compared with GG carriers. n = 3 experiments. *P < 0.05, ***P < 0.001.

CD74/CXCR4 complex promotes immune cell survival, inflammation and apoptotic inhibition; CXCR2 and CXCR4 promote chemotaxis of neutrophil and monocytes [48,49]. As MIF interferes with the host control of inflammation and immunity, the detailed study on the role of MIF in pathogenesis of inflammatory diseases will fulfills the lacunae exist in the literature related to regulatory mechanism of inflammatory and immune responses. Therefore, MIF signaling is the control system of inflammatory and immune responses during infection.

The most striking result of this study states that leptospirosis patients carrying at least one C allele at −173G/C SNP also had an increased risk of developing severe leptospirosis and significantly higher serum MIF than those with G allele. The primary requirements of the research on diagnostic, prophylactic and therapeutic approaches of leptospirosis are better understanding of the disease process. Also as a part of this effort, we conducted this case-control study to provide new insights into leptospirosis pathogenesis and help to identify the patients at increased risk.

5. Conclusion

The evidence from this study intimates that MIF gene promoter polymorphism −173G/C SNP might be contributing to the genetic risk of leptospirosis susceptibility and severity and the upregulation of MIF gene expression and serum MIF levels. Thus, the MIF -173G/C genotyping of leptospirosis patients may be helpful to guide stratify the leptospirosis patients in order to institute prophylaxis and personalized treatment under outbreak situations. These results also showed the significance of considering MIF gene polymorphisms to perceive the intended mechanisms of host immune response to leptospirosis and also extend the knowledge of the role of host genetic factors in developing disease susceptibility and severity.

CRediT authorship contribution statement

Krishnamoorthi Sumaiya: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Kalimuthusamy Natarajaseenivasan:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Invited Article

Macrophage migration inhibitory factor (MIF): A multifaceted cytokine regulated by genetic and physiological strategies

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ABSTRACT

Macrophage migration inhibitory factor (MIF) is a proinflammatory cytokine encoded within a functionally polymorphic genetic locus. MIF was initially recognized as a cytokine generated by activated T cells, but in recent days it has been identified as a multipotent key cytokine secreted by many other cell types involved in immune response and physiological processes. MIF is a highly conserved 12.5 kDa secretory protein that is involved in numerous biological processes. The expression and secretion profile of MIF suggests that MIF to be ubiquitously and constitutively expressed in almost all mammalian cells and is vital for numerous physiological processes. MIF is a critical upstream mediator of host innate and adaptive immunity and survival pathways resulting in the clearance of pathogens thus playing a protective role during infectious diseases. On the other hand, MIF being an immune modulator accelerates detrimental inflammation, promotes cancer metastasis and progression, thus worsening disease conditions. Several reports demonstrated that genetic and physiological factors, including MIF gene polymorphisms, posttranslational regulations, and receptor binding control the functional activities of MIF. Taking into consideration the multi-faceted role of MIF both in physiology and pathology, we thought it is timely to review and summarize the expressional and functional regulation of MIF, its functional mechanisms associated with its beneficial and pathological roles, and MIF-targeting therapies. Thus, our review will provide an overview on how MIF is regulated, its response, and the potency of the therapies that target MIF.

Contents

Abbreviations: AMPK, Adenosine monophosphate activated protein kinase; AP-1, Activator protein 1; CD, Cluster of Differentiation; COX, Cyclo-oxygenase; CXCR, Chemokine (CXC motif) receptor; DAMP, Damage-associated molecular patterns; ERK1/2, Extracellular signal-regulated kinase 1/2; FoxO3a, Forkhead transcription factors of the O3 class; GC, Glucocorticoids; GR, glucocorticoids receptor; GILZ, Glucocorticoid-induced leucine zipper protein; HDACi, Histone deacetylase inhibitor; IBD, Inflammatory bowel disease; IFN, Interferon; IL, Interleukin; JAB, c-Jun activation domain-binding protein-1; JNK, c-Jun N-terminal kinase; LPS, Lipopolysaccharide; MAPK, Mitogen-activated protein kinase; MIF, Macrophage migration inhibitory factor; MKP-1, MAP kinase phosphatase-1; MMP, Matrix metalloproteinase; NK cells, Natural killer cells; OxMIF, Oxidized MIF; PARP, PAR - Poly (ADP-ribose) polymerase; PGE_2, Prostaglandin E_2; PI3K/Akt, Phosphoinositide 3-kinase/Akt (protein kinase B); PLA_2, Phospholipases A_2; PTMs, Posttranslational modifications; RA, Rheumatoid arthritis; RANKL, Receptor activator of nuclear factor kappa B ligand; ROS, Reactive oxygen species; SNP, Single nucleotide polymorphism; SOD1, Superoxide dismutase; Src kinase, Sarcoma kinase; STR, Short tandem repeat; STZ, Streptozotocin; T1D, Type 1 diabetes; T2D, Type 2 diabetes; TLR, Toll-like receptor; TNF - α, Tumor necrosis factor - α; VEGF, Vascular endothelial growth factor.

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1. Introduction

Macrophage migration inhibitory factor (MIF) is a homo-trimeric protein that acts as a pleiotropic pro-inflammatory cytokine. It is involved in various functions, including leukocyte recruitment, inflammation, immune responses, cell proliferation, tumorigenesis, and counter-regulation of glucocorticoids (GC). The *MIF* gene is located on chromosome 22 (22q11.23) of the human genome and contains 3 exons (107, 172, and 66 bp) and two introns (188 and 94 bp). Almost six decades ago, migration inhibition activity was identified during a delayed-type hypersensitivity study (Bloom & Bennett, 1966), and in 1989, researchers first cloned human MIF that facilitated the characterization of the biological activities of MIF (Weiser, Pozzi, & David, 1991). In 1993, MIF was discovered to be a secreted pro-inflammatory protein (Bernhagen et al., 1993), and subsequently, the physiological and pathological roles of MIF and MIF receptors were elucidated (Leng et al., 2003).

Initially, MIF was recognized as T cell lymphokine. However, rapidly growing evidence revealed MIF to be broadly expressed in various cell types and tissues, including cells of the immune and nervous systems (Calandra, Spiegel, Metz, & Bucala, 1998), pituitary cells (Bernhagen et al., 1993), epithelial cells (He et al., 2006), endothelial cells (Pellowe et al., 2019), smooth muscle cells (Fan et al., 2017), synovial fibroblasts (Abe, Shimizu, Ohkawara, & Nishihira, 2000), and mesenchymal cells (Park et al., 2009). MIF is an evolutionarily conserved low molecular weight protein (~12.5 kDa) consisting of 114 amino acid residues. MIF is a ligand for four membrane receptors, namely Cluster of differentiation 74 (CD74), Chemokine (CXC motif) receptor (CXCR) 2, CXCR4, and CXCR7. Based on the inflammatory context and cell types, MIF binds to an individual receptor or receptor complexes that determine the functional activity of MIF (Alampour-Rajabi et al., 2015; Schwartz et al., 2009). The established signaling receptor complexes are CD74/CD44 (Shi et al., 2006), CD74/CXCR4, CXCR4/CXCR7 (Alampour-Rajabi et al., 2015), and CD74/CXCR2 (Bernhagen et al., 2007).

MIF is a multifaceted controversial cytokine, and therefore, the role of MIF in pathological conditions appears to be diverse with both protective and detrimental effects. MIF is a pro-inflammatory cytokine and immunomodulator that is rapidly released from both immune and non-immune cells in response to various stimuli. Lipopolysaccharides (LPS) of Gram-negative bacteria (Roger, Glauser, & Calandra, 2001), exotoxins of Gram-positive bacteria (Calandra et al., 1998), tumor necrosis factor-α (TNF-α) (Lan et al., 1997), GC (Leech et al., 1999), angiotensin II (Rice et al., 2003), glucose (Toso, Emamaullee, Merani, & Shapiro, 2008), insulin (Waeber et al., 1997), phorbol myristate acetate (Rossi et al., 1998), and ultraviolet B irradiation (Shimizu, Abe, Ohkawara, & Nishihira, 1999) have all been reported to augment the MIF expression and secretion. Among these inducers LPS is a potent pro-inflammatory stimulus that upregulates MIF in a time and dose-dependent manner in various cell types. LPS-induced MIF plays a critical role in lethal endotoxemia by stimulating TNF-α secretion. MIF counteracts the anti-inflammatory and immunosuppressive activity of GC. GC stimulate the MAP kinase phosphatase-1 (MKP-1) () that leads to the inhibition of inflammatory mediators, thereby achieving anti-inflammatory properties. The functional activity of MIF drives the inflammation targeted cascade ↑Akt/↓Nuclear FoxO3a (Forkhead transcription factors of the O3 class)/↓GILZ (GC-induced leucine zipper protein)/↓MKP-1 to override the action of GC (Fan et al., 2014). Due to its broad regulatory properties, MIF is a potential mediator of many inflammatory and immune diseases, including sepsis (Bozza et al., 2004), rheumatoid arthritis (Kim et al., 2011), diabetes (Sanchez-Zamora et al., 2010), solid tumors/cancer (Nobre et al., 2017), acute respiratory distress syndrome (Donnelly et al., 1997), hepatic inflammatory diseases (Marin et al., 2017), multiple sclerosis (Benedek et al., 2017), systemic lupus erythematosus (Tu et al., 2019), psoriasis (Bezdek et al., 2018), and dengue (Chuang, Chen, & Yeh, 2015).

Several studies proposed the mechanism of MIF in the pathogenesis of diseases in part by enhanced production of inflammatory molecules (TNF-α, nitric oxide, IL-1, IL-6, IL-8, and cyclo-oxygenase (COX)). MIF regulates the LPS-mediated host responses in an autocrine manner by upregulating the Toll-like receptor 4/myeloid differentiation factor 2 (TLR4-MD2) expression (Roger, Froidevaux, Martin, & Calandra, 2003). During infection, MIF is secreted in response to endotoxin/exotoxin and increased GC levels. MIF counter-regulates the immunosuppressive effect of GC on inflammatory cytokine production. The immunosuppressive effects of GC are inhibition of TNF-α mRNA translation *via* the suppressed activity of *c-jun* N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and inhibition of LPS-induced arachidonic acid release. After secretion, MIF activates the p44/42 ERK (extracellular signal-regulated kinase) MAP kinase and cytosolic phospholipases A2 ($cPLA_2$), an important pro-inflammatory cascade factor. MIF-induced $cPLA_2$ activation produces inflammatory components, including prostaglandins, arachidonic acid, and leukotrienes. The release of arachidonic acid activates JNK/SAPK, which is responsible for the translation of TNF-α mRNA. Thus, MIF plays a key role in the inflammatory cascade and acts as an upstream regulator of inflammatory cytokine production (Mitchell, Metz, Peng, & Bucala, 1999; Sampey, Hall, Mitchell, Metz, & Morand, 2001). MIF triggers inflammatory responses by MIF signaling pathways during infections. Thus, MIF has emerged as a promising drug target of many diseases.

The potential drug target strategies of MIF include small molecule, anti-MIF antagonist (Kok et al., 2018), neutralizing anti-MIF antibodies, nanobodies (Sparkes et al., 2018), genetic ablation, and deficiency of MIF (Xu et al., 2016). Despite its role in the pathogenesis of diseases, MIF also contributes to essential physiological activities of cells, especially cell proliferation and differentiation by regulating cellular signaling through intracellular and extracellular processes. MIF encoded within polymorphic genetic loci, and the MIF promoter region has two polymorphisms, namely, -173 G/C single nucleotide polymorphism (SNP) (rs755622) and microsatellite polymorphism, -794 $CATT_{(5-8)}$ (rs5844572). The functional genetic polymorphism of the MIF gene modulates the susceptibility and severity of infectious and autoimmune diseases (Assis et al., 2016; Tong et al., 2017). The overexpression of MIF during several diseases and its functional polymorphism makes MIF a promising biomarker to predict the status of the clinical course in patients. In this article, we reviewed the regulation of MIF expression, its dual consequences in physiology and pathology, its downstream signaling cascade and MIF-directed therapeutic strategies through a five-part discussion. The first part highlights the ubiquitous expression of MIF across various cell types; the second part summarizes the literature regarding beneficial effects of MIF and its mechanism of action; the third part explained the mechanistic role of MIF in the pathogenesis of diseases; fourth part discusses the rationale and strategy by which MIF achieves their multifaceted effects; and the fifth part focus on the therapeutic concepts of MIF.

2. MIF expression in different cell types and tissues

MIF was primarily recognized as a product of T lymphocytes, but studies show endotoxin-induced T cell-deficient mice to have circulatory

MIF. Rapidly growing evidences unraveled MIF to be constitutively expressed in multiple cell types. MIF secreting cells consist of baseline MIF expression to maintain the normal physiology of cells but are elevated under antigenic stimulation during diseased conditions. Most immune cells produce MIF and hypersecretion of MIF is associated with several diseases. Studies report the serum MIF concentration in healthy controls to be in the range of 0.2–8.3 ng/ml. Whereas the mean concentration of elevated serum MIF broadly varies upon different pathological conditions; dengue shock syndrome (102 ng/ml), esophageal squamous cell carcinoma (100 ng/ml), pulmonary tuberculosis (19.84 ng/ml), polymyositis (50 ng/ml), vitiligo (32.96 ng/ml), periodontitis (71.8 ng/ml) and severe sepsis (103.7 ng/ml) (Emonts et al., 2007; Farag, Hammam, Habib, Elnaidany, & Kamh, 2018; Ganganna, Subappa, & Bhandari, 2020; Ren et al., 2005; Yamada et al., 2002; Zou, Jin, & Li, 2018).

Macrophages are the predominant source of MIF. Recent study proved that autophagy-deficient monocytes and macrophages release markedly increased levels of MIF in response to LPS. The authors also show the increased MIF secretion to depend on mitochondrial ROS in these autophagy-deficient monocytes and macrophages (Lee et al., 2016). Upon pneumococcal infection, MIF was expressed by primary human monocyte-derived macrophages and THP-1 macrophages. In this case, phosphorylation of p38 MAPK (Mitogen-activated protein kinase) in macrophages was required for both local and systemic MIF expression (Das et al., 2014). Unstimulated human circulating eosinophils contain preformed cytosolic MIF. Eosinophils stimulated with phorbol myristate acetate and physiological stimuli such as C5a and IL-5 cause significant secretion of MIF in a concentration and time-dependent manner (Rossi et al., 1998). MIF is also pre-formed in human neutrophils and released only after secondary necrosis for maintaining and modulating the immune defense and inflammatory function (Roth, Solbach, & Laskay, 2016). Chen et al. reported MIF to be constitutively expressed by mast cells and secreted during degranulation (Chen, Centola, Altschul, & Metzger, 1998). Mast cells produce MIF to regulate innate immune responses (Calandra & Roger, 2003), mast cell-mediated fibrogenic activities, and non-scleroderma-related fibrosis (Ningyan, Xu, Hongfei, Jingjing, & Min, 2015). TNF-α stimulated pPlatelet-derived MIF mRNA and protein was reported in cardiovascular diseases (Wirtz et al., 2015). Growing evidence found that circulating MIF concentration reflects the pathological state of patients. Apart from this, blood cells also have constitutive and induced MIF expression. MIF was detected in white blood cells, plasma fraction (Lehmann et al., 2005), red blood cells (Karsten, Hill, & Herbert, 2018), and in platelets (Strussmann et al., 2013).

MIF is produced by different types of endothelial and epithelial cells. TNF-α stimulated endothelial cells (primary human microvascular cells) secrete MIF in a time-dependent manner to enhance neutrophil extravasation (Pellowe et al., 2019). Human hepatic sinusoidal endothelial cells (HHSECs) is known to release MIF in order to facilitate the proliferation, chemotaxis and apoptotic resistance in colorectal cancer cells (Hu et al., 2015). Human umbilical vein endothelial cells (HUVECs) secrete MIF in response to LPS induction, and its expression depends on the time and dose of the stimulant. After 12 h of LPS exposure, MIF levels reached the maximum in cells and decreased after 24 h. Nishihira et al. suggested that upregulated MIF mRNA expression may play a potential role in systemic inflammatory reactions (Nishihira, Koyama, & Mizue, 1998). Cheng et al. revealed that upon TNFα stimulation MIF expression is enhanced in HUVECs. The increased expression of MIF in turn promotes leukocyte recruitment by upregulating the expression of E-selectin, ICAM-1, VCAM-1, IL-8 and monocyte chemoattractant protein-1 (MCP-1) (Cheng et al., 2010). Gastric epithelial cells significantly increased the expression of MIF during gastric inflammation and cancer (He et al., 2006). MIF is also known to be overexpressed in the exosomes derived from the pancreatic ductal adenocarcinoma (PDAC). Kupffer cells selectively uptake these exosomes and promotes the transforming growth factor β (TGFβ) secretion and fibronectin production thus creating a pre-metastatic niche. The PDAC patients with elevated MIF levels in exosomes later develop liver metastasis evidencing MIF to serve as a prognostic marker for PDAC liver metastasis (Costa-Silva et al., 2015).

MIF is highly expressed throughout the central nervous system and peripheral nervous system. Neural cells including astrocytes, microglia, oligodendrocytes, neurons, Schwann cells, epithelial cells of choroid plexus, and ependymal cells abundantly express MIF (Nishibori et al., 1996; Nishio, Minami, Kato, Kaneda, & Nishihira, 1999; Ogata, Nishihira, Suzuki, Nagashima, & Tashiro, 1998; Su et al., 2017). Astrocytes express MIF protein, which increases the growth factors, promotes oligodendrocyte migration and proliferation, and neural protection (Cruz-Martinez et al., 2014). Because neural cells are in contact with cerebrospinal fluid (CSF), the secreted MIF was observed in CSF, similar to the serum MIF concentration (Ogata et al., 1998).

Human hepatocellular carcinoma cell, HuH7 challenged with ethanol enhances MIF mRNA expression in 4 h, while stimulation with LPS takes 24 h for achieving the same MIF mRNA levels. In contrast, ethanol-challenged THP-1 cells have no significant MIF mRNA expression and MIF secretion, whereas MIF mRNA and protein release occurred earlier after 4 h of LPS stimulation (Marin et al., 2017), indicating the expression and secretion entirely depends on the cell type and the stimuli. In alcoholic liver disease and alcoholic hepatitis, serum and hepatocytic MIF expression were notably increased (Akyildiz et al., 2010). In hepatoma cells, expression of MIF and MIF-induced hepatopoietin was observed (Li, Lu, Xing, Zhu, & He, 2004), Zhang et al. reported that in hepatocytes, MIF mRNA was weakly expressed and in intrahepatic sinusoids moderate expression of MIF was observed (Zhang et al., 2005).

When compared to cultured normal dermal fibroblasts, fibroblasts from skin wound lesion secrete ~3 fold increased MIF into the culture medium in response to LPS. Also, the MIF mRNA expression was also observed to be increased at 1 h and reached the maximum at 3 h and decreased thereafter (Abe et al., 2000). MIF was reported to be expressed in the basal layer of skin epidermis (Shimizu, Ohkawara, Nishihira, & Sakamoto, 1996) and keratinocytes (a source of cytokines under stress) and during inflammatory skin diseases (Yoshihisa, Rehman, Kondo, & Shimizu, 2016). MIF was observed to be expressed in the cytoplasm of human keratinocytes and epidermis and was shown to be secreted during immune reactions and inflammatory processes involving cutaneous immunity (Shimizu et al., 1999). *In vivo* studies by Shimizu et al. proved that MIF production was induced by various levels of ultraviolet B irradiation (0–125 mJ per cm^2) in keratinocytes. After 48 h of UVB exposure MIF levels significantly increased in cell free supernatants of cultured keratinocytes.

MIF mRNA and protein expression were observed in corneal tissues, aqueous humor, and serum of corneal incision rats. MIF mRNA expression was observed to be increased at 6, 24, and 48 h post-corneal injury and significantly diminished after 72 h (Matsuda, Tagawa, Matsuda, & Nishihira, 1997). Waeber et al., for the first time reported that insulin-secreting pancreatic islet β-cells produce MIF, and its expression is regulated by glucose (Waeber et al., 1997). MIF was observed to be co-localized with insulin in the secretory granules of the β-cells. Insulin-targeted cells such as myocytes, cardiomyocytes, and adipocytes secrete MIF constitutively or by stimulation with TNF-α. The variation in the level of adipocyte MIF is site-specific. For example, ten times higher level of MIF was reported in subcutaneous tissue and omentum when compared to mammary adipocytes (Toso et al., 2008).

3. Multifaceted activity of MIF

MIF is implicated in several biological functions, including leukocyte recruitment, inflammation, immune responses, cell proliferation, tumorigenesis, and counter-regulation of GC associated with either physiological or pathological processes (Bloom & Bennett, 1966). Fig. 1 describes the MIF-mediated pathways involved in the multifunctional activity of MIF. The broadly varied biological functions of MIF are carried

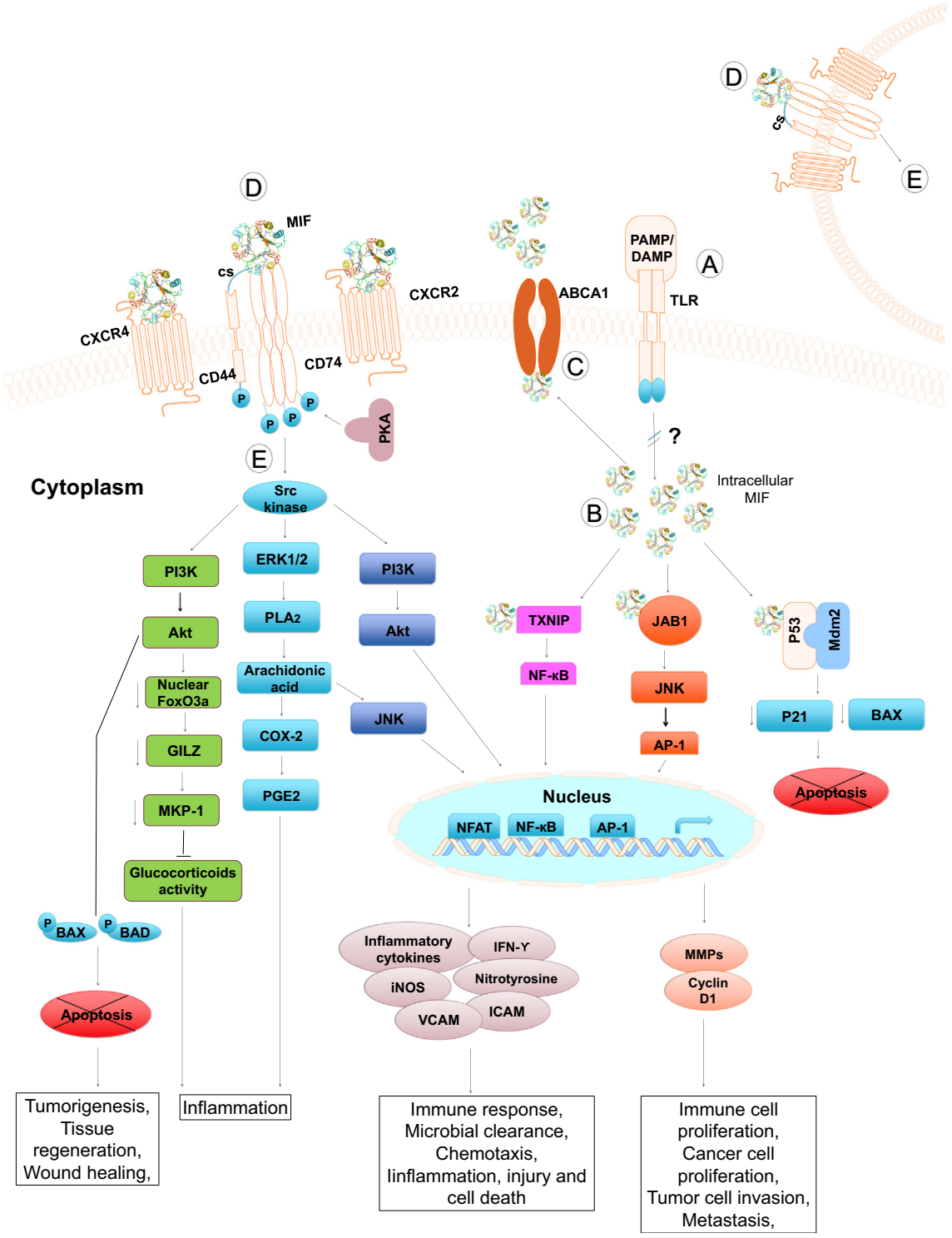

Fig. 1. The multifunctional activity of Macrophage migration inhibitory factor. A) The binding interaction between TLR agonists (PAMP/DAMP) and Toll-like receptor activates the MIF expression. B) The intracellular MIF can directly bind to the cytoplasmic proteins such as TXNIP, JAB1 and p53 to exhibits the biological functions. C) Intracellular MIF also secreted into the extracellular environment through ABCA1 transporter, D) The secreted MIF can binds either to its cognate (CD74) and non-cognate receptors (CXCR2 & CXCR4) to activates the receptor complexes, E) Activated MIF receptors regulates the signal transduction pathways (PI3K/Akt pathway, ERK1/2 pathway, COX-2/PGE2 pathway, NF-κB pathway, JNK pathway and p53 pathway) through activating the signaling cascades (MIF/TXNIP/NF-κB, MIF/JAB1/JNKAP-1, MIF/p53/↓P21/↓BAX, Src kinase/ERK1/2, PLA_2/Arachidonic acid/COX-2/PGE2, Src kinase/PI3K/Akt/Nuclear FoxO3a/GILZ/MKP-1, Src/PI3K/Akt, Src kinase/PI3K/Akt/pBAX-pBAD). The pathways enhance the translocation of transcription factors (NFAT, NF-κB and AP-1) that induces the gene expression responsible for both physiological and pathological processes. CD74 - Cluster of Differentiation 74, CD44 - Cluster of Differentiation 44, CXCR - Chemokine (CXC motif) receptor, ABCA1 - ATP Binding Cassette Subfamily A Member 1, PAMP - Pathogen-associated molecular pattern molecules, DAMP - Damage-associated molecular patterns, PKA - Protein kinase A, Src kinase - Sarcoma kinase, MKP-1 - MAP kinase phosphatase-1, GILZ - Glucocorticoid-induced leucine zipper protein, COX - Cyclo-oxygenase, ERK1/2 - Extracellular signal-regulated kinase 1/2, PI3K/Akt: Phosphoinositide 3-kinase/Akt (protein kinase B), $cPLA_2$ - Cytosolic phospholipases A2, PGE_2 - Prostaglandin E_2, JNK - c-Jun N-terminal kinase, DC-STAMP - Dendritic cell-specific transmembrane protein, NF-κB - Nuclear factor Kappa B, AP-1 - Activator protein 1, iNOS - Inducible nitric oxide synthase, ICAM - Intercellular Adhesion Molecule, VCAM - vascular cell adhesion molecule, IFN – Interferon, MMP - Matrix metalloproteinase, BAX – Bcl2 - associated X protein, BAD - Bcl2 associated agonist of cell death, TXNIP - Thioredoxin-interacting protein, JAB1 - c-Jun activation domain-binding protein-1.

Table 1
The multifunctional activity of MIF and its relevance in diseases.

Disease/pathological implications	Contribution of MIF	Mechanism of action	References
Infectious diseases	Innate and adaptive immunity	Activation of macrophages for the production of pro-inflammatory cytokines Upregulates the cytokine receptors and other inflammatory mediators such as nitric oxide, COX-2, PGE_2 and ROS Upregulates TLR4 expression	(Calandra et al., 1995)
		Activation and proliferation of T and B cells Chemotaxis of immune cells	(Starlets et al., 2006)
		Enhance antigenic recognition, presentation and clearance	(Bernhagen et al., 2007; Klasen et al., 2014)
	Detrimental inflammation	Prolonged stimulation of MIF secretion inhibits the immunosuppressive effects of glucocorticoids by ↑Akt/ ↓Nuclear FoxO3a/ ↓GILZ/↓MKP-1 signaling cascade Mediates cytokine storm Enhances LPS signaling pathway Upregulates the TLR4-MD2 expression	(Beishuizen et al., 2001; Delaloye et al., 2012; Fan et al., 2014; Lue, Kleemann, Calandra, Roger, & Bernhagen, 2002; Roger et al., 2003)
Tissue Injury	Worsen the tissue damage	MIF/CD74 complex enhances the infiltration and activation of CD68-positive macrophages to mediate inflammation and matrix production Facilitates renal fibrosis Increases TNF-α production, vascular permeability and hemorrhage	(Amaral et al., 2007; Lu et al., 2016)
	Tissue regeneration and wound healing	MIF/CD74 complex stimulates proliferative and survival pathways such as Akt/ERK pathway Reduces oxidative stress and apoptosis Inhibition of PARP cleavage	(Li et al., 2019; Rassaf et al., 2014)
Cancer	Apoptosis inhibition and cell survival	Stabilizes the binding of p53-Mdm2 Downregulates the Bax and p21 gene expression Cell cycle interference by suppress the activity of p53 Upregulates COX-2 and PGE_2 and enhances the transition of inflammation to cancer Overexpression of angiogenic mediators: IL-8, bFGF, VEGF Activates the metastatic cascade MIF/AKT/ERK/Cyclin D1/MMP-2	(Conroy et al., 2010; Jung et al., 2008; Nobre et al., 2017; Zhang et al., 2016)
Neurological diseases	Survival of motor neurons	Inhibits the mitochondrial accumulation and toxicity of misfolded SOD1 Inhibits the deposition of SOD1 on outer mitochondrial membrane and activation of pro-apoptotic mitochondrial pathway Promotes Brain-derived neurotrophic factor (BDNF) expression Increased MMP level; Inhibits PARP cleavage; decreases neuron loss	(Li et al., 2019; Nakahara et al., 2019; Tafuri, Ronchi, Magri, Comi, & Corti, 2015)
	Neurodegeneration and cognitive deficits	Upregulation of pro-inflammatory cytokines; neuroinflammation; hyper-phosphorylation of tau and neuronal injury in Alzheimer's disease (AD) Aggregation of amyloid β (A β) protein and mediates toxicity in AD	(Bacher et al., 2010; Nasiri et al., 2020)
Diabetes	Glucose metabolism	Upregulation of insulin gene transcription factor Induces the TNF-α dependent synthesis of fructose 2,6-bisphosphate and 6-phosphofructo-2-kinase Stimulates insulin secretion	(Benigni et al., 2000; Waeber et al., 1997)
	β cell destruction	stimulates the TNF-α, IL-1β, IL-18, iNOS, TGF-β and ROS production Inhibits IL-10 expression Induces apoptosis and insulitis	(Cvetkovic et al., 2005; Sanchez-Zamora et al., 2010; Stosic-Grujicic et al., 2008)
	Insulin resistance	Production of resistin in adipocytes	
Rheumatoid Arthritis	Enhances pathological features of RA	induces the MMPs gene transcription by N-terminal proline residue of MIF protein RANKL mediated osteoclast formation Mediates inflammation by stimulating PLA_2, PGE_2 and COX-2	(Onodera et al., 2000; Pakozdi et al., 2006; Sampey et al., 2001)
Viral infections	HIV replication	Enhances Long terminal repeats mediated transcription Mediates Viremia	(Arjona et al., 2007; Assuncao-Miranda et al., 2010; Regis et al., 2010)
	Inflammation and lethality	Activates macrophages to secretion of TNF-α and PGE_2 Neuroinvasion of virus	

(continued on next page)

Table 1 (*continued*)

Disease/pathological implications	Contribution of MIF	Mechanism of action	References
Fungal infections			
i) Psoriasiform dermatitis	Increases pathological features (Erythema, Skin infiltration and desquamation)	Keratinocyte hyperproliferation	(Bezdek et al., 2018; Xu et al., 2019)
		Inflammatory cell infiltration	
		Dermal angiogenesis	
ii) *Aspergillus fumigatus* keratitis	Increased inflammatory responses	Activates IL-23 pathway	
		Inflammatory cytokine expression: TNF-α and IL-6	
Protozoan infections			
i) Toxoplasmosis	Cell mediated Immunity	Classical activation of Ly6C	(Holowka & Bucala, 2020; Ruiz-Rosado Jde et al., 2016; Satoskar, Bozza, Rodriguez Sosa, Lin, & David, 2001; Stijlemans et al., 2014; Stijlemans et al., 2016; Weiser et al., 1991)
		Differentiation of inflammatory $Ly6C^{high}$ monocytes into TNF and iNOS producing dentritic cells (TipDCs)	
ii) Leishmaniasis	Anti-parasitic effect	Stimulates IFN-γ-producing NK cells	
		Activates macrophages and promotes the sustained expression of TNF-α, ROS and nitric oxide.	
		IFN-γ *induced macrophage* leishmanicidal effect	
		Enhances Type 1 inflammation	
iii) African Trypanosomiasis	Immunopathogenicity development	Recruitment of monocytes and neutrophils lead to tissue damage	
		Iron accumulation in liver reduces erythropoiesis	
		Enhances hemodilution, Red blood cell clearance and anemia	

out *via* several signaling pathways which are discussed in upcoming sections in relevance to physiology and pathology. In brief, the signaling cascade Src kinase/PI3K/Akt/NuclearFoxO3a/GILZ/MKP-1 and Src kinase/ERK1/2,PLA_2/Arachidonic acid/COX-2/PGE2 promotes inflammation, Src kinase/PI3K/Akt/pBAX-pBAD promotes the tumorigenesis and tissue regeneration, MIF/TXNIP/NF-κB/inflammatory cytokine promotes the chemotaxis and various immune responses, MIF/JAB1/JNK/AP-1/CyclinD1 promotes the immune cell proliferation, tumor cell invasion and cell death, MIF/p53/Mdm2/↓P21/↓BAX inhibits apoptosis and tumorigenesis. These differential effects of MIF are controlled by host regulatory mechanisms and will be discussed later in Section 5 of the review. The detailed mechanisms of multifaceted activity of MIF in physiology and pathology are presented in Table 1.

3.1. Beneficial effects of MIF

3.1.1. Regulation of immune response and microbial clearance

Cytokines are the key players that organize the innate and adaptive immune responses and promote host inflammatory responses that suppress invading pathogens. Rapidly growing evidence shown that MIF positively regulates the macrophage responses during infection and enhances the production of pro-inflammatory cytokines and other inflammatory components such as TNF-α, IFN-γ (Interferon-γ), IL-1β, IL-2, IL-6, IL-8, nitric oxide, and COX2 (Calandra & Roger, 2003) to achieve the clearance of pathogen (Das et al., 2014). Especially in tuberculosis, *Mycobacterium* encounters the immune response by immune escape mechanism through their complex membrane-mediated protection from host enzymatic action. MIF overcome the immune escape of pathogen and inhibit the intracellular pathogen survival by promoting cytokine and reactive oxygen production and regulating the expression of pattern recognition receptor dectin-1 (Das et al., 2013). MIF implicates early innate immune response to parasitic infections including malaria, Chagas disease, leishmaniasis, trypanosomiasis, and toxoplasmosis. MIF enables the expression of proinflammatory cytokines and their receptors, parasite recognition, macrophage-mediated microbial clearance, and efficacious antigen presentation (Rosado Jde & Rodriguez-Sosa, 2011).

3.1.2. Renal protection

The binding of MIF and soluble CD74 receptor protects the kidney from renal ischemia-reperfusion injury in post-cardiac surgery. After surgery, circulating MIF concentration was increased significantly in patients thus reducing the risk of developing post-operative complications such as acute kidney injury (AKI). Increased cell death by necroptosis and ferroptosis and increased tubular cell injury was observed in MIF-deficient mice. At the same time, administration of recombinant MIF after experimental ischemia-reperfusion reduced the cell death and oxidative stress and protected the renal tubular epithelial cells and decreased the incidence of AKI in mice (Stoppe et al., 2018). This clearly proves MIF to be renal protective at least in patients with post-cardiac surgery complications.

3.1.3. Cardioprotection

The first *in vivo* evidence regarding the cardioprotective role of MIF in the post-ischemic heart was studied by Koga *et al* (Koga et al., 2011). MIF plays a key role in maintaining cellular redox homeostasis (Kleemann et al., 1998). MIF deficient cardiac fibroblast show increased intracellular ROS production in response to oxidative stress, while the rMIF expression reduced the oxidative stress and their effects significantly. MIF deficient heart has increased mitochondrial damage resulting in increased cytochrome *c* release, an indicator of apoptosis (Koga et al., 2011). Ischemia triggered the MIF expression and release for maintaining a normoxic state of heart muscles. MIF modulated adenosine monophosphate-activated protein kinase (AMPK) and thus play a key role in ischaemic tolerance. In this regard, it was shown that MIF deficiency is functionally detrimental to the

heart during ischemia-reperfusion (I/R). In addition, during I/R, MIF develops the early adaptive responses in the heart (Miller et al., 2008). Studies show accumulated intracellular MIF and its S-nitrosylation at cysteine 81 to reduce oxidative stress and myocardial apoptosis. Additionally, secreted MIF activates the cardioprotective AMPK and ERK signaling pathways through its binding receptor, CD74 (Luedike et al., 2012; Miller et al., 2008; Rassaf, Weber, & Bernhagen, 2014).

3.1.4. Neuroprotection

Neuronal cell death and neuroinflammation are notable pathological hallmarks of neurodegeneration. Proinflammatory cytokines and their inflammatory cascade are primarily associated with the degeneration of neurons (Amor, Puentes, Baker, & van der Valk, 2010). MIF has a neuroprotective effect in Alzheimer's and Parkinson's disease, the most common neurodegenerative disorders. In Parkinson's disease, MIF inhibits the inflammatory response, apoptosis and stimulates autophagosomes. The regulation of inflammatory responses was shown to be driven by increased anti-inflammatory cytokine IL-10 and reduced the proinflammatory cytokine TNF-α. Additionally, MIF significantly reduces the cleavage of poly (ADP-ribose) polymerase (PARP), downregulates pro-apoptotic gene (Bax) expression, and increases mitochondrial membrane potential to inhibit apoptosis (S. Li et al., 2019). In Alzheimer's disease, neurons release MIF as a defensive component. Overexpressed MIF protects neurons from amyloid β protein stimulated neurotoxicity in an autocrine fashion. MIF initiates the cell survival signals and improves cognitive performance during Alzheimer's disease (S. Zhang et al., 2019).

In 2006 Cherepkova et al. demonstrated the peptide binding property and chaperone-like effects of MIF for the first time in brain tissue (Cherepkova, Lyutova, Eronina, & Gurvits, 2006). Mutant SOD1 misfolding and loss of motor neurons cause amyotrophic lateral sclerosis. But, experimentally upregulated intracellular MIF levels and its chaperone activity rescued the neurons from mutant SOD1-induced cell death (Israelson et al., 2015; Leyton-Jaimes et al., 2016). The increased expression of MIF directly inhibited mutant SOD1 and its association with mitochondria and endoplasmic reticulum, thus exerting its protective role. Though MIF was shown to exert protective role in SOD1 mutant, unfortunately the actively synthesized MIF in motor neurons was reported to be rapidly cleared (secretion, degradation, and transport) from the perikarya of motor neurons, leading to low accumulation of intraperikaryal MIF accompanied by accumulation of misfolded SOD1 (Israelson et al., 2015). The *in vivo* studies illustrated that elimination of intracellular MIF expedited disease onset and progression and minimized the lifespan of SOD1 (G85R) mutant mice.

3.1.5. Maintenance of immune-privileged sites

The downregulation of primary histocompatibility complex class I chain-related A gene (MICA) and the local expression of potent immunosuppressants are the key strategies to maintaining immune-privileged sites. However, the cells with negative or lower expression of MHC class I are attacked by natural killer (NK) cells, but the hair follicles and corneal endothelium are not constantly subjected to NK cell attack. Independent studies from two groups show MIF expression to be significantly higher and widespread throughout the corneal endothelium and epithelium of normal anagen scalp hair follicles. However, well decreased MIF expression in hair follicles of Alopecia areata (condition result from damage of hair follicle immune privilege) was observed evidencing the role of MIF as a natural killer cell inhibitory factor or immunosuppressive cytokine (Apte, Sinha, Mayhew, Wistow, & Niederkorn, 1998; Ito et al., 2008). Interestingly, the authors also reported murine rMIF to inhibit the NK cell-mediated lysis of corneal endothelium in a dose-dependent manner. Thus MIF plays a crucial role in repressing unfavorable NK cell activity by inhibiting the release of perforin granules and cytolysis (Ito et al., 2008).

3.1.6. Regeneration of cells and wound healing

The first report (Abe et al., 2000) on wound healing property of MIF described the biphasic increase of MIF mRNA and protein at 3 and 24 h after injury. MIF governs the chemotaxis to achieve the migration and infiltration of fibroblasts and keratinocytes into the wound site, promotes the development of granulation tissue and neovascularization, which are the most critical events in the wound healing process. Fibroblasts regenerate the damaged tissue in the wound site by synthesizing extracellular matrix components and contracting the granulation tissue. MIF impregnated gelatin microbeads technology-based investigations provide significant evidence for the wound healing property of MIF (Zhao et al., 2005). The elevated expression of MIF in Schwann cells of peripheral nerve tissue plays a significant role in the acceleration of nerve regeneration after the peripheral nerve injury. During the process of nerve regeneration, MIF is involved in axon regeneration and obstruction of Schwann cell apoptosis by suppressing the activity of an apoptotic protein, p53 (Nishio et al., 1999; Nishio, Nishihira, Ishibashi, Kato, & Minami, 2002). MIF expression in the injured cornea promotes the corneal wound healing by initiating the regeneration of epithelium after the 6 h of injury and completes the regeneration process at 48 h (Matsuda et al., 1997).

The overexpression of MIF and its cell surface receptor CD74 was found in epithelial cells of inflamed intestinal tissue of mouse that mimick inflammatory bowel disease (IBD). MIF-mediated CD74 signaling activates the Akt/ERK proliferation pathway and promotes the regeneration of epithelial cells and mucosal healing in the IBD model. Thus, MIF/CD74/Akt/ERK cascade transforms epithelial cell inflammation into regeneration. Most IBD patients are resistant to currently using clinically approved drugs like TNF blockers, novel approaches that focus on tissue regeneration to treat the IBD patients effectively is warranted. Farr et al. proposed that the triggered activation of the CD74 signaling pathway can serve as an effective strategy for treating IBD.

3.1.7. Regulation of insulin secretion

The constitutive MIF expression of pancreatic β-cell was turned to overexpression by physiological stimulator, glucose, and insulin in a concentration and time-dependent manner. The secreted MIF positively regulates insulin release, and carbohydrate metabolism by autocrine or paracrine action and thus prevents hyperglycemia. An *in vivo* study addressed the significant negative impact of MIF neutralization on insulin secretion by pancreatic β-cells (Waeber et al., 1997). Apart from its role in preformed insulin secretion, MIF enhances insulin gene transcription by regulating the transcription factor, PDX-1 (pancreatic duodenum homeobox - 1) (Stojanovic, Saksida, & Stosic-Grujicic, 2012). MIF stimulates glucose uptake and glycolysis by enhancing the 6-phosphofructo-2-kinase activity and fructose 2,6-biphosphate production (Benigni et al., 2000).

3.2. Detrimental effects of MIF

MIF is involved in the pathogenesis of diseases and mediates the disease severity. MIF is associated with several inflammatory and autoimmune diseases. Here we will discuss the remarkable pathological involvements of MIF.

3.2.1. Sepsis

Sepsis is a leading cause of mortality in hospitalized patients' and results from the immense inflammatory and immune system responses that contributes to tissue damage and multi-organ dysfunction. Several lines of clinical evidence revealed the significant contribution of MIF in sepsis. The intraperitoneal injection of *E. coli* increased the MIF concentration in peritoneal exudate fluid and plasma rapidly at 3-6 h post-injection (Calandra et al., 2000). Increased MIF level was also detected in the plasma of patients with severe sepsis when compared to healthy controls. All non-survivors with sepsis have very high levels of MIF in

plasma (>1000 pg/ml) suggesting MIF as an acute indicator of fatal outcome of patients in intensive care (Beishuizen, Thijs, Haanen, & Vermes, 2001; Bozza et al., 2004). Although the cytokines TNF and IL-1 have been established as key mediators of septic shock, anti-TNF and anti-IL-1 based therapies have no beneficial effects on lethality. Some preclinical studies have been recommended for more immunologic monitoring of targets with a well-known pathological role in septic shock to improve the therapeutic efficacy (Abraham, 1999; Opal, 2003; Vincent, Sun, & Dubois, 2002). The neutralization of MIF by anti-MIF antibody and MIF deficiency protected the experimental model by diminishing the TNF-α production and neutrophil accumulation. The administration of recombinant MIF markedly enhanced LPS-mediated lethality by 85% in an experimental model (Bernhagen et al., 1993; Bozza et al., 1999; Calandra et al., 2000). This evidence strongly defined a central role of endogenously secreted MIF in the pathogenesis of septic shock.

3.2.2. Rheumatoid arthritis (RA)

RA is an autoimmune chronic inflammatory disease characterized by proliferation and invasion of fibroblast-like synoviocytes that cause bone and cartilage destruction, leukocyte infiltration and adhesion, and increased resistance to apoptosis. As a pro-inflammatory cytokine, MIF exacerbates the disease and drives the pathological hallmarks of RA. Immunosorbent and immunohistochemistry data from previous studies illustrated that baseline concentration of MIF was significantly elevated in serum, synovial fluid, and synovial tissue of patients with RA and ankylosing spondylitis, a chronic inflammatory form of arthritis (Kim et al., 2011; Leech et al., 1999; Ranganathan et al., 2017). The predominant MIF expression was mainly due to the increased T cell infiltration and activation and the production of excessive cytokines, which are the essential pathological characteristics of RA (Klareskog, Forsum, Wigren, & Wigzell, 1981; Young, Adamson 3rd, Vaughan, & Fox, 1984). Pakozdi et al., and Onodera et al., discussed the typical pathological features of RA driven by MIF. MIF induces the RA synovial fibroblast matrix metalloproteinases (MMP), MMP-1 (Interstitial collagenase), MMP-2 (Gelatinase A), and MMP-3 (Stomelysin-1) expression in a dose-dependent manner and is primarily responsible for the degradation of connective tissue in RA by destroying extracellular matrix components. Mainly, the N-terminal proline residue of MIF protein induced the MMPs gene transcription. The prolonged elevated productions of MMPs are achieved by MIF-mediated angiogenesis of RA synovial fibroblast. In addition, they assessed the MIF-induced specific signaling pathways by which MIF upregulates MMPs. MIF is known to activate the protein kinase C (PKC), JNK (c-Jun N-terminal kinase), IL-1β, TNF-α, *c-jun*, and *c-fos* gene. MIF-induced phosphorylation of PKC and JNK are followed by the upregulation of jun and fos that activates the tetradecanoylphorbolacetate (TPA) mediated activator protein 1 (AP-1), an important transcription factor of the MMP gene (Onodera et al., 2000; Pakozdi et al., 2006). Thus, MIF-mediated signal transduction explicitly targets the promoter region of MMPs thereby, MIF plays a notorious role in the cytokine network involved in the pathogenesis of RA.

MIF-mediated increased osteoclast formation breaks the balance between the activity of osteoblast (bone formation) and osteoclast (Bone resorption), another pathological feature of RA. The elevated MIF in RA synovial tissues stimulated the nuclear factor kappa B ligand (RANKL) receptor activator that positively regulates osteoclast formation. MIF activates the RANKL induced phosphorylation of transcription factors, NFκB-p65, ERK1/2 (Extracellular signal-regulated kinase ½), and NFATc1 followed by the upregulation of osteoclast related gene expression including TRAP, CTR, OC-STAMP (Osteoclast stimulatory transmembrane protein), and DC-STAMP (dendritic cell-specific transmembrane protein). Kim et al., described that MIF-induced IL-1β is an intermediate mediator of RANKL expression, and also the blockade of IL-1β partially reduced the RANKL expression and osteoclastogenesis. MIF enhanced the RANKL expression also by PI3 kinase, p38 MAPK, JAK-2, NF-κB (Nuclear factor Kappa B), and AP-1 specific signal transduction pathway. The interaction between the network of components, including MIF, osteoclasts, RANKL, IL-1β, and synovial fibroblasts, are inevitable for the osteoclast formation in RA (Gu et al., 2015; Kim et al., 2011). Additionally, MIF-mediated inflammation in RA was shown to be achieved by stimulating PLA_2, PGE_2 (Prostaglandin E_2), and COX-2 through IL-1β (Sampey et al., 2001).

3.2.3. Diabetes

As MIF has a well-established role in autoimmunity and inflammation, MIF may play a vital role in developing Type 1 diabetes (T1D) and Type 2 diabetes (T2D), respectively. T1D is marked by the infiltration of T lymphocytes in the islets of Langerhans and the destruction of β cells (Hayakawa et al., 1991). MIF expression was markedly increased in serum of diabetic patients and *in vitro* and *in vivo* experimental models (Herder et al., 2008; Yabunaka et al., 2000). Cvetkovic et al., reported the role of MIF in immune-mediated β cell destruction in multiple low doses of streptozotocin (MLD-STZ) (glucose analog stimulate diabetic phenotype) induced experimental mice model. This study revealed the MIF induced clinical and histopathological features of type 1 diabetes such as insulitis and hyperglycemia. Anti-MIF antibody and genetically deficient MIF reversed the STZ stimulated hyperglycemia and maintained the normoglycemic condition. MIF induced immunological events in T1D is characterized by enhanced T cell proliferation, cell-cell adhesion, higher density of cell adhesion receptor, CD11b of spleen mononuclear cells, and increased frequency of IL-2 receptor. MIF stimulates TNF-α, IFN-γ, and iNOS (Inducible nitric oxide synthase) production, nitrotyrosine formation, and ROS, whereas it inhibits the production of IL-10. In this context, the histopathological analysis indicated the overexpression of MIF in pancreatic islets and destruction of β cells. MIF expression in β cells accompanied the autoimmune T1D progression (Cvetkovic et al., 2005; Stosic-Grujicic et al., 2008). MIF expression in lymphocytes of the spleen also mediates the autoimmune T1D (Bojunga et al., 2003).

Several studies have shown that circulating MIF increases in T2D patients and depicts the involvement in the progression and severity of T2D (Herder et al., 2006; Sanchez-Zamora et al., 2010; Toso et al., 2008). MIF increased the blood glucose level, polyuria, high food intake, and weight loss continually in STZ induced $MIF^{+/+}$ mice compared with $MIF^{-/-}$ mice. Further, STZ induced $MIF^{+/+}$ mice displayed glucose resistance; produced the inflammatory cytokines, IL-1β, IL-6, and TNF-α that exacerbate T2D; stimulated the production of resistin in adipocytes and leads to insulin resistance. Whereas STZ induced TD2 in $MIF^{-/-}$ mice showed a gradual decrease of blood glucose level by 7 weeks, significantly less inflammatory cytokine production. MIF antagonist CPSI-1306 exhibits similar effects on STZ induced TD2 mice as in $MIF^{-/-}$ mice. Additionally, the clinical findings also revealed the role of MIF in the pathogenesis of T2D in humans (Sanchez-Zamora et al., 2010).

Herder et al., carried out a sizeable case-cohort study and analyzed baseline MIF level to determine the incident T2D. In the context, the baseline MIF concentration significantly differed between the diabetic and non-diabetic females, whereas no difference was observed among males. The higher blood concentration of MIF was associated with an increased risk of developing T2D. Increased serum MIF levels were found to be associated with several other risk factors including, high systolic blood pressure and high TC/HDL (Total cholesterol/high-density lipoprotein) in women with incident T2D. It was also observed that the association between MIF levels and T2D was influential in obese than non-obese women (Herder et al., 2008). Based on the different fat depots, MIF release from adipocytes is associated with the body mass index, and the metformin treatment decreases the MIF concentration in plasma of obese (Dandona et al., 2004; Herder et al., 2008; Skurk et al., 2005).

The blockade of MIF by ISO-1 ameliorates diabetic nephropathy in T2D and also reduced the MIF induced pathological hallmarks such as hyperglycemia, increased albumin excretion rate, increased inflammatory cytokines IL-1β, and TNF-α, increased accumulation of extracellular matrix and macrophage activation in the kidney (Wang et al., 2014).

3.2.4. Cancer

In past decades, the impact of MIF in the progression of cancer is underestimated (Chesney et al., 1999; Mitchell et al., 1999; Takahashi et al., 1998). MIF has been overexpressed and display a key role in almost all types of solid tumors and human cancers including, breast, endometrium, esophagus, bladder, ovarian, cervical, colorectal, prostate, lung, liver, head and neck cancer, gastric, pancreatic, renal carcinoma, neuroblastoma, and glioblastoma by interacting with several cellular signaling pathways and exerts homeostatic imbalance that causes negative impacts on the immune system, thus leading to tumor growth and metastasis enhancement. The functional activities of MIF can create a favorable microenvironment for the growth and outspread of the tumor. In this context, MIF disrupts the cell cycle regulation, downregulates the anti-tumor immune surveillance, stimulates the proliferation of tumor cells, upregulates the COX-2 and PGE2, and induces angiogenesis and metastasis (Conroy, Mawhinney, & Donnelly, 2010; Nobre et al., 2017).

MIF accomplish the increased risk of onset and progression of cancer by several mechanisms (Nobre et al., 2017), including 1) cell cycle interference, MIF can directly interact with p53 and stabilizes the binding of p53 to its inhibitor, Mdm2. Inhibition of p53 leads to the decreased expression of p21 and Bax protein, thereby inhibiting the cell cycle arrest, apoptosis, and malignant transformation (Jung, Seong, & Ha, 2008), 2) isuppression of anti-tumor immunity, MIF activates the myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs), which acts together to inhibits the cytotoxic T cells and natural killer cell activity and thus allows the tumor cells to escape anti-tumor immunity (Nobre et al., 2017), 3) tumor cell proliferation and angiogenesis, MIF stimulates the tumor cells for the overexpression of growth mediators or angiogenic factors such as IL-8, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) through the activation of the MAPK signaling pathways, including MAPK/PI3/Akt, p38 MAPK and ERK 1/2 pathways (Zhang et al., 2016). Xu et al. described the positive correlation of MIF expression with VEGF expression and tumor microvessel density (Xu et al., 2008). 4) metastasis, MIF upregulates the pro-metastatic mediators: Cyclin D1 and Matrix metalloprotease (MMP) to facilitate the tumor cell invasion and metastasis. MMP-2 degrades the extracellular matrix that allows the tumor cell entry into the bloodstream. Nuclear and cytoplasmic cyclin D1 promotes the Cdk4 regulated tumor cell migration and phosphorylates the cytoplasmic and membrane proteins associated with cell invasion. Wang et al., described that MIF/AKT/ERK/CyclinD1/MMP-2 cascade drives the metastasis of pancreatic ductal adenocarcinoma.

Richard et al., showed the increased expression of MIF and its cell membrane receptor, CD74, in the cancer cells and the stroma of breast cancer tissues, and they suggested MIF as a prognostic marker for breast cancer (Richard et al., 2014). Recently, Lee et al., highlighted the overexpression of MIF and CD74 in ovarian cancer tissues and recommended the MIF and CD74 as a biomarker and therapeutic target due to the suspected involvement of MIF in ovarian cancer progression. The binding of MIF to its functional receptor CD74 exerts several downstream effects i) activate the Akt and AMPK proteins by phosphorylation (Bozzi et al., 2017), ii) enhance the MAPK signaling pathway that involved in tumorigenesis (Leng et al., 2003), and iii) downregulate the JNK mediated cell death (Bozzi et al., 2017). The overexpression of CXCR4 and MIF was shown in the human non-small cell lung cancer cell line, A549. MIF/CXCR4 axis promotes the tumorsphere formation, cell proliferation, and epithelial-mesenchymal transition. Also it was shown that exogenous addition of recombinant MIF stimulates IL-6 production. In turn, IL-6 augments MIF expression. Thus, the cascade of MIF/CXCR4/IL-6 mediates the NSCLC (Non-small cell lung carcinoma) progression and crosstalk with fibroblasts (Jager et al., 2020).

3.2.5. Viral infections

MIF is believed to be involved in the pathogenesis of viral infection. Viral infections can increase the MIF release. In turn, the proinflammatory activity of MIF accelerates several viral infections by modulating host immune response and amplifies the pathological features of infection. MIF-associated viral infections are HIV, Dengue, Influenza, cytomegalovirus (CMV), Ebola, West Nile virus (WNV), and Japanese encephalitis. In HIV infection, primary peripheral blood mononuclear cells secreted abnormal levels of MIF and plasma MIF levels were reported to be elevated. The envelope protein gp120 of HIV influences the MIF release through ATP Binding Cassette Subfamily A Member 1 (ABCA1) transporters. In addition, elevated MIF enhances HIV replication by activating the long terminal repeats mediated transcription. Thereby, MIF favors HIV infection and proliferation. Additionally, antiretroviral therapy significantly decreased MIF expression and secretion, substantiating its role in HIV infection and disease progression (Delaloye et al., 2012; Regis et al., 2010). In dengue infection, macrophages and hepatocytes induce MIF secretion to trigger the pathogenic events. MIF increased i) the viremia and splenic viral load by enhancing viral replication, ii) production of inflammatory mediators such as TNF-α and PGE_2, iii) macrophage inflammatory response, iv) thrombocytopenia, and v) pathological features like vascular hyperpermeability (Assuncao-Miranda et al., 2010; Chen et al., 2006; Chuang et al., 2011). MIF promotes the abnormal inflammatory responses or immunomodulation and cytokine storm against Influenza virus infection (Hou et al., 2009), Ebola virus infection (Wauquier, Becquart, Padilla, Baize, & Leroy, 2010), Human CMV infection (Bacher, Eickmann, Schrader, Gemsa, & Heiske, 2002), and Japanese encephalitis (Suzuki et al., 2000). WNV infection-mediated MIF protein expression facilitates the neuroinvasion of virus by regulating the blood-brain barrier permeability and thereby increase the viral load in the brain and lethality (Arjona et al., 2007).

4. Biological strategies that influence the activity of MIF

The dichotomous activities (beneficial and disease exacerbating) of MIF and diversity of MIF functions are tightly regulated by several processes and host genotype, as depicted in Fig. 2. Researchers unraveled that genetic polymorphism, epigenetic mechanisms, post-translational modifications (PTMs), and types of binding receptors may determine the activity of MIF in physiology and pathology *via* appropriate signaling events. These strategies maintains the versatile function of MIF.

4.1. MIF regulation by genetic polymorphism

About 200 genetic polymorphisms are identified in human *MIF* gene, of which 2 functionally important polymorphisms are described in the promoter region of *MIF* gene (Garcia-Orozco et al., 2020). G/C SNP is the polymorphism of G to C transition located at −173 (rs755622) in the untranslated 5′ flanking region, whereas CATT5-8 is a microsatellite repeat located at −794 (rs5844572) in the upstream region of G/C SNP (Barton et al., 2003). The existence of MIF -794 $(CATT)_6$, $(CATT)_7$, $(CATT)_8$ nucleotide repeats, and -173*C MIF genetic variants are identified as the higher MIF expression gene variants, and the $(CATT)_5$ repeat and -173*G allele is the low expression gene variants. These polymorphisms are associated with host susceptibility and severity of infectious diseases and immune disorders. Indeed, MIF gene polymorphisms have regulated the level of MIF mRNA and MIF serum levels (Matia-Garcia et al., 2015). In most cases high expression MIF genotype contributes to disease worsening, but recent studies showed that high expression MIF gene variants markedly reduced the susceptibility of few diseases. Thereby the correlation between MIF polymorphism and disease severity must be relied on the appropriate role of MIF in each individual diseases (Bucala, 2013). In addition, MIF polymorphism determines the morbidity and mortality rate of the host against several diseases (Baugh et al., 2002). MIF gene polymorphism and elevated serum MIF may be a critical biomarker and severity predictor for complex diseases (Okazaki et al., 2018). Thus, MIF genotype analysis may serve as early severity predictor and helps to classify the high risk population.

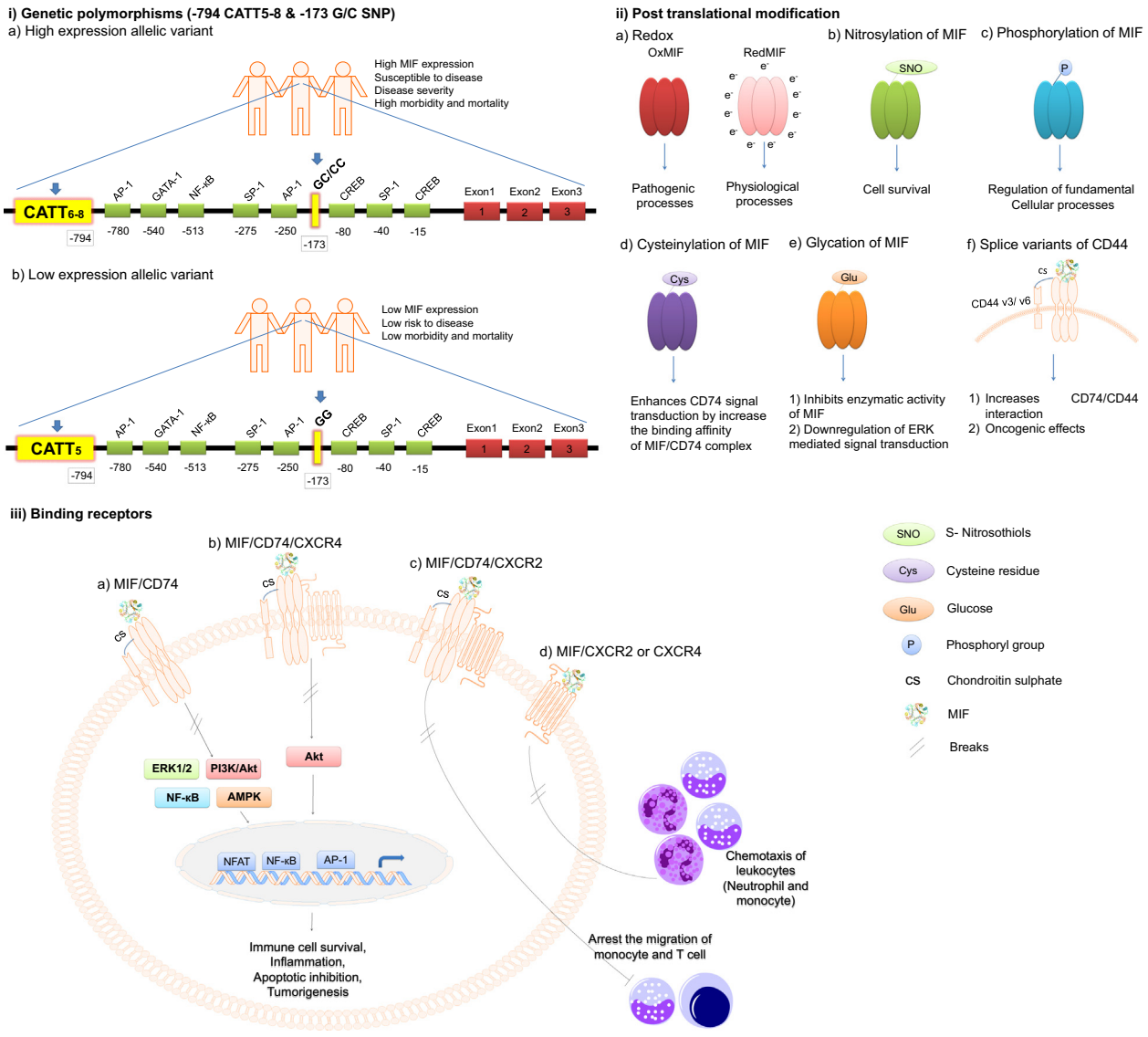

Fig. 2. Biological strategies influence the activity of MIF. i) Two functional MIF gene polymorphisms are -794 CATT repeats and -173 G/C single nucleotide polymorphism. a) Individuals having high expression allelic variant (-794 $CATT_{6-8}$ and -173 GG) are susceptible to diseases, High risk of disease outcome, morbidity and mortality. b) Individuals having low expression allelic variant (-794 $CATT_5$ and -173 GC/CC) exhibits low risk of disease outcome, morbidity and mortality. ii) Post-translational modifications (PTM): The modifications of the residues of MIF protein based on cellular microenvironment and type of PTM are a) Redox, b) Nitrosylation, c) Phosphorylation, d) Cysteinylation and e) Glycation and f) Splice variants of CD44. iii) Different MIF receptors and receptor complexes determine the functional diversity of MIF.

4.1.1. MIF -794 CATT short tandem repeats (STR)

MIF 5'-CATT STR has 5–8 repeats of tetra nucleotides located in functionally polymorphic locus of MIF. The higher number of CATT repeats ($CATT_{6-8}$) upregulates the expression of MIF mRNA and serum protein by CATT repeat length dependent binding of the transcription factor pICBP90 (Yao et al., 2016). The high expression MIF alleles play a key role in the progression of inflammatory diseases, autoimmunity, and human tumors (Castaneda-Moreno et al., 2018; Chuo, Lin, Yin, & Chen, 2021; Garcia-Orozco et al., 2020; Savva et al., 2016) and it could be a promising marker for disease severity. The association between 5'-CATT STRs and disease susceptibility and severity was observed in several diseases including Rheumatoid arthritis (Baugh et al., 2002), pneumococcal meningitis (Savva et al., 2016), Vitiligo (Garcia-Orozco et al., 2020), tuberculosis (Li, Zeng, & Deng, 2012) Multiple sclerosis (Castaneda-Moreno et al., 2018), cancer (Chuo et al., 2021), ulcerative colitis (Shiroeda et al., 2010) and psoriasis (Chhabra et al., 2021). Though, extracellular stimuli is known to trigger MIF expression and direct the pathogenesis of diseases, MIF polymorphic variants can also steer the host response to infection by prolonged MIF expression and subsequent disease outcome in patients (Baugh et al., 2002). High MIF expression allele (-794 $CATT_7$) activated MIF gene expression and secretion is primarily involved in inflammatory and immune diseases severity, thus representing a systemic marker of appropriate diseases.

*4.1.2. MIF -173*G/C SNP*

The genotype and allele frequencies of MIF -173 G/C polymorphism are significantly varied between healthy control and diseased cases. Recent studies revealed that the distribution of −173 G/C polymorphism was strongly correlated with disease susceptibility and severity. The carriage of allele C associated genotypes (GC and CC) had increased risk of disease onset and progression. The genotypes GC and CC occur more frequently in patients with several diseases including with inflammatory and autoimmune diseases including inflammatory polyarthritis (Barton et al., 2003), coronary artery disease (Du et al., 2020), pulmonary tuberculosis (Tong et al., 2017), pneumococcal meningitis (Savva et al., 2016), vitiligo (Garcia-Orozco et al., 2020), inflammatory bowel

disease (Shen et al., 2013), Behcet's disease (Nursal et al., 2018), multiple sclerosis (Castaneda-Moreno et al., 2018), psoriatic arthritis (Morales-Zambrano et al., 2014), and cancer (Chuo et al., 2021). Thus, genotypes GC and CC are considered as potential genotypic risk factor and may serve as a biomarker to predict the high risk diseases. In contrast, the genotype GG have no effects and frequently occur in healthy controls (Barton et al., 2003). But, a recent study revealed the genotype GG to be associated with increased risk of heart failure (El-Mahdy, Saleem, Essam, & Algowhary, 2021). Though the allele frequency of MIF -173 G/C polymorphism cannot be generalized to all diseases, the results from these recent studies prove the G/C polymorphism as an essential criterion to be considered while developing diagnostic markers and therapeutic strategies (Savva et al., 2016). MIF gene polymorphism can transcriptionally regulate MIF expression in patients and determine the varied disease outcome. G/C polymorphism in MIF promoter region is known to generate the binding site for the transcription factor, activator protein 4 (AP-4) and result in increased MIF transcript and protein levels (Donn, Shelley, Ollier, Thomson, and British Paediatric Rheumatology Study, G, 2001). Additionally, MIF gene polymorphisms are correlated with the CpG island hypermethylation (CIHM) of tumor suppressor genes such as $p14^{ARF}$ and $p16^{INK4\alpha}$, and thus leads to gene silencing. The ulcerative colitis (UC) patients carrying MIF -173*C allele is found to be associated with CIHM of $p14^{ARF}$ gene. Whereas patients carrying MIF -794 $(CATT)_7$ repeats are associated with CIHM of $p16^{INK4\alpha}$. The MIF gene polymorphism mediated aberrant mutation increased the risk of developing colitis associated cancer in UC patients. Thus, high risk MIF gene polymorphic allele may be a potential biomarker to identify the UC patients at high risk for developing carcinogenesis (Sakurai et al., 2020).

4.2. Transcriptional regulation

The molecular mechanism that regulate the transcription of *MIF* have been investigated in several studies and reviewed elsewhere (Okazaki et al., 2018). Putative binding sites for activator protein (AP-1), NFκB, glucocorticoid receptor (GR) have been identified in the *MIF* promoter. The GR has proven to be effective in inducing MIF transcription. The *MIF* promoter has two functional binding sites for NFκB (-2538/-2528 and -1389/-1380). The transcription factors CREB and AP-1 is known to interact with proximal CRE (-20/-11) and SP1 (-42/-34) sites to regulate the transcriptional activity of the constitutive promoter (Baugh et al., 2002). In monocytic cells, the CRE and Sp1 sites facilitate the induction of *MIF* expression when treated with microbial products (Roger, Ding, Chanson, Renner, & Calandra, 2007). In CEM-C7 T cells, the CRE site also regulates glucocorticoid-induced MIF expression, but this activity can be eliminated by the disruption of the distal GR element consensus (at -742) (Leng et al., 2009). In endometrial cells, the recruitment of NFκB to the promoter region activates MIF transcription in response to TNF-α, and IL-1β secretion (Veillat et al., 2009).

4.2.1. Epigenetic regulation

Epigenetic mechanisms regulate the gene expression at either transcription, post-transcription, translation and post-translation levels. Histone deacetylation by the histone deacetylase (HDAC) typically reduces the recruitment of transcription factors that are essential for driving basal gene transcription (Jankauskas, Wong, Bucala, Djudjaj, & Boor, 2019). Nevertheless, HDAC inhibitors (HDCAi) are known to impair the transcription of *MIF* and to have significant impact on the *in vivo* and *in vitro* protein expression. For instance, inhibition of HDAC by TSA is known to impair the recruitment and interaction of transcription factors Sp1, CREB and RNA polymerase II to proximal *MIF* promoter (Lugrin et al., 2009). Although the molecular mechanism through which HDAC inhibitors (HDACi) affect gene expression are not fully understood, the poor accessibility of the transcription machinery can explain the reduced expression of MIF upon TSA treatment (Lugrin et al., 2009). Another study showed treatment with MS-275 and suberoylanilide hydroxamic acid (SAHA) to have a potent suppressive effects on MIF expression (Choo, Ho, Tanaka, & Lin, 2013). VPA is another HDAC inhibitor used to treat epilepsy and bipolar disorders. All three HDCAi: VPA, SAHA and TSA is known to downregulate MIF mRNA in different tumor cells lines and also in mouse bone marrow derived macrophages. In contrast to the MIF downregulating effect of HDACi, a recent study suggested the increased serum MIF levels in schizophrenia (SCZ) to be associated with the activity of antipsychotic drug, clozapine (a HDACi). Clozapine increased the expression of MIF mRNA and protein by enhancing the acetylation of histone H3 at lysine 27 (Okazaki et al., 2018). Results from these studies indicate existence of HDACi-specific activation/inhibition of MIF gene expression.

4.3. miRNA regulation

MicroRNAs (miRNAs) are a short non-coding RNAs that regulate the gene expression by promoting mRNA degradation or by translational repression at the 3' UTR of target genes. miRNAs and cytokines are known to be involved in cellular signaling that drives the physiological events and disease progression (Gorabi et al., 2020). Because of its multifaceted role and with the recent advances, miRNA have emerged as promising biomarkers and epigenetic regulators. miRNAs are known to regulate MIF expression. For instance, miR-654 was identified as a negative regulator of MIF and exerts its effect by binding to 3′-UTR of MIF. The suppression of MIF expression by miR654 reduces the MIF-dependent AKT and ERK phosphorylation and downstream pro-inflammatory cytokine expression in lupus nephritis (Tu et al., 2019). MiR-451 MIF and downregulates the mRNA and protein expression in the experimental model of gastric cancer. LRNA9884, a long non-coding RNAs (lncRNA) longer than 200 nucleotides was observed to be upregulated in AKI and LRNA9884 stimulates MIF expression and mediate the NF-κB associated inflammatory cytokine production. The regulation of MIF by LRNA9884 was observed to aggravate the renal damage in AKI (Zhang et al., 2020). Thus, the epigenetic marks related to appropriate diseases may involve in the development of disease diagnosis and prediction of severity.

4.4. Post-translational regulations

4.4.1. Redox dependent conformational isoform of MIF

MIF has two immunologically distinct conformational isoforms; oxidized MIF (OxMIF) and reduced MIF (RedMIF). OxMIF is the disease-related form that is specifically expressed only in the inflammatory state of acute and chronic diseases, and it is circulated in plasma to promote disease severity. Total MIF was moderately circulated in healthy control individuals, whereas oxMIF protein in healthy control was scarce. In a diseased state, total MIF concentration consists of two-third oxidized MIF which expression is inflammatory tissue-specific but not significantly in circulation. Schinagl et al., analyzed the distribution of oxMIF in plasma and specific tissues of solid tumors such as pancreatic, colorectal, ovarian, prostate, head and neck, breast, and lung cancer. The less considerable plasma oxMIF level was noticed in all cancer types except ovarian cancer; however, the total MIF of plasma was elevated in most cancer patients tested. Inversely, the level of oxMIF protein was detected significantly in almost several cancer tissues. MIF-mediated inflammatory progression was significantly reduced in anti- oxMIF treatment *in vivo* during inflammatory bowel disease (Thiele et al., 2015). These studies clearly show that oxidized state is responsible for detrimental effects of MIF that reflects the fact that redMIF is the ubiquitously expressed isoform involved in essential physiological processes and pathological conditions. In addition, the specific neutralization of oxMIF provides better therapeutic efficiency than against total MIF.

4.4.2. CD44 splice variant isoform

CD44 is an integral member/co-receptor of the CD74 receptor complex. The alternative splicing of the exons of the CD44 gene produces splice variants named CD44v1 to CD44v10. Among these, v3 and v6 isoforms are highly associated with clinical manifestations of diseases as well as the cells that overexpressed these variants are greatly oncogenic invasive. The expression of splice variant isoforms (CD44v3 and v6) is hugely higher in T cells of systemic lupus erythematosus (SLE) patients (Crispin et al., 2010) and fibroblast-like synoviocytes (FLS) of RA patients (Wibulswas et al., 2002). The expression of CD44v3 and v6 variants lead to an enhancement of cellular invasion. Invasion and infiltration of T cells into the target site are responsible for the abnormal inflammatory response and disease severity. High MIF expressing genetic polymorphism regulates the oncogenic CD44 splice variant expression and CD74-CD44 interaction for invasive activity in RA (Yoo et al., 2016).

4.4.3. S-Nitrosylation of cysteine residues

S-Nitrosylation is a redox-dependent, reversible posttranslational protein modification that can play an essential role in regulating dynamic processes. Cys-81 of MIF is selectively S-nitrosylated during redox stress. Commonly cysteine residues are highly reactive groups, and this modification regulates the cardioprotective role of MIF by exacerbating the MIF's oxidoreductase activity. S-nitrosylation of MIF blocks the binding interaction between MIF and JAB1 (c-Jun activation domain-binding protein-1), promoting anti-apoptotic activity and increases the MIF expression to execute its beneficial effects (Luedike et al., 2012). Additionally, nitrosylated Cys-81 based MIF and p53 interaction inactivate p53 to inhibit apoptosis (Jung et al., 2008).

4.4.4. Phosphorylation and cysteinylation

Other essential modifications are phosphorylation and cysteinylation. Cysteinylation at Cys-60 of MIF, in general, defined as an attachment of free second cysteine to unpaired cysteine residue by the formation of disulfide bonds. Whereas, the attachment of phosphoryl group to Ser-91 of MIF is involved in signal transduction and is known to regulate many cellular processes. Cysteinylation of Cys-60 facilitates the bioactivity of MIF by processes, including 1) increases the affinity of MIF to its receptors on activated target cells, T and B lymphocytes. Cysteinylation of Cys-60 allows a conformational alteration in α2 helix region thus enhancing its interaction with its receptor, 2) immunosuppressive activity. Interestingly, phosphorylation at Ser-91 inactivates the cysteinylated MIF. Thus, cysteinylated MIF is the bioactive isoform that exhibits beneficial effects (Watarai et al., 2000).

4.4.5. Glycation and glucose modification (oxidation) of MIF

In diseased condition, MIF is glycated in which the N-terminal amino acid residues of MIF are linked to glucose. Glycation and oxidation is the pathological hallmark of AD. Glycated MIF is protected from protease-mediated degradation. Also, glycation results in the absolute inhibition of enzymatic activity such as tautomerase and oxidoreductase *in vitro*, and negatively regulate ERK phosphorylation. Therefore, glycated and OxMIF exacerbates the disease and implicates hyperglycemia, dysregulated immune response, and oxidative stress (Kassaar et al., 2017).

4.5. Binding receptors

4.5.1. CD74 receptor

CD74 is a cell membrane cognate receptor with high affinity and it directs the role of MIF in pathological conditions. During injury, MIF signaling pathways are activated by CD74 and drive epithelial cell regeneration and healing. Activated MIF and CD74 provoke the proliferative and survival pathways such as ERK1 and 2, PI3K-Akt, NFκB, and AMPK pathways to heal injured cells (Leng et al., 2003). Moreover, activated CD74 plays an essential role in B cells proliferation and survival mechanisms. Activated CD74 upregulates the expression of Cyclin E and BCL-XL (B cell survival factor), indicating that CD74 promotes the cell division of B cells and apoptosis inhibition. Thus, CD74 acts as a survival receptor (Starlets et al., 2006). MIF-CD74 complex requires a co-receptor CD44 as a signaling component, especially for ERK phosphorylation (Shi et al., 2006). Inversely, MIF-CD74 interaction increases the ERK1/2 activation in prostate cancer leads to the worsening of cancerous condition (Meyer-Siegler, Iczkowski, Leng, Bucala, & Vera, 2006). Furthermore, the MIF-CD74 complex downregulates cell surface Fas receptor expression and desensitize lymphoma cells to Fas-mediated apoptosis and Fas-dependent chemotherapies (Berkova et al., 2014). Liu et al., demonstrated that co-regulation of CD74 and CD44 enhanced breast cancer cells invasion and migration by phosphorylating the metastasis-associated actin-binding protein, Cofilin 1 (CFL-1). Therefore, the outcomes of signaling pathways regulated by the MIF-CD74 complex depend on the type of pathological conditions (Liu et al., 2016).

4.5.2. CXC motif chemokine receptor (CXCR)

The upregulation of MIF expression induces the heteromeric receptor complexes by interacting between the cognate and non-cognate receptors of MIF, and indeed, MIF can bind directly with the non-cognate receptor. Each receptor complex carries out different biological functions in response to cellular MIF. MIF triggers the CD74/CXCR4 complex formation and activate the Akt signaling in T lymphocytes (Schwartz et al., 2009). CD74/CXCR2 and CD74/CXCR4 complex arrests monocytes and T cells by stimulating adhesion molecule expression. At the same time, the direct interaction of MIF with CXCR2 and CXCR4 elicits chemotaxis of leukocytes such as neutrophils and monocytes, thereby exacerbating the effects of MIF during inflammatory and atherogenic conditions (Bernhagen et al., 2007). The direct binding of MIF and CXCR7 stimulates the B- cell migration, ERK1/2 signaling, and zeta-chain associated protein kinase (ZAP)-70 (Alampour-Rajabi et al., 2015). Thus, the pattern of binding receptors of MIF determines the versatile functions of MIF.

5. MIF targeting therapies

As the multifaceted functions of MIF are being described in the pathogenesis of inflammatory and autoimmune diseases, in recent years, MIF has emerged as an attractive and novel therapeutic target of diseases. Generally, neuroendocrine axis is an essential regulatory system that directs the inflammatory responses, indeed MIF is a primary secreted protein of anterior pituitary cells upon stimulation which increased the attention of researchers to focus on evaluation of MIF functions and MIF targeting therapies (Bernhagen et al., 1996). Since the pathological role of MIF are highlighted in cancer progression, nowadays MIF based therapeutic strategies are the progressive area of research in oncology. MIF targeting therapies has three major approaches which are anti-MIF neutralizing antibodies, indirect destabilization of MIF and MIF antagonist or small molecule inhibitors (O'Reilly, Doroudian, Mawhinney, & Donnelly, 2016). Recent studies defined that anti-MIF antibody, antibodies against MIF receptors (anti-CD74, anti-CXCR4, anti-CXCR2), and small molecule inhibitors could potentially impact MIF mediated biological activity and its associated pathogenesis. MIF targeting drug developments are more effective at improving the treatment strategies in relevant clinical situations. Some antibody-based therapeutic strategies have advanced into clinical trials such as Imalumab (Phase II trial) (Liu et al., 2015), BaxB01, BaxG03, BaxM159 (Research and pre-clinical models) (Hussain et al., 2013), NbE10-NbAlb8-NbE10 (half-life extended nanobody) (Research and pre-clinical model) (Sparkes et al., 2018), Milatuzumab (Phase II trial) (Haran et al., 2018), and MEDI3185 (Research and pre-clinical models) (Cheng et al., 2009), and Ulocuplumab (phase Ib/II trial)

Table 2
MIF based therapeutic concepts for pathological conditions.

Pharmacological approaches	Targeting diseases/pathologic conditions	Mechanism of action	References
Antibody based therapy			
Anti-MIF antibodies	Tuberculosis	Inhibited the development of delayed-type hypersensitivity reactions	(Bernhagen et al., 1996)
	Collagen type II induced Arthritis	Decreased the production of anti-collagen type II IgG2a antibodies Increased the immune response of T lymphocytes to Collagen type II Delayed the onset as well as decreased the frequency of arthritis	(Mikulowska et al., 1997)
	Sepsis	Provide survival benefit to infection model by decreased the magnitude of bacteremia and TNF-α concentration in plasma	(Calandra et al., 2000)
	Clostridium difficile infection	Reduced the disease severity and mortality by reducing tissue and plasma MIF levels and minimizing clinical features such as weight loss, diarrhea and intestinal tissue inflammation	(Jose et al., 2018)
	Staphylococcus toxic shock syndrome	Inhibited the excessive inflammation by reduced the lymphocyte activation, spleen cell proliferation and enlargement	(Calandra et al., 1998)
	Cancer	Imalumab (BAX69) regulate the steady level of circulating Ox MIF and total MIF Anti-tumor activity and anti-MIF activity Anti-MIF antibodies BaxG03, BaxB01 and BaxM159 inhibits reduced the growth and invasion of prostate cancer cells by prevents the MIF induced activation of ERK1/2 and AKT Increased activation of Caspase 3/7	(Hussain et al., 2013; Mahalingam et al., 2020)
	Peripheral osteolytic lesions	Prevents the MIF induced homing of CXCR4+ osteoclast precursor cells to osteolytic lesions Minimize bone resorption	(Movila et al., 2016)
	Atherosclerosis	Neutralizing anti-MIF mAB (clone III.D.9) diminishes the circulating inflammatory mediators (Fibrinogen, MIF and IL-6) and local aortic inflammatory mediators (ICAM-1, MMP-2, TNF, IL-12, and CD40L) Minimizes the inflammation by downregulates the transcription factors, phospho-c-Jun and C/EBP-β	(Burger-Kentischer et al., 2006)
Anti-MIF nanobodies (NbE10-NbAlb8-NbE10)	Septic shock	Inhibits tautomerase activity, TNF-α mediated inflammatory responses, glucocorticoid counteraction Interfere with inflammatory end-organ damage and minimize the lethality	(Sparkes et al., 2018)
Anti-CD74 antibodies	Tumorigenesis	Milatuzumab: Anti-proliferative effects and stimulates extrinsic apoptotic pathway by activates Caspase-8 and -3 Inhibits the activation of proliferative NF-κB pathway	(Ghoochani et al., 2016; Stein et al., 2004; Stein et al., 2009)
	Brain tumorigenesis	Inhibits glioma growth and stimulate M2 to M1 shift of microglia by enhance the IFN-γ secretion Maintain microglial proinflammatory M1 function	
Anti-CXCR4 antibody	Tumorigenesis	MEDI3185 inhibits tumor growth in hematologic and ovarian tumors by prevents the binding of CXCR4 to chemokine stromal derived factor-1 (SDF-1) and blocks the CXCR4 mediated signaling Ulocuplumab (BMS-936564/MDX-1338) inhibited tumor growth by stimulates the apoptosis in acute myeloid leukemia, multiple myeloma, hematologic and solid tumor malignancies Ulocuplumab stimulates apoptotic cell death by enhanced ROS production Inhibited the CXCR4/CXCL12 pathway Monoclonal antibody 7D4 decelerates proliferation of CXCR4 expressing glioma cells 6H7 and 7D4 inhibited the SDF-1α induced cell migration	(Z. Cheng et al., 2009; Kashyap et al., 2016; Kuhne et al., 2013; Peng, Oganesyan, Wu, Dall'Acqua, & Damschroder, 2015)
Anti-CXCR2 antibody	Angiogenesis Multiple Sclerosis	Inhibited neovascularization in pancreatic cancer cells Proliferation of oligodendrocyte progenitor cells in demyelinating lesions Accelerate remyelination and promote myelin repair	(Wente et al., 2006) (Liu et al., 2010)
Combined Anti-MIF and Anti-CXCR4 antibodies	Ischemia	Blocks the MIF mediated chemotaxis of endothelial progenitor cells to ischemic tissues under hypoxic conditions	(Simons et al., 2011)
Small molecule inhibitors			
Repertaxin (CXCR2 inhibitor)	Intestinal ischemia and reperfusion injury	Suppressed the elevation of tissue and serum TNF-α Reduced the vascular permeability and neutrophil influx in intestine and lungs Reduced the tissue injury, systemic inflammation and reperfusion mediated lethality	(Souza et al., 2004)
AV411 (Ibudilast; 3-isobutyryl-2-isopropylpyrazolo-[1,5-a]pyridine)	Inflammatory, autoimmune and neurological diseases	Blocks catalytic (tautomerase activity) and chemotactic functions of MIF	(Cho et al., 2010)

(continued on next page)

Table 2 (*continued*)

Pharmacological approaches	Targeting diseases/pathologic conditions	Mechanism of action	References
N-benzyl-benzoxazol-2-ones	Rheumatoid arthritis	Inhibited MIF- dependent phosphorylation of ERK1/2 in human synovial fibroblast by blocks the MIF- CD74 mediated pathways	(Hare et al., 2010)
N-acetyl-p-benzoquinone imine (NAPQI)		Inhibits the tautomerase activity of MIF by reacts with catalytic Pro-1 to disrupt the biological active site of MIF	(Senter et al., 2002)
4-iodo-6-phenylpyrimidine (4-IPP)	Cancer	Blocks catalytic activity of MIF and lung adenocarcinoma cell migration	(Winner et al., 2008)
(*S,R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1)	Sepsis, chronic obstructive pulmonary disease	Inhibited the tautomerase and pro-inflammatory activity of MIF Inhibited the TNF-α release from macrophages Improved the survival rate and reduce disease progression Reduces cell proliferation and invasion, secretion of MIF protein	(Al-Abed et al., 2005; Korf et al., 2017; Meyer-Siegler et al., 2006; Russell et al., 2016)
	Prostate cancer	Reduces tumor volume and tumor angiogenesis Interferes with disease pathogenesis by inhibits the T cell activation, cytokine and chemokine responses	
	Autoimmune diabetes	Delayed the onset and development of diabetes	
p425	Inflammation	Inhibits MIF-CD74 interaction and tautomerase activity Blocks proinflammatory activity of MIF by inhibits the secretion of IL-6, IL-8 and MMP-3	(Bai et al., 2012)
SCD-19	Lung cancer	Reduced tumor volume by 81% and attenuate the MIF mediated lung cancer growth	(Mawhinney, et al., 2014)
	Inflammation	Inhibited LPS mediated PGE_2 and TNF-α secretion	
CPSI-1306	Bladder cancer	Minimize the growth and progression of cancer by inhibits the MIF mediated expression of angiogenic factors	(Choudhary et al., 2013)

(Ghobrial et al., 2020). The overview of the developments of MIF-based pharmacological approaches is summarized in Table 2.

5.1. Anti-MIF antibodies

Many preclinical studies and clinical trials were performed to evaluate the safety and biological activity of anti-MIF antibodies. Nanobodies (Nbs) are small single domain antigen binding fragments derived from camelid heavy chain antibodies, which have the advantages over conventional antibodies including minimum inflammatory responses due to lack of Fc region, high tissue penetrability, increased stability and solubility (Vincke et al., 2009). Sparkes and his colleagues developed the bioengineered Nbs against MIF for sepsis treatment. Among the investigated seven families of 11 different anti-MIF Nbs (NbB5, NbC10, NbA2, NbD2, NbD4, NbF10, NbH5, NbE5, NbH9, NbD12, and NbE10), NbE5 and NbE10 showed the lead inhibitory effect on proinflammatory activity of MIF. Further, four multivalent nanobodies (NbE10-NbAlb8, NbE10-NbE10, NbE10-NbAlb8-NbE10 and NbE10-NbE10-NbAlb8) were constructed to extend the serum half-life of Nbs, and suggested that the prophylactic and therapeutic administration (intraperitoneal) of NbE10-NbAlb8-NbE10 exhibits promising inhibitory effects on MIF (Sparkes et al., 2018).

The clinical trial (Phase I) on oxMIF antibody, Imalumab (BAX69) investigated the safety, pharmacokinetics (PK), tolerability and anti-tumor activity of antibody in patients with advanced solid tumors. The maximum tolerated dose of Imalumab was 37.5 mg/kg for every two weeks (intravenous administration) and the dose limited toxicity was allergic alveolitis in solid tumor patients. Imalumab is a fully human, anti-MIF monoclonal antibody. Imalumab had specific interaction with β sheet of oxMIF containing the oxidoreductase motif (Cys^{57}-Ala-Leu-Cys^{60}) of MIF. Imalumab exhibited the significant tissue penetration, activation of apoptotic pathways and provided the 26% of stable disease in treated patients (Kerschbaumer et al., 2012; Mahalingam et al., 2020). The phase Ib/IIa proof of concept study on imalumab in metastatic colorectal cancer patients will be to administer the 7.5 mg/Kg of antibody combined with 5- fluorouracil or leucovorin and the safety, tolerability, ORR, PK and overall survival rate will evaluated (Z. Liu et al., 2015). BaxG03, BaxB01 and BaxM159 are the recombinant human monoclonal antibodies developed against MIF. BaxB01 binds to the oxidoreductase motif of MIF, while BaxG03 and BaxM159 binds to C-terminus end of MIF protein to inhibit the tumor promoting activity of MIF in prostate cancer. The route of administration was intravenous, intraperitoneal and subcutaneous (Hussain et al., 2013). Several studies investigated the therapeutic role of anti-MIF antibodies in diseases including tuberculosis (Bernhagen et al., 1996), Collagen type II induced arthritis (Mikulowska, Metz, Bucala, & Holmdahl, 1997), sepsis (Calandra et al., 2000), *Clostridium* infection (Jose et al., 2018), cancer (Hussain et al., 2013), and atherosclerosis (Burger-Kentischer et al., 2006). The detailed mechanistic roles of antibodies in these diseases are presented in Table 2.

5.2. Antibodies directed against MIF receptors

About one third of marketed drugs are based on ligand-receptor related therapeutic targets (Shi et al., 2021). The binding of MIF and its receptors drives the MIF mediated signaling pathways. Recent studies suggested that blocking or inactivation of MIF-receptor complex could be a promising therapeutic strategy for diseases especially cancer. The cognate receptor of MIF, CD74 has overexpressed in most cancers and emerged as a leading therapeutic target of cancer. Milatuzumab is a humanized mouse monoclonal LL1 (anti-CD74) antibody. The preclinical studies on milatuzumab assessed the safety and anti-tumor activity and reported that no mortality and organ toxicity with single dose of 250 μg/Kg as well as with multiple doses of 50 μg/Kg (Stein, Smith, Chen, Zalath, & Goldenberg, 2009). The cross linked milatuzumab with bortezomib, doxorubicin or dexamethasone enhanced the apoptotic

pathway and anti-proliferative activity. The combined milatuzumab and bortezomib increased the median survival in mouse models of multiple myeloma (MM) (Stein et al., 2007).

Most clinical trials on milatuzumab were performed with patients of MM and few studies were dealt with chronic lymphocytic leukemia (CLL) and Non-Hodgkins lymphoma (NHL). The phase I clinical trial on milatuzumab was a multicentre, dose escalation study in advanced MM patients. Intravenous administration of 8 doses of milatuzumab was performed for 4 consecutive weeks. Milatuzumab showed no dose limiting toxicity even at the administration of higher dose (16 mg/Kg), and also showed low accumulation and rapid clearance from serum. At the end of 12 week post treatment, 26% of stable disease was reported in patients with progressive disease. The median B cell concentration in bloodstream was significantly reduced by 34%, but no effects on T lymphocytes and monocytes were observed (Kaufman et al., 2013). There are reports on the Phase I-II trial of milatuzumab in frail patients of refractory CLL (Haran et al., 2018) and combined milatuzumab and veltuzumab (humanized anti-CD20 antibody) in relapsed and refractory B cell NHL (Christian et al., 2015). In the unique patient centered study, Milatuzumab was administered at a dose of 120 mg/m^2 twice weekly for 12 weeks. Reported responses are reduced spleen size in patients having enlarged spleen, reduced level of BCL2 protein in CLL cells, increased counts of white blood cells, improved quality of life and stable disease (Haran et al., 2018). In another clinical trial II, escalating doses at 8, 16 and 20 mg/Kg milatuzumab for 4 weeks and 200 mg/m^2 veltuzumab was administered in patients of NHL and assessed the tolerability and activity of combined antibodies. Most of the toxicity reactions are limited and manageable and the overall response rate (ORR) was 25% in this phase II trial (Christian et al., 2015).

CXCR2 and CXCR4 are the non-cognate receptor of MIF. The inhibition of the binding interaction of MIF and these receptors exhibited the improvement of diseases. Studies reported anti-CXCR2 antibody to have an inhibitory effect on neutrophil chemotaxis, experimental autoimmune encephalomyelitis, angiogenesis and demyelination of multiple sclerosi(Liu et al., 2010; Wente et al., 2006) (Shi et al., 2021). Ulocuplumab (BMS-936564/MDX-1338) is a fully humanized anti-CXCR4 antibody which inhibits the binding interaction between CXCR4 and CXCL12. The pre-clinical study on ulocuplumab characterizes the antibody for its therapeutic potential against acute myeloid leukemia (AML), NHL, CLL and MM. MDX-1338 interacts with CXCR4 expressing cells and inhibits the CXCL12 mediated cellular mobilization, calcium flux and promotes anti-tumor activity through apoptosis (Kuhne et al., 2013). The phase I trial was conducted to demonstrate the tolerability, safety, pharmacodynamics, PK, and anti-cancer activity of antibody and combined ulocuplumab with lenalidomide and dexamethasone or bortezomib and dexamethasone in relapsed or refractory MM. The antibody was administered at the escalating doses of 1, 3 and 10 mg/kg. The ORR of all subjects was 50%. Mobilization of leukocytes and plasma cells were detected after each ulocuplumab infusion (Ghobrial et al., 2020). The recent phase I trial on ulocuplumab and ibrutinib (small molecule inhibitor of Bruton's tyrosine kinase with antineoplatic activity) measured the safety and dosage of antibodies to target the mutated CXCR4 in Waldenstrom macroglobulinemia patients. Ibrutinib (420 mg) was administered orally once per day until progression, while ulocuplumab was intravenously administered for 2 to 4 times per cycle for 6 cycles. The most common adverse effects are reversible thrombocytopenia, rash and skin infections, which is not associated with dose escalation (Treon et al., 2021). The phase Ib/II trial assessed the safety and tolerability of ulocuplumab alone and combined antibodies in relapsed or refractory MM. Neutropenia (43.3%) and thrombocytopenia (37.5%) are the common acceptable adverse effects observed in treated patients. The combined antibodies provide the 55.2% of response rate and 72.4% of clinical benefit rate (Ghobrial et al., 2020).

5.3. Small molecule inhibitors

A number of disease models suggested promising inactivation of MIF with small molecule inhibitors. Most of the MIF inhibitors bind the hydrophobic region of MIF that contains the conserved amino acids which promotes the biological activity of MIF. (*S*,*R*)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) is the first small molecule antagonist of MIF and is known to inhibit the proinflammatory activity of MIF. ISO-1 binds to enzymatic active site (Tautomerase) and inhibits the activity by competing with the substrate for catalytic site and modifies the binding interactions between MIF and its binding partners of inflammatory pathways (Al-Abed et al., 2005; Korf et al., 2017). Previous studies found the role of ISO-1 in disease improvement and suggested that ISO-1 is an attractive candidate for therapeutic studies of diseases where MIF has been implicated (Al-Abed & VanPatten, 2011). Repertaxin is a novel low molecular weight inhibitor of CXCL8 and CXCR2 receptor. Repertaxin reduces the inflammatory events and lethality in ischemia- reperfusion model (Souza et al., 2004). AV411 (Ibudilast; 3-isobutyryl-2-isopropylpyrazolo-[1,5-a]pyridine) is a nonselective allosteric anti-inflammatory drug which inhibits the chemotactic and catalytic activity of MIF (Cho et al., 2010). N-benzyl-benzoxazol-2-ones is a receptor antagonist of MIF that blocks the enzymatic activity and MIF-CD74 binding interactions (Hare et al., 2010). *N*-acetyl-p-benzoquinone imine (NAPQI) covalently binds to MIF and reacts with catalytic pro-1 of MIF. NAPQI binding results in a modified form of MIF protein with low binding capacity and proinflammatory activity (Senter et al., 2002). 4 - iodo-6-phenylpyrimidine (4-IPP) is an irreversible inhibitor of MIF which binds at the nitrogen of N-terminal proline of MIF (Winner et al., 2008). P425 is a novel sulfonated azo compound which acts as an allosteric MIF inhibitor and binds MIF by hydrophobic interactions and block the formation of MIF-CD74 complexes (Bai et al., 2012). CPSI-1306 is a low molecular weight isoxazoline which reduced the relapses of cancer and may have therapeutic implications in high grade bladder cancer (Choudhary et al., 2013). The mechanism of action of each small molecule inhibitors is summarized in Table 2. The discussed studies suggested that MIF directed therapies are one of the powerful pharmacological strategies and a novel class of drug to find the wide application in the treatment of inflammation associated complex diseases.

6. Conclusion

The expression of the MIF gene is an essential part of the host response to infection. MIF is implicated in a range of diseases and exerts both physiological and pathological effects. The microenvironment of cells, MIF-specific genetic variants of the host, and functional MIF receptors determine the functionality of MIF. Identification of elevated MIF might be a novel and early diagnostic marker for several diseases. We anticipate MIF-based therapeutic concepts will be appreciated for broad range of disease management in near future. Also, during an outbreak situation, identifying host genetic susceptibility and disease progressing risk factors by analyzing the MIF allele distribution can stratify the patients to provide priority-based treatment. This review highlighted the potential of MIF as a diagnostic marker and therapeutic target for early screening and treatment of diseases.

Declaration of competing interest

The authors declare no conflicts of interest.

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RESEARCH ARTICLE
Clinical Science and Epidemiology

MicroRNAs Regulated by the LPS/TLR2 Immune Axis as Bona Fide Biomarkers for Diagnosis of Acute Leptospirosis

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ABSTRACT Leptospirosis remains a significant human health issue due to its systemic complications. Therefore, biomarkers that are more effective are urgently needed for the early diagnosis of leptospirosis. MicroRNAs (miRNAs) are evolutionarily conserved regulatory RNAs that have shown the potential to be used as biomarkers for diagnosis, prognosis, and therapy of infectious diseases. In this study, we performed an unbiased screen using the miRNome miRNA array to identify circulating miRNAs with the potential to serve as authentic biomarkers for early diagnosis of leptospirosis. Because leptospiral lipopolysaccharide (LPS) is the predominant leptospiral antigen and plays a vital role in immunological and biological activities, we used LPS treated and untreated *in vitro* (THP1 cells) and *in vivo* (BALB/c mice) surrogate models to identify the LPS-specific miRNAs. Differential expression analysis revealed 18 miRNAs to be associated strongly with LPS stimulation in THP1 cells. Of these, three (miR-let-7b-5p, miR-144-3p, and miR-21-5p) were observed to be present at increased levels *in vivo*. The identified miRNAs were validated for their biomarker potential using serum samples from leptospirosis-negative patients and patients with confirmed cases of leptospirosis. Identified miRNAs were able to discriminate the acute leptospiral infection from other febrile diseases with a test sensitivity and specificity of 93.2% and 88.19%, respectively. Gene functional enrichment and protein-protein interaction (PPI) network analysis revealed that the identified miRNAs play important roles in disease signal transduction, signaling by interleukins, the stress-activated protein kinase signaling cascade, the mitogen-activated protein kinase (MAPK) signaling pathway, and the cellular response to a transforming growth factor β (TGF-β) stimulus with a notable interconnection between these biological processes.

IMPORTANCE Here, we used miRNAs that are differentially regulated by the LPS/TLR2 immune axis to devise a miRNA-based diagnosis for leptospirosis. The study established the role of the circulating stable miRNAs (miR-21-5p, miR-144-3p, and miR-let-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs can be used to diagnose acute leptospirosis and also to differentiate leptospiral infection from other bacterial and spirochetal infections, as proved by the use of human clinical samples. Thus, our findings indicate that miRNAs can play a crucial role in the diagnosis of infectious diseases, like leptospirosis, that are generally misdiagnosed.

KEYWORDS leptospirosis, LPS, microRNA, TLR2, biomarkers, PPI network

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Leptospirosis is a zoonotic disease caused by pathogenic serovars of spirochetal bacteria belonging to the genus *Leptospira* (1). Although leptospirosis has long been recognized as an important endemic disease in tropical countries, it is now becoming a more common problem in highly populated urban centers (2). The Leptospirosis Burden Epidemiology Reference Group (LERG) report included a systematic literature review that estimated the overall global annual incidence of endemic and epidemic human leptospirosis at 5 and 14 cases per 100,000 population, respectively (57). Leptospirosis ranges in severity from a mild flu-like illness to hepatorenal impairment, multiorgan failure, and septic shock with pulmonary hemorrhages. Clinically, it is typified by diverse symptoms that include fever, myalgia, headache, severe muscle pain, ocular disorders, meningitis, jaundice, renal failure, and pulmonary hemorrhage, and it is often confounded with other entities such as influenza and dengue fever. In this regard, early and definitive diagnosis of leptospirosis is critical for preventing its severe clinical complications.

We and others have determined that immunity against *Leptospira* depends on the production of circulating antibodies directed against serovar-specific lipopolysaccharide (LPS) (3, 4). *Leptospira* produces an atypical LPS that differs from LPS of Gram-negative organisms in several biochemical, physical, and biological properties (5). LPS is known to activate macrophages by a mechanism that involves the serum protein LPS-binding protein and a plasma membrane receptor complex comprising at least CD14 and one or more members of the Toll-like receptors (TLRs) (6–8). Among the 10 TLRs present in human, a large body of evidence supports TLR4 as the predominant receptor mediating LPS activation (9, 10). In contrast, it was shown recently that leptospiral LPS activates human cells through CD14 and TLR2 recognition (11, 12), whereas both TLR2 and TLR4 contribute to the activation of murine cells (11, 12). The recognition of LPS by TLRs results in subsequent recruitment of the intracellular adaptor protein MyD88 through homotypic Toll–interleukin 1 receptor (IL-1R) domains, leading to intracellular signaling through NF-κB and mitogen-activated protein kinase (MAPK) activation. The ultimate result of this LPS recognition by TLRs is a proinflammatory cytokine and chemokine response. However, when high levels of leptospiremia occur during infection, innate immune mechanisms eventually trigger tissue-based and systemic responses to infection that lead to severe outcomes, such as a sepsis-like syndrome or organ failure (13–15). Therefore, it seems probable that mechanisms exist to regulate the host immune response. Although several regulators have been proposed, microRNAs (miRNAs) are recognized as important regulators of immune response (16–18) and as fine tuners of TLRs (19–21).

miRNAs are 22-nucleotide-long small noncoding regulatory RNA molecules that regulate gene expression posttranscriptionally through complementary base pairing with thousands of messenger RNAs and play important roles in regulating diverse physiological, developmental, and pathophysiological processes (22). Alterations of miRNA expression profiles have been observed in various diseases, including cancer and cardiovascular, neurological, inflammatory, and autoimmune diseases. A growing body of evidence supports the key role of miRNA in the activation of both innate and adaptive immune response (23). Studies have shown differential expression of miRNA following LPS stimulation in various types of immune cells (23, 24). Several early and late LPS-responsive miRNAs have been reported (23, 24). Thus, identification of an miRNA repertoire responsive to TLR2-mediated LPS signaling will be a valid biomarker for the diagnosis of human leptospirosis. Identification of dysregulated miRNAs in infectious diseases is emerging to help devise novel diagnostics, prevention measures, and therapy (25). Additionally, extracellular miRNAs were identified to be circulating in the blood, thus raising the possibility of finding a connection between specific miRNA levels in serum and various disease states (26–28). Also, biochemical analyses indicate that miRNAs are resistant to RNase activity, extreme pH, extreme temperatures, extended storage, and large numbers of freeze-thaw cycles (26, 29, 30), making their isolation and analysis straightforward. Compared to protein-based biomarkers, miRNAs are homogenous and can be easily detected by qPCR, while low abundance and

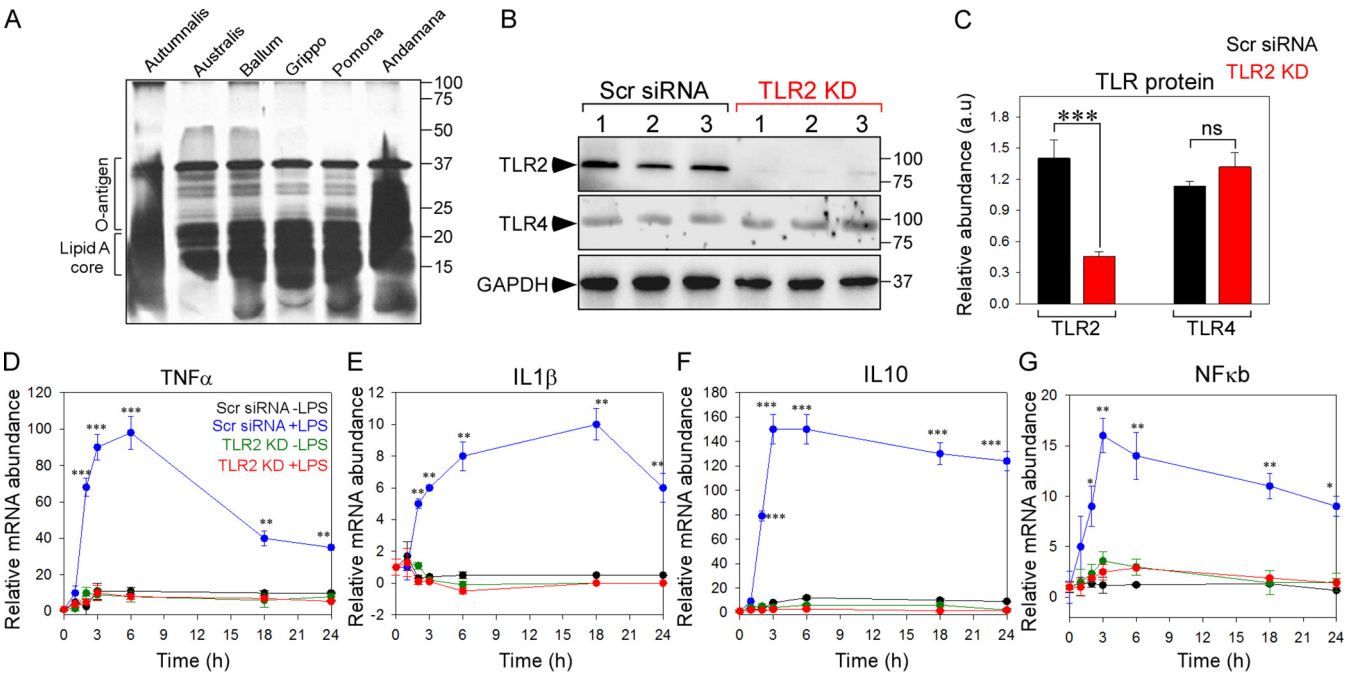

FIG 1 Extracted leptospiral LPS stimulates cytokine production in THP1 monocytes. (A) Representative SDS-gel image stained with silver stain. The electrophoretic mobility patterns of leptospiral LPS indicate apparent molecular masses between 14 and 37 kDa. (B) Representative Western blot analysis of the protein extracted from control (Scr siRNA) and TLR2 knockdown (KD) THP1 cells probed for antibodies specific for TLR2, TLR4, and GAPDH. The blot shows decreased expression of TLR2 and no changes in TLR4. (C) Quantification of the protein abundance of TLR2 and TLR4 from panel B by densitometric analysis, normalized to GAPDH. (D to G) Cytokine mRNA levels were measured in Scr siRNA and TLR2 KD cells treated with (blue and red) or without (black and green) LPS. Data are means ± SEM. ***, $P < 0.001$; **, $P < 0.005$; *, $P < 0.01$.

posttranslational modifications of protein markers affect the accuracy of the diagnosis (31). In this regard, the use of miRNAs that regulate LPS-TLR immune axis as novel biomarkers represents a new approach for early diagnosis of leptospirosis.

To that end, in the present study we used *in vitro* and *in vivo* models to identify the miRNAs that were differentially expressed during leptospiral LPS stimulation through the TLR2 axis. The identified miRNAs were validated for its efficacy to diagnose early leptospirosis using clinical samples. Additionally, we also predicted (i) the target genes, (ii) the potential functions of the differentially expressed miRNAs by gene ontology (GO) enrichment analysis, and (iii) a protein-protein interaction (PPI) network to identify the hub genes. The present study is the first to identify miRNAs specific for leptospiral LPS stimulation and provide a novel perspective on the involvement of these miRNAs in the pathological mechanism of leptospirosis.

RESULTS

Leptospiral LPS stimulates cell death and cytokine production. We first extracted and purified LPS from pathogenic (*Leptospira interrogans* serovar Australis strain AHF651, *L. interrogans* serovar Autumnalis strain N2, *L. kirschneri* serovar Grippotyphosa strain D22, *L. interrogans* serovar Pomona strain H3, and *Leptospira borgpetersenii* serovar Ballum strain BDU51) and nonpathogenic leptospiral strains (*Leptospira biflexa* serovar Andamana strain CH11). The concentration of the purified leptospiral LPS was between 575 and 625 μg/ml, and the LPS was free from protein, DNA, and RNA contaminations. The electrophoretic mobility patterns of leptospiral LPS evidenced apparent molecular masses between 14 and 37 kDa (Fig. 1A). Because leptospiral LPS is recognized by TLR2 in human cells due to differential recognition of the atypical lipid A moiety (11, 12), we transiently knocked down TLR2 in THP1 cells. Transient knockdown significantly decreased the levels of TLR2 in THP1 cells with no change in the TLR4 levels (Fig. 1B and C). Then, we verified the biological activity of leptospiral LPS using control (scrambled [Scr] small interfering RNA [siRNA]) and TLR2 knockdown cells.

First, we measured the cytotoxicity of leptospiral LPS by MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. Increased cytotoxicity was observed in THP1 cells stimulated with pathogenic LPS compared to unstimulated or nonpathogenic LPS stimulation (Fig. S1A to 1F). Additionally, knocking down TLR2 in THP1 cells protected them against LPS-mediated cell death. Because we observed that LPS extracted from *L. interrogans* serovar Autumnalis strain N2 caused increased cytotoxicity compared to LPS from other pathogenic serovars, LPS from Autumnalis was used for further analysis. We then measured the mRNA levels of the proinflammatory mediators in control and TLR2 knockdown THP1 cells stimulated with Autumnalis LPS for various time points. In control THP1 cells, mRNA levels of tumor necrosis factor alpha (TNF-α), NF-κB, IL-1β, and IL-10 increased within 3 h of LPS stimulation. On the other hand, TLR2 knockdown significantly inhibited the elevated cytokine mRNA levels (Fig. 1D to G). This confirmed that extracted leptospiral LPS activated human cells through TLR2 recognition. Further, to better validate the reactivity of the extracted LPS *in vivo*, cytokines were measured by extracting mRNA from the whole blood of BALB/c mice at different time intervals (0, 4, 7, 14, and 21 days) after intraperitoneal injection of leptospiral LPS in phosphate-buffered saline (PBS). Consistent with the increase in cytokine mRNA levels in THP1 cells, at day 3 LPS postinjection, there was an increase in mRNA expression of proinflammatory mediators, further validating the potency of the extracted LPS (Fig. S2A to D).

miRNome miScript microarray analysis of miRNAs. After confirming the ability of leptospiral LPS to prime immune responses through TLR2-dependent signaling, we next asked whether any of the positive or negative regulators of the TLR-signaling cascade could be used as biomarkers for the diagnosis of leptospirosis. Such a TLR regulation is primarily known to be achieved by the activation or repression of a large array of genes and microRNAs (miRNAs). Primarily, we used a human miRNome profile panel to identify LPS-responsive miRNAs that are differentially expressed in Scr siRNA and TLR2 knockdown THP1 cells. Analysis of the miRNA profile of the extracellular media of control and TLR2 knockdown cells with or without LPS treatment showed ~18 miRNAs to be upregulated (>10-fold) in THP1 cells treated with LPS compared to untreated controls (Table S1 and Fig. 2A), whereas knocking down TLR2 normalized the upregulated miRNA levels, indicating that these miRNAs are specific to the TLR2-LPS immune axis and thus can serve as biomarkers (Table S1 and Fig. 2A).

To further validate these miRNAs as authentic biomarkers for early diagnosis of leptospirosis, we adopted a murine model of LPS stimulation. Time-dependent changes in miRNAs expression profiles were analyzed in serum of mice injected with LPS. Compared to control animals, ~7, 2, 6, and 5 miRNAs showed >10-fold upregulation at days 4, 7, 14, and 21, respectively (Table S2 and Fig. 2B). Because we were interested in analyzing the miRNAs that are expressed and circulating in serum during early stages of leptospiral infection, we narrowed our miRNA categories to those that were upregulated day 4 and day 7. Of the miRNAs upregulated at day 4 or day 7, three (miR-21-5p, miR-144-3p, and miR-let-7b-5p) shared similarities with the human miRNome profile of the THP1 cells exposed to leptospiral LPS.

miRNAs for early diagnosis of leptospirosis. Because miR-21-5p, miR-144-3p, and miR-let-7b-5p shared similarities between human and mouse miRNome profiles and were upregulated during the early stage of leptospiral LPS administration, we next validated these three miRNAs for their diagnostic potential. Semiquantitative PCR was performed with serum samples to validate the diagnostic efficacy of the miRNAs. The fold changes of circulating miR-21-5p, miR-144-3p, and miR-let-7b-5p in the serum of confirmed cases of leptospirosis were significantly higher ($P < 0.001$) than those in healthy controls and persons diagnosed with other febrile illness (Fig. 3A to C).

To confirm the statistical relevance of the miRNA-based serum markers, a receiver operating characteristic (ROC) curve was established, and the area under the curve (AUC) was determined (Fig. 3D to G). AUC values for miR-21-5p, miR-144-3p, miR-let-7b-5p, and all three miRNAs combined were 0.92, 0.91, 0.94, and 0.92 respectively.

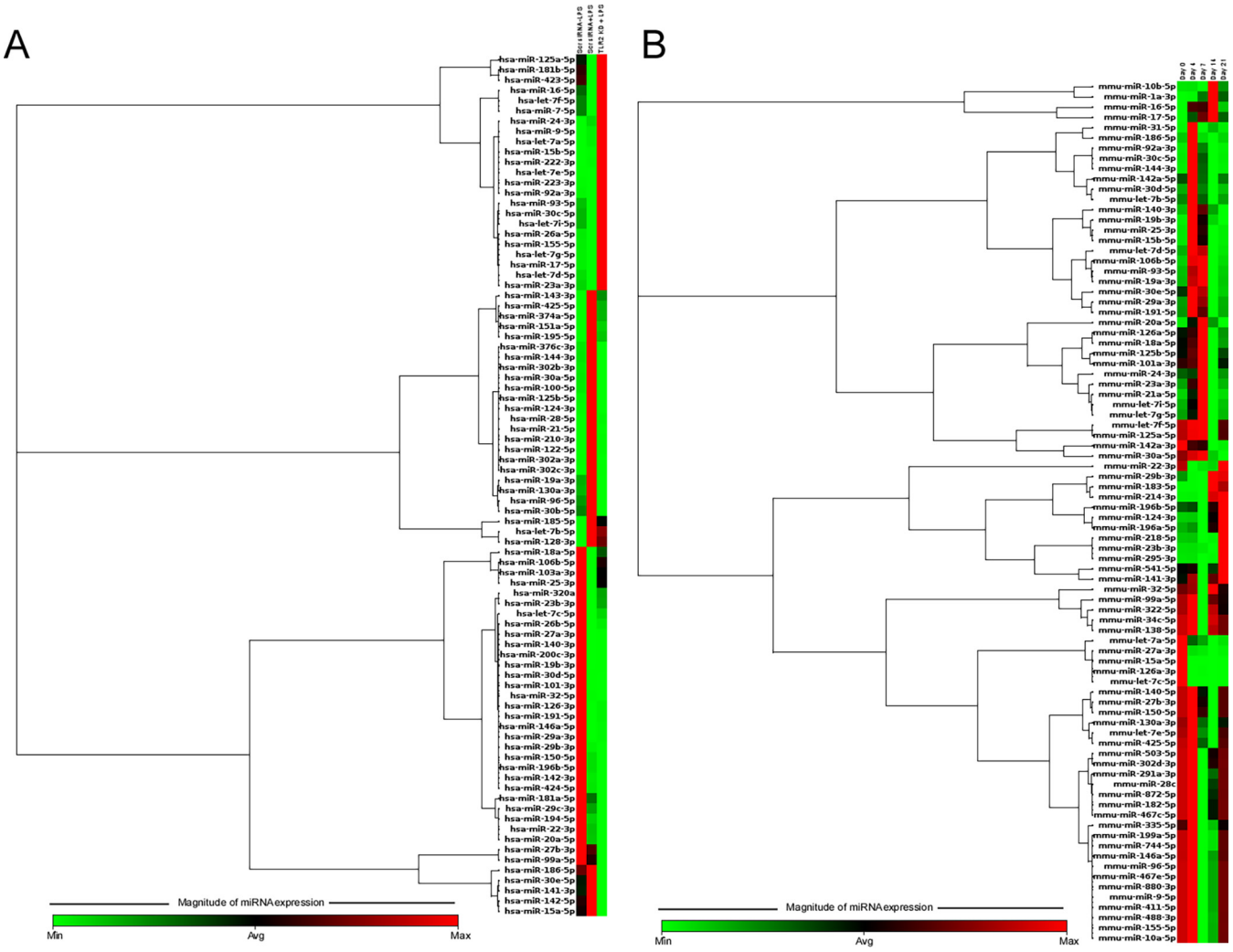

FIG 2 *In vitro* and *in vivo* miRNome miScript microarray analysis of miRNAs upregulated upon LPS treatment. (A) Hierarchical clustering of the differentially expressed miRNAs in control and TLR-2 KD THP1 cells after LPS stimulation. (B) Hierarchical clustering of the differentially expressed miRNAs in control and LPS-injected mice at different time intervals. Red represents miRNAs with an average fold change of 3, and green represents miRNAs with an average fold change of −3, relative to controls.

Quantifying the levels of serum miRNAs is reliable and reproducible, because the sums of the diagnostic sensitivity and specificity were 93.2% and 88.19%, respectively. The high sensitivity and considerable specificity suggest that the signature miRNAs might be potential candidates for diagnosis of acute leptospirosis.

Functional enrichment analysis of miRNA target genes. Because these miRNAs have diagnostic potential and were modulated by the TLR2 axis, we next asked whether these miRNAs target the genes that are involved in immune regulation. In both DIANA-microT-CDS and TargetScan, totals of 993, 521, and 1,611 genes were predicted to be the target genes of miR-let-7b-5p, miR-21-5p, and miR-144-3p, respectively. Gene ontology enrichment analysis revealed that miR-21-5p, miR-144-3p, and miR-let-7b-5p play important roles in disease signal transduction, signaling by interleukins, regulation in actin cytoskeleton, stress-activated protein kinase signaling cascade, MAPK signaling pathway, FoxO signaling pathways, cellular response to a transforming growth factor β (TGF-β) stimulus, and small-RNA loading onto the RNA-induced silencing complex (RISC) (Fig. 4A to C). Notably, all these biological processes are interconnected (Fig. 4D to F). Our data imply that the genes involved in the target signaling pathways may serve as potential diagnostic and therapeutic targets for leptospirosis in the future.

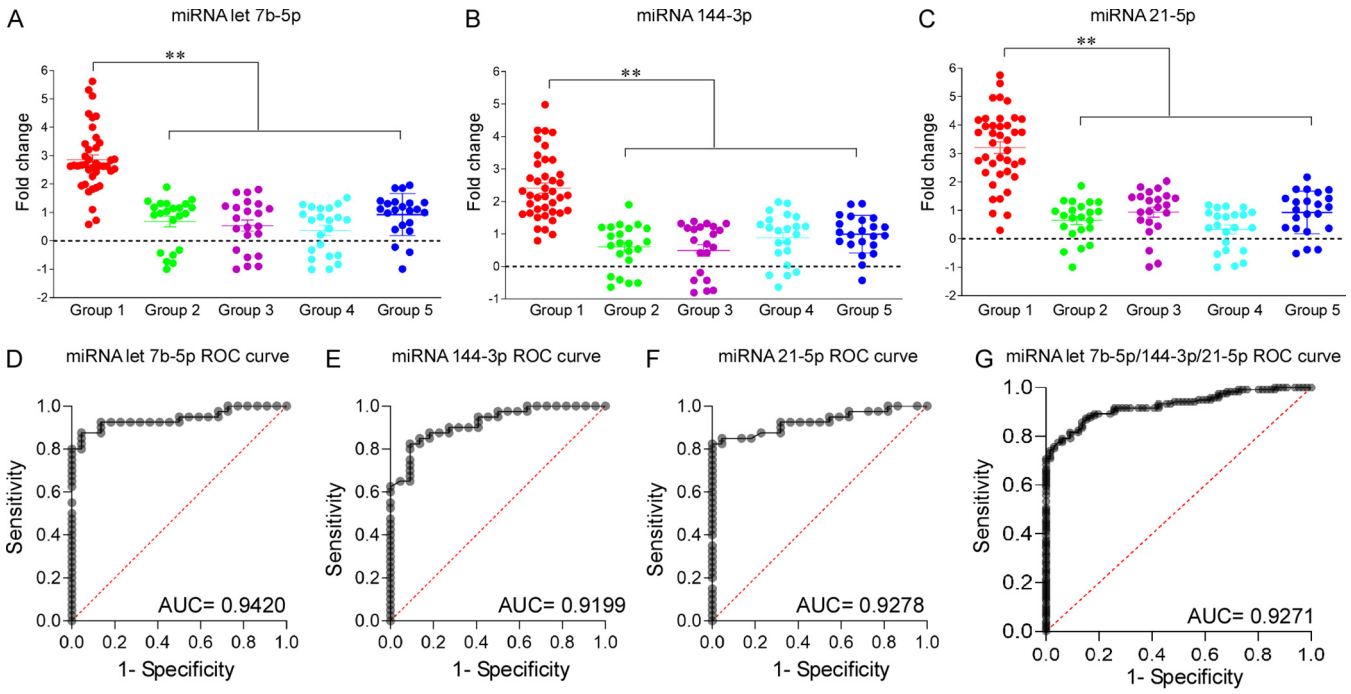

FIG 3 Analysis of the diagnostic potential of identified miRNAs for early diagnosis of leptospirosis. Total RNA was isolated from serum samples of patients and controls. Group 1, patients with laboratory-confirmed cases of leptospirosis; groups 2 to 4, patients suspected of having leptospirosis but identified as having other illnesses, including dengue (group 2), typhoid (group 3), and malaria (group 4); group 5, seronegative healthy controls. (A to C) qPCR analysis was used to measure the fold change in the miRNA levels between confirmed leptospiral cases and controls. The relative fold changes of miR-let-7b-5p (A), miR-144-3p (B), and miR-21-5p (C) showed the diagnostic potential of the identified miRNAs. Data points (dots) represent mean fold changes in individual patient's serum. The horizontal line represents the cutoff value. **, $P < 0.005$. (D to G) Plots of the sensitivity (true-positive rate) versus 1−specificity (false-positive rate) for miR-let-7b-5p (D), miR-144-3p (E), miR-21-5p (F), and all three miRNAs (G). AUC values indicate the fold change in leptospirosis cases (test group) versus normal samples (control group).

DISCUSSION

Monocytes belong to a subset of circulating white blood cells that can further differentiate into macrophages and dendritic cells (32). *In vivo* and *in vitro* studies have shown that monocytes and their derivatives function as essential components of the innate immune system that mediate host defense and serve as the first line of resistance to microbial attack (33). This innate immunity is triggered by the recognition of pathogen-associated molecular patterns (PAMPs), like LPS, through pattern recognition receptors (PRR); one such conserved PRR is TLR. Indeed, the pleiotropic potential of monocytes suggests that their cellular functions must be tightly regulated in a manner that is distinct from that of more differentiated myeloid cell types to enable appropriate, context-dependent responses.

MicroRNAs (miRNAs) are a large class of small noncoding RNAs that posttranscriptionally regulate mRNAs and subsequently influence essential cellular functions through modulation of gene expression at the RNA or protein level (34). Several miRNAs in myeloid cells were previously identified to impact immune responses, although results have varied due to different cell types, assay methods, and culture conditions (35, 36). Despite differences in cell type, both oligonucleotide-based microarray technology (21, 37, 38) and next-generation sequencing (NGS) technologies (23) indicated that the classic miRNAs hsa-mir-155, hsa-mir-9, and hsa-mir-146a are upregulated in LPS-treated monocytes, suggesting that these miRNAs are critical for promoting pan-myeloid function in response to LPS (21, 23, 37, 38). In another independent study, miRNA profiles of whole blood in mice exposed to LPS demonstrated a significant alteration of the multiple miRNAs (let-7d, miR15b, miR16, miR25, miR92a, miR103, miR107, and miR451) in a dose- and time-dependent fashion. Additionally, LPS from different Gram-negative bacteria, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Serratia marcescens*,

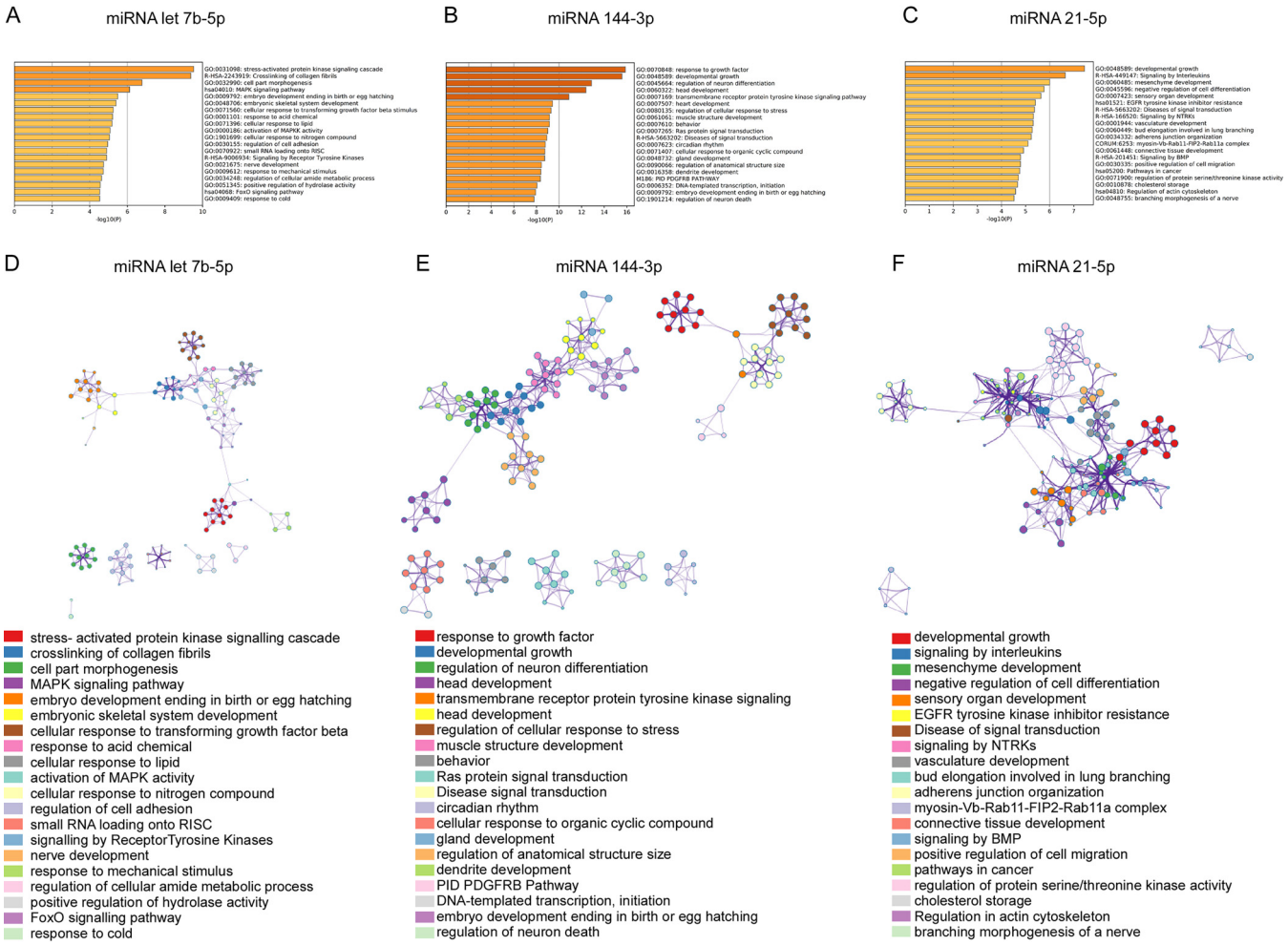

FIG 4 Functional enrichment analysis of miRNA target genes. (A to C) Top 20 clusters from Metascape pathway enrichment analysis of miR-let-7b-5p (A), miR-144-3p (B), and miR-21-5p (C) and the display of the associated genes of miR-let-7b-5p (D), miR-144-3p (E), and miR-21-5p (F) as a network. Nodes of the same color belong to the same cluster, and terms with a similarity score of >0.3 are linked by an edge.

induced the upregulation of similar miRNAs (24). However, in the present study, stimulation of monocytes (THP1 cells) or whole animals with leptospiral LPS did not induce the upregulation of any of these known signature miRNAs. Rather, 18 different miRNAs (Table S1 and Fig. 2A) were upregulated by stimulation of THP1 cells with leptospiral LPS. Additionally, knocking down TLR2 in THP1 cells normalized the expression of these miRNAs.

The difference in the miRNAs that are upregulated by leptospiral LPS stimulation could be explained by differences in the structure of the leptospiral lipid A. Lipid A is the anchor moiety of LPS in the bacterial membrane and is the active component of LPS responsible for its toxic activity and functions. Although the structure of lipid A from Gram-negative bacteria shows some variability between bacterial species (39), all lipid A moieties are known to stimulate the TLR4 complex. However, leptospiral LPS activated human cells through TLR2 instead of TLR4 (12). The leptospiral lipid A moiety structure was recently deciphered, revealing that it possesses some peculiar characteristics compared to lipid A of Gram-negative bacteria (40), including one phosphate residue that is capped with a methyl group, a lack of a 4′ phosphate group, and four amide-linked acyl chains, longer than in enterobacterial lipid A, with two secondary unsaturated acyl chains. Additionally, of these 18 miRNAs upregulated by leptospiral LPS, 6 (hsa-miR-185-5p, hsa-let-7b-5p, hsa-miR-28-5p, hsa-miR-376c-3p, hsa-miR-195-5p, and hsa-miR-100-5p) were found to be upregulated in bone marrow-derived

mesenchymal stem cells stimulated with TLR2 agonist (PAM3CSK4 cells) (41). This further confirms that the identified miRNAs are upregulated by leptospiral LPS through the TLR2 immune axis, and these miRNAs can be significant signature molecules to differentiate leptospiral infection from other bacterial infections with which it is often confused. Because other spirochetes, like *Borrelia* and *Treponema*, do not possess LPS, identification of leptospiral LPS-stimulated miRNAs could differentiate leptospiral infection from other spirochetal infections.

A recent work that examined the microtranscriptome of murine macrophages J774A.1 stimulated with virulent, attenuated, and saprophyte strains of *Leptospira* showed differential expression of 29 miRNAs. Of these 29 miRNAs, the highest fold change was observed for miR155-5p in cells stimulated with pathogenic *L. interrogans* (42). We did not see an upregulation of this miR155-5p, which could be due to different factors, including but not limited to the difference in the assay method, the cell type, and the culture conditions. Moreover, our study identified the miRNAs that are regulated by LPS stimulation in an *in vivo* mouse model. In this regard, our study is the first to validate the efficacy of miRNAs for the diagnosis of leptospirosis.

Early diagnosis of leptospiral infection is critical for preventing further complications, including multiorgan failure and hemorrhages. Although routine clinical methods, including isolation of leptospires, microscopic agglutination test (MAT), enzyme-linked immunosorbent assay (ELISA), and PCR, are standard methods for diagnosis of leptospirosis, these techniques are time-consuming and require special expertise that might delay treatment. Also, until now, potential biomarkers have included acute phase proteins, cytokines, and chemokines, but they are not sufficient to distinguish other bacterial infections from leptospiral infection. The miRNAs identified in our study are promising biomarkers for early leptospiral infection. In fact, miRNA-based biomarkers are already being used for diagnosis of tuberculosis (43, 44). Because we were interested in developing biomarkers for the early identification of leptospirosis, we characterized the circulating miRNAs in a time-dependent manner (4, 7, 14, and 21 days) in the whole blood of mice after LPS stimulation. The miRNAs miR-21-5p, miR-144-3p, and miR-let 7b-5p shared similarity with the human miRNome profile and were also expressed during the early stage of leptospiral infection (Fig. 2). We validated the diagnostic potential of these three miRNAs using patients' serum samples. The diagnostic sensitivity and specificity were calculated to be 93.2% and 88.19%, respectively (Fig. 3). Despite accumulating evidence of miRNAs circulating in the blood and body fluids, and the origin and functions of these circulating extracellular miRNAs remain poorly understood (45). Most circulating miRNAs are part of larger lipid or lipoprotein complexes (26, 27, 29, 45). Currently, little is known regarding the biological roles of these molecules at distant sites in the body (46). Extracellular miRNAs may be mediators of cell-cell communications (47, 48) or show hormone-like effects, leading to widespread responses (30, 49). The expression of circulating miRNAs is thought to reflect extrusion of miRNAs from relevant remote tissues or organs or disease processes (30). Indeed, the present study did not show that the miRNAs that are differentially expressed in the circulation are expressed in other tissues. Thus, the source of circulating miRNAs not yet understood, and future studies will be performed to clarify the origin and physiological roles of these circulating miRNAs.

However, attempts were made to identify the physiological role of these miRNAs in cell function using GO analysis. Gene ontology enrichment analysis revealed that miRNAs play important roles in disease signal transduction, signaling by interleukins, the stress-activated protein kinase signaling cascade, the MAPK signaling pathway, FoxO signaling pathways, and cellular response to TGF-β stimulus (Fig. 4A to C). These biological processes are in fact, interconnected (Fig. 4D to F). We propose that these miRNAs fine-tune the gene expression of key components in the pathway under the impact of LPS stimulation. However, this hypothesis does not rule out other possible functions for these miRNAs, since dozens of the targets, validated or predicted, will be still outside the LPS-TLR immune axis pathway. Therefore, in light of the increasing knowledge about the role of miRNAs in disease, the promise they hold for

therapeutics and diagnostics should not be underestimated (50). Our study provides evidence that miRNAs play a crucial role in the diagnosis of infectious diseases like leptospirosis that are generally misdiagnosed.

The major challenge of this study is that the miRNAs were tested for its diagnostic potential in a very small patient cohort and without a random sampling scheme. Also, we consider the analysis of miRNAs in the human clinical samples biased, as it is just a confirmation of the serum miRNAs by PCR. In order to use these identified miRNAs as the best prognostic markers, our plan is to enroll a large cohort of patients with laboratory-confirmed leptospirosis. Additionally, comparing the serum miRNA profiles of greater numbers of healthy individuals and patients with other confirmed febrile illnesses will validate the prognostic use of these identified miRNAs. This approach is expected to overcome the problem of definitive diagnosis of acute leptospirosis in the near future.

Conclusions. The current work established the relationship between miRNAs and innate immunity and established a detailed profile of circulating miRNAs in THP1 cells and mouse sera after exposure to leptospiral LPS. Further, these findings also established the role of the circulating miRNAs (miR-21-5p, miR-144-3p, and miR-let-7b-5p) as an early diagnostic marker for leptospirosis. These miRNAs will facilitate diagnosis of acute leptospirosis and differentiation of leptospiral infection from other bacterial and spirochetal infection.

MATERIALS AND METHODS

Leptospiral strains and media. The pathogenic leptospiral strains *L. interrogans* serovar Australis strain AHF651, *L. interrogans* serovar Autumnalis strain N2, *L. kirschneri* serovar Grippotyphosa strain D22, *L. interrogans* serovar Pomona strain H3, and *L. borgpetersenii* serovar Ballum strain BDU51, isolated from ailing human subjects, and nonpathogenic *L. biflexa* serovar Andamana strain CH11, isolated from water sources, were obtained from the WHO Reference Centre for Leptospirosis, Regional Medical Research Centre, ICMR, Port Blair, Andaman Islands (India). Strains were maintained by regular subculturing in Ellinghausen-McCullough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) in a shaking incubator (100 rpm) at 30°C. The pathogenicity of the strains was maintained by passaging the isolates in immunocompromised (cyclophosphamide-treated) BALB/c mice ~15 times (51). These pathogenic mouse-adapted challenge strains (MACS) were used for all the experiments.

Cells and cell culture. THP-1 cells obtained from NCCS, Pune, India, were cultured in RPMI medium containing 10% fetal bovine serum (FBS) and 2 mM glutamine (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Patients and case definition. In total, 46 serum samples collected during the early phase of illness (0 to 10 days after the onset of disease) were selected from a bank of samples from 376 laboratory-confirmed cases of leptospirosis (a positive IgM ELISA or MAT titer of $\geq$1:160 or isolation of leptospires from the blood). A total of 22 seronegative healthy controls matched with respect to age ($\pm$5 years) and sex and 66 patients who were hospitalized with a clinical suspicion of leptospirosis and subsequently diagnosed as having other illness, including dengue (22), malaria (22), and typhoid (22), based on laboratory evidence were also included. Informed written consent was obtained from both case patients and controls before sampling, and the study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (DM/2014/101/49) as well as receiving permission from the Directorate of Health Services (reference no. 5796/TV 1/07), Tamil Nadu, India.

Extraction of leptospiral LPS. Leptospires were collected from mid-log-phase cultures by centrifugation at 10,000 $\times$ *g* for 30 min and then washed three times with sterile 1$\times$ Dulbecco's phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA) before LPS extraction. Crude LPS was extracted following standard hot phenol-water extraction as described previously (4). The phenol phase that contained the LPS was dialyzed extensively against Milli-Q water (8 changes for 4 days) to remove phenol. Purified LPS was pelleted by ultracentrifugation (120,000 $\times$ *g* for 4 h at 4°C), dissolved in Milli-Q water, and lyophilized. Lyophilized crude material was resuspended in 10 ml of Milli-Q water and treated with DNase and RNase (Sigma-Aldrich, St. Louis, MO, USA), followed by proteinase K digestion (Sigma-Aldrich, St. Louis, MO, USA). LPS was quantified by the phenol-sulfuric acid method using sucrose as a standard (52). Protein was determined by bicinchoninic acid (BCA) kit (Sigma-Aldrich, St. Louis, MO, USA). Total and inorganic phosphorus were determined as described previously. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% polyacrylamide gel using a discontinuous buffer system. The extracted LPS was mixed with 2$\times$ SDS-PAGE sample loading buffer (Bio-Rad, Hercules, CA, USA) and boiled for 5 min before loading. Electrophoresis was carried out in a vertical electrophoretic minicell unit (Bio-Rad, Hercules, CA, USA) for 2 h at 100 V using the Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS [pH 8.3]) (53). A modified silver staining procedure was performed to detect the LPS bands on SDS-PAGE (4, 54) and documented using a gel documentation system (Bio-Rad, Hercules, CA, USA).

RNA interference and LPS stimulation. THP-1 cells (0.5×10^6/well) grown in six-well plates were transfected with pools of 4 distinct siRNAs against TLR2 (On-TargetPlus SmartPool; Dharmacon, USA) (50 nM) using the RNAiMAX transfection reagent (Thermo Fisher Scientific, USA). As a control, nontargeting (scrambled) siRNA (Scr siRNA) duplexes (Dharmacon, USA) were used. After 72 h posttransfection, the cells were exposed to 1 μg/ml of leptospiral LPS. Cells and extracellular media were harvested at different time intervals for further experiments.

Western blotting. Cell extracts were prepared from THP-1 cells transfected with Scr and TLR2 siRNA using radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.25% deoxycholic acid, 1 mM EDTA, 1% NP-40, protease and phosphatase inhibitor cocktail; Thermo Scientific). Protein concentrations were quantified using the Pierce 660nm protein assay reagent. Equal amounts of protein (25 μg/well) were separated on 4 to 12% bis-Tris polyacrylamide gels, transferred to a nitrocellulose membrane using a V20 semidry blotter (Scie-Plas, United Kingdom), and probed with antibodies specific for TLR2 (1:1,000; Sigma-Aldrich), TLR4 (1:1,000; Santa Cruz), and GAPDH (1:5,000; Santa Cruz).

Mice and LPS stimulation. Ten- to twelve-week-old BALB/c mice were injected intraperitoneally (i.p.) with either PBS or leptospiral LPS (10 μg/g body weight) reconstituted in PBS. After different intervals (0, 4, 7, 14, and 21 days) post-PBS-LPS injection, blood samples with or without heparin were collected by cardiac puncture. Blood samples collected with heparin were gently mixed with RNAlater and used for RNA extraction (cytokine profiling). In order to obtain serum, samples collected without heparin were left overnight at 4°C, and the serum was separated by centrifugation at 3,000 rpm for 5 min and used for total RNA extraction (miRNA profiling). A minimum of 6 mice/cohort were used in the present study. Animal experiments described in this study were carried out in strict accordance with the recommendations approved by the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), the Bharathidasan University Ethics Committee in Animal Experimentation (BDU/IAEC/2011/29), and the Bharathidasan University Institutional Biosafety Committee (BT/BS/17/29/2000 PID).

Cytokine and chemokine profiling. RNA was isolated from THP1 cells and mouse blood samples using a PureLink RNA minikit or RiboPure blood kit (Thermo Fisher Scientific, Wilmington, DE, USA), respectively. cDNA was synthesized using iScript reverse transcription supermix (Bio-Rad, Hercules, CA, USA). Quantitative reverse transcriptase PCR (qRT-PCR) was performed with a CFX96 Touch real-time PCR detection system (Bio-Rad, Hercules, CA, USA). Semiquantitative real-time PCR was performed using SYBR green master mix (Bio-Rad, Hercules, CA, USA) with a 25-μl reaction volume (50 ng cDNA, 12.5 μl master mix, 0.5 μM concentration of each primer). Primers used in the present study are listed in Table S3. The fold change of each target mRNA was normalized to β-actin and GAPDH.

Microarrays and miRNA profiling. Total RNA was isolated from extracellular media of control and TLR2 knockdown cells, THP1 cells, or mouse serum (Norgen Biotek Corp., Canada). Reverse transcription of RNA (500 ng) was performed using a miScript reverse transcription kit (Qiagen, Hilden, Germany). Briefly, with miScript HiSpec buffer, poly(A) tails were added to mature miRNAs and converted to cDNA by reverse transcriptase with oligo(dT) priming. The cDNAs were further used in miRNA profiling using the human and mouse whole miRNome miScript miRNA qPCR arrays (v.16 miRNAs; Qiagen) per the manufacturer's procedure. All experiments were performed in triplicate.

Biomarker signature. For the identification of signature miRNAs expressed during the acute phase of leptospiral infection, we systematically compared miRNA profiles at different days after LPS administration. Day 0, days 4 to 7, and days 14 to 21 were considered control, early, and convalescent phases of leptospiral infection, respectively. To identify miRNAs for early diagnosis of leptospirosis, we devised 5 comparison groups: (i) control versus day 4, (ii) control versus day 7, (iii) control versus day 14, (iv) control versus day 21, and (v) days 4 to 7 versus days 14 to 21.

Validation of differentially regulated miRNA in clinical cases. Based on the miRNome miRNA array, we identified three signature miRNAs (miR-21-5p, miR-144-3p, and miR-let-7b-5p) that could have potential for early diagnosis of leptospirosis. We validated the efficacy of these miRNAs using patients' serum samples. A serum-plasma RNA purification minikit (Norgen Biotek Corp., Canada) was used for the extraction of RNA. The RNA was reverse transcribed using a miScript II RT kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. cDNA obtained from the RT reaction was diluted 1:10 with RNase-free water, and real-time qPCR was performed using the miScript SYBR green PCR kit (Qiagen, Hilden, Germany) that contains the miScript universal primer (reverse primer). MystiCq microRNA qPCR assay primers specific for miR-21-5p, miR-144-3p, and miR-let-7b-5p (Sigma-Aldrich, St. Louis, MO, USA) were used as forward primers. miRNA expression was normalized to the internal control SNORD25 (Sigma-Aldrich, St. Louis, MO, USA). Real-time qPCRs were performed at 95°C for 15 min, followed by 40 cycles of 94°C for 15 s, 60°C for 30 s, and 70°C for 30 s. Each reaction was carried out in triplicate in CFX96 detection system (Bio-Rad, Hercules, CA, USA), and data normalization was performed using the $\Delta\Delta C_T$ method.

Prediction of target genes. For miRNAs miR-21-5p, miR-144-3p, and miR-let-7b-5p, we predicted the target genes using two different target prediction databases: TargetScan 7.1 (http://www.targetscan.org/vert_72/) and DIANA-microT-CDS (http://snf-515788.vm.okeanos.grnet.gr/). The threshold was set to 0.7, and we retained only the gene sets that were significant at a nominal *P* value threshold of 0.01.

Gene ontology enrichment analysis. Gene ontology (GO) was used for gene functional enrichment analysis using Metascape software (http://metascape.org). The resulting GO terms with *P* values of <0.05 were considered significantly enriched in the differentially expressed miRNAs.

PPI network. A PPI network was constructed using Cytoscape software (version 3.6.1) to investigate the role of these miRNAs in the pathogenesis of leptospirosis (55). An integrated score of >0.4 (the default threshold in the STRING database; http://www.string-db.org [56]) was defined to construct the PPI network.

Statistical analysis. All data were normally distributed and are presented as means and standard errors of the means (SEM). In the case of single-mean comparisons, data were analyzed by Student's *t* test or, when not normally distributed, a nonparametric Mann-Whitney U test. Differences in means among multiple data sets were analyzed using 1-way analysis of variance (ANOVA) with the Tukey test. *P* values less than 0.05 were considered significant. The diagnostic potential of the miRNAs was evaluated by calculating the area under the receiver operating characteristic (ROC) curve and AUC. For ROC curves, the cutoff value was defined to maximize the sum of sensitivity and specificity. The data were computed with SigmaPlot 11.0 software or GraphPad Prism 8.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, PDF file, 0.1 MB.

FIG S2, PDF file, 0.1 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, DOCX file, 0.02 MB.

TABLE S3, DOCX file, 0.02 MB.

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Biochemical analysis of leptospiral LPS explained the difference between pathogenic and non-pathogenic serogroups

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ABSTRACT

Lipopolysaccharide (LPS) is the major surface antigen of *Leptospira*. In this study, the genes involved in the LPS biosynthesis were analyzed and compared by bioinformatics tools. Also, the chemical composition analysis of leptospiral lipopolysaccharides (LPS) extracted from 5 pathogenic serovars like Autumnalis, Australis, Ballum, Grippotyphosa, Pomona, and the nonpathogenic serovar Andamana was performed. Methods used were Limulus amebocyte lysate assay (LAL), gas chromatography-mass spectrometry (GC-MS), fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance spectroscopy (NMR). LAL assay showed a significantly higher level of endotoxicity among pathogenic serovars (~0.490 EU/mL) than that of nonpathogenic Andamana (~0.102 EU/mL). FAMES analysis showed the presence of palmitic acid (C16:0), hydroxy lauric acid (3-OH-C12:0), and oleic acid (C18:0). Palmitoleic acid (C16: 1), and 3- hydroxy palmitate (3-OH-C16:0) was detected only in pathogenic serovars. In contrast myristoleic acid (C14:1) and stearic acid (C18:0) were present in Andamana. FTIR analysis revealed C–O–C stretch of esters, 3°ROH functional groups and carbohydrate vibration range were similar among pathogenic serovars. The NMR analysis reveals similarity for 6 deoxy sugars and methyl groups of Autumnalis, Australis, and Ballum. Further, the presence of palmitoleic acid and 3-hydroxy palmitate may be the significant pathogen-associated predisposing factor. This mediates high osmolarity glycerol (HOG) mediated stress response in leptospiral LPS mediated pathogenesis.

1. Introduction

Leptospirosis is a common, globally important neglected zoonotic infectious disease, caused by spirochetes of the genus *Leptospira*. Leptospirosis is a disease of public health importance in tropical and subtropical countries [1]. Conventionally the genus *Leptospira* was classified into 24 serogroups that have been further classified into ≥250 pathogenic serovars based on the immunological characterization of surface lipopolysaccharide (LPS) by microscopic agglutination test. Recently, phylogenetic analysis revealed that *Leptospira* can be divided into three lineages that correlate with the level of pathogenicity of the species: pathogenic (P1), intermediate (P2), and saprophytic (S1 and S2), [2]. The emerging serodiversity among leptospires relies on the changes in the genetic organization of the LPS biosynthesis genes [3]. LPS are complex, amphipathic biomolecules that offer biological protection to the pathogen from lysozymal degradation and anti-microbial effects of many peptide antibiotics. The acyl chains present in the LPS molecules are highly saturated thus serving as tight and an efficient barrier for most of the hydrophobic moieties [4]. LPS increases the negative charge of the cell membrane and maintains membrane

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integrity. Unlike all other human-infecting spirochetes, *Leptospira* is unique as it possesses LPS in the outer membrane. Leptospiral LPS plays a vital role in immunological and biological activities during the infection [5] and is considered to be the predominant leptospiral antigen.

Leptospiral LPS is composed of three major components: (i) the O antigen or somatic antigen which contains the polysaccharide. They are hydrophilic and contains longer carbohydrate chains than the core region and serve as antigenic determinants. Because O- antigen is exposed on the surface, it serves as a primary target during the host immune response. (ii) Lipid A, the membrane-anchored portion, is the hydrophobic endotoxic domain of the LPS. It consists of free fatty acids, esterified 3-hydroxy fatty acids and N-acetyl glucosamine (NAG) subunits. *Leptospira* can store and associate fatty acids (hydroxylauric, palmitic, and oleic acids) with their endotoxins [6–9] which is believed to be essential for the pathophysiological implications such as the development of osmolarity stress. Additionally, any unusual modifications that occur in the lipid A region lead to altered Toll-like receptor-mediated innate immune responses in the host system [10–13]. (iii) The core saccharide of LPS consists of 10–15 carbon length oligosaccharides and is further divided into two segments. The inner core oligosaccharides are covalently bound with the lipid A moiety and the outer core oligosaccharides are proximal to the O-antigen. The common sugars present in this region are heptose, galactose, glucose, and an eight-carbon signature molecule 3-deoxy-D-mannooctulosonic acid (KDO). Non-carbohydrate molecules like phosphate, amino acids, and ethanolamines are also present in the core region. Each serogroup has highly specific sugars in this domain.

Recently, we evaluated the serological specificity of the LPS by IgM-ELISA, dot-blot and ICG-LFA and developed a single LPS array for serogroup specific diagnosis [14]. Remarkably, in our study except for Ballum LPS, no other LPS showed cross-reactivity to heterologous sera confirming serogroup specificity. Since we observed cross-reactivity of only Ballum LPS, we hypothesized this cross-reactivity to be a result of the antigenic structural similarities of the Ballum LPS with Autumnalis/Australis. Therefore, the genes involved in the LPS biosynthesis of pathogenic and non-pathogenic serovars were analyzed and compared by bioinformatics tools. Also, an in-depth understanding of the biochemical composition of LPS will be informative to know about the pathogen-associated structural composition of leptospiral LPS. We performed a biochemical and structural assessment of both pathogenic and non-pathogenic LPS using FT-IR, GC-MS and NMR analysis.

Toll-like receptors (TLRs) are recognized as primary immune sensors for the pathogenic bacterial components and to stimulate an innate immune response to clear the microbial burden from the host. Previous studies have shown that both TLR2 and TLR4 are involved in the clearance of *Leptospira* [10]. However, it remains unclear how the TLRs are involved in the recognition of different species, strains, structurally and biochemically varied pathogenic, and non-pathogenic leptospiral LPS. Furthermore, the binding of LPS to TLR4 triggers the activation of the serine/threonine kinases in mammalian cells. Among them, MAP kinase family (p42/44 MAP kinase and p38) plays a major role in cellular activation and regulates the translation of cytokines, adhesion and migration molecules [15,16]. The yeast high osmolarity glycerol protein (Hog1) is both functionally and structurally similar to the mammalian p38. Also, the yeast Hog1 MAP kinase responds specifically to increased extracellular osmolarity caused by LPS that referred to as the bacterial endotoxin [17]. The Glycerol-3-phosphate dehydrogenase [NAD (+)] 1 (GPD1) catalyzes the production and accumulation of glycerol during hyperosmotic stress conditions to prevent turgor of the cells. The expression of GPD1 is regulated by HOG pathway [18]. Since p38 mediates LPS effects in mammalian cells, we also tested the effect of leptospiral LPS in *Saccharomyces cerevisiae* wild and mutant (Hog1Δ) model system.

2. Materials and methods

2.1. Strains, media, and ethics

In this study, totally six strains from four *Leptospira* specieswere used. Pathogenic leptospiral strains (*Leptospira interrogans* serovar Australis strain AHF651, *Leptospira interrogans* serovar Autumnalis strain N2, *Leptospira borgpetersenii* serovar Ballum strain BDU51, *Leptospira kirschneri* serovar Grippotyphosa strain D22, and *Leptospira interrogans* serovar Pomona strain H3 isolated from ailing human subjects and non-pathogenic *Leptospira biflexa* serovar Andamana CH11 isolated from water source were used for this study [19]. The pathogenicity of the strains was maintained by passaging the isolates in immunocompromised (cyclophosphamide treated) BALB/c mice ~15 times [20]. The MACS (mouse-adapted challenge strains) were used for all the experiments. The strains were obtained from the WHO Reference Centre for Leptospirosis, Regional Medical Research Centre, ICMR, Port Blair and maintained by regular sub-culturing in Ellinghausen-McCullough-Johnson-Harris (EMJH) bovine serum albumin-Tween 80 medium (Difco Laboratories, USA) in a shaking incubator (100 rpm) at 30 °C).

Saccharomyces cerevisiae strains used in this study were wild-type W303-1A (Mata *ade 2-1 can 1–100 his 2-11,15 leu 2–3,112 trp1-1 ura3-1*) and its derivative YSH 444 (*hog1*Δ: TRP1) mutant. These strains were obtained from Dr. Stefan Hohmann, Gothenberg University, Sweden as a kind gift. Yeast cells were grown at 30 °C in yeast nitrogen base (YNB) medium supplemented with the appropriate selective amino acids and bases and 2% glucose. Growth was assessed by measuring the optical density at a wavelength of 640 nm (*A*640 nm). THP-1 cells obtained from NCCS, Pune, India were cultured in RPMI media containing 10% FBS, 2 mM glutamine (Sigma Aldrich, USA), and supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified 5% CO_2 atmosphere. These strains/cells were maintained at the Medical Microbiology Laboratory, Bharathidasan University, Tiruchirappalli, Tamilnadu.

The studies involving human participants were reviewed and approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (DM/2010/101/14). The patients/participants [legal guardian/next of kin] provided written informed consent to participate in this study. The Institutional Animal Ethics Committee (IAEC) of Bharathidasan University (BDU/IAEC/25/2013) approved the study protocols for animal experiments.

2.2. Profiling of genes involved in the LPS biosynthesis

Identification and characterization of LPS on genes level will give a great insight into their structural characterization and confirmation. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/pathway.html) was used to identify the leptospiral genes involving in lipopolysaccharide biosynthesis pathway. The resulted genes from pathogenic *L. interrogans* and non- pathogenic *L. biflexa* were further used to identify the similar proteins from other leptospiral species such as pathogenic *(L. borgpetersenii, L. kirschneri, L. noguchii, L. santarosai, L.weilii, L.alexanderi, L.alstonii, L.kmetyi),* intermediate (*L. broomii, L. fainei, L. inadai, L. licerasiae, L. wolffii)* and nonpathogenic (*L. meyeri, L. terpstrae, L.wolbachii, L. yanagawae, L. idonii)* using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).

2.3. Extraction of leptospiral LPS

Leptospires were collected from 25 mL of mid-log cultures (~2x10^8 cells/mL) by centrifugation at 14,000 rpm for 30 min and then washed three times with sterile 1X Dulbecco's phosphate buffered saline (PBS) (Corning, Manassas, VA, USA) before LPS extraction. Crude LPS was extracted following the standard hot phenol-water extraction as described previously [14,21]. The phenol phase that contained the LPS

was dialyzed extensively against Milli-Q water (8 changes for 4 days) to remove phenol and then lyophilized. Purified LPS was pelleted by ultracentrifugation (120,000g for 4 h at 4 °C), dissolved in Milli-Q water and lyophilized. The lyophilized crude material was re-suspended in 10 ml of Milli-Q water, treated with DNase and RNase (Sigma-Aldrich, St. Louis, MO, USA) followed by proteinase-K digestion (Sigma-Aldrich, St. Louis, MO, USA). LPS was quantified by the phenol/sulfuric acid method using sucrose as a standard [22] and *E. coli* O111:B4 LPS (Sigma-Aldrich, St. Louis, MO, USA) was used as control. Protein was determined by bicinchoninic acid (BCA) kit (Sigma-Aldrich, St. Louis, MO, USA). Total and inorganic phosphorus were determined as described previously [23,24]. SDS-PAGE was performed on a 12% polyacrylamide gel using a discontinuous buffer system. The extracted LPS were mixed with 2X SDS-PAGE sample loading buffer (BioRad, Hercules, CA, USA) and boiled for 5 min before loading. Electrophoresis was carried out in a vertical electrophoretic mini-cell unit (Bio-Rad, Hercules, CA, USA) for 2 h at 100 V using the Tris-glycine running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3) [25]. Modified silver staining was performed to detect the LPS bands on SDS-PAGE [14,26] and documented in a gel documentation system (Bio-Rad, Hercules, CA, USA).

2.4. Determination of endotoxicity by limulus amebocyte lysate assay (LAL)

The level of endotoxin present in the LPS samples was determined by LAL assay using E-Toxate kit (Sigma-Aldrich, St. Louis, MO, USA). To compare the leptospiral and *E. coli* LPS, we performed a dose-response curve. Varying concentration of LPS (1, 10, 100, and 1000 ng/ml) in 100 µl Milli-Q was mixed with 100 µl of E-TOXATE working solution and incubated for 1 h at 37 °C. Endotoxin standard solutions were prepared by diluting the stock standard (4000 EU/ml) and endotoxin-free water (Sigma-Aldrich, St. Louis, MO, USA) served as a negative control. After 1 h incubation, the tubes were inverted, and the gelation was observed.

2.5. KDO (2-keto-3-deoxyoctonate) assay

The thiobarbituric acid reaction was used to determine the KDO present in the LPS extract. KDO (1 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) was used as standard. 10 µg/ml of leptospiral LPS was boiled with 0.5 N H_2SO_4 for 20 min, then 50 µl of 0.123 M periodate reagent was added and allowed to stand for 10 min at room temperature. 200 µl of 0.308 M sodium arsenite reagent and 800 µl of 0.0416 M thiobarbituric acid reagent was added and boiled at 100 °C for 10 min. 1.5 ml of 95% butanol reagent was added and centrifuged at 2000 rpm for 5 min. The optical density of the upper butanol layer was measured at 552 nm and 509 nm using ELISA reader (Biorad, Hercules, CA, USA).

2.6. GC-MS analysis of fatty acid methyl ester (FAME)

Lyophilized LPS samples (50 µg) were subjected to methanolysis with 1 M BF3 methanol (Sigma-Aldrich, St. Louis, MO, USA) for 16 h at 80 °C. The samples were placed on ice and slowly evaporated under a dry nitrogen stream until almost half of the sample volume had evaporated. To this reaction mixture, cold half saturated NaCl was added followed by the addition of 1 ml chloroform. The samples were vortexed at high speed for 1 min with a 20-sec pulse, and then allowed to settle for 1 min, and then centrifuged at 2000 rpm for 2 min (Eppendorf, Germany) to separate organic and aqueous layers. The lower organic phase was carefully removed to a glass hydrolyzing tube, and the aqueous phase was extracted twice with 0.5 ml of chloroform. Chloroform fractions were pooled then washed with 2.0 ml of cold Milli-Q water twice and the organic layer was dried down. FAME derivatized samples were analyzed by Shimadzu QP-2010 plus GC-MS (Shimadzu, Chiyoda-ku, Tokyo, Japan) using a Restek-5 ms capillary column. Ultrapure Helium was used as a carrier gas with a flow rate of 1.21 ml/min and the following temperature gradient (100 °C for 3 min, Ion source temperature 230 °C, interface temperature 260 °C, solvent cut time 4.50 min). The injector and transfer line temperatures were maintained at 250 °C and 280 °C respectively. Fatty acids were identified by their characteristic EI fragmentation pattern and corresponding retention times and the percentage composition calculated from the area under the respective peaks.

2.7. Fourier transform infrared (FT-IR) analysis of functional group

The intergroup characterization of the leptospiral LPS was performed using FTIR analysis [27]. Lyophilized leptospiral LPS 10 µg/ml were analyzed by FTIR spectrometer equipped with a mercury, cadmium telluride A detector and KBr optics (Varian, Palo Alto, CA, USA). Spectra were recorded using 8 scans at 4 cm^{-1} resolution and units of log(1/R) over the entire mid-IR wavenumber region of 4,000-650 cm^{-1}. Datapoint resolution was approximately 1 point per wavenumber. A background spectrum was recorded before every sample spectrum and subtracted with the sample spectra.

2.8. Nuclear magnetic resonance (NMR) spectroscopy

One dimensional ^{1}H and ^{13}C NMR experiments were performed using a Bruker Avance III 400 MHz spectrometer, equipped with 5 mm smart probe. LPS were prepared by dissolving 10 µg in 0.5 mL of sterile Milli-Q water containing 5% D_2O. Protons of hydroxyl, carboxyl, amine, and amide groups were exchanged with deuterium and simple spectra were obtained. The spectra were acquired in D_2O at 25 °C, except for ^{13}C, which was acquired at 35 °C. The chemical shifts are reported in ppm. The resonances were measured relative to internal acetone [$(CH3)_2CO$ δ^{1}_{H} 2.225; δ^{13}_{C} 30.89]. The data were acquired and processed with standard Bruker software (Mnova v7.1).

2.9. Cell viability and plasma membrane permeability assay

The cell viability assay in *S. cerevisiae* (WT and Hog1Δ) and THP1 cells were performed with 1 µg/ml of leptospiral LPS [28]. The *S. cerevisiae* was grown in YNB for overnight at 30 °C. The cells were then divided and incubated with leptospiral serovars LPS and 0.8 M NaCl was used as a positive control. To quantify the number of viable cells 100 µL aliquotes were taken and plated on the YNB agar plate. The number of colonies was counted after 3 days of incubation at 30 °C. Plasma membrane permeability was measured by acridine orange/ethidium bromide (AO/EB) double staining [29]. The THP1 cells were grown in RPMI medium for overnight at 30 °C with 5% CO_2. The cells were incubated with LPS for 12 h. Then the cells were harvested by trypsin digestion and resuspended in 0.1 mL of serum-free RPMI medium containing 4 µL of AO/EB staining solution (Sigma-Aldrich, USA). After 1 min at 25 °C, AO/EB treated cell suspension was observed under inverted fluorescent microscope (Nikon, Japan). Apoptotic cells were distinguished from non-apoptotic cells based on nuclear condensation and fragmentation.

2.10. qRT-PCR analysis

Total mRNA was extracted from LPS treated and untreated yeast ($5X10^7$ cells/mL) and THP1 ($2x10^7$ cells/mL) cells using RNeasy mini kit as per manufacturer instructions (Qiagen, USA). The mRNA from each condition were reverse transcribed using Transcriptor first-strand cDNA synthesis kit as per manufacturer instructions (Roche Diagnostics, Lisbon, Portugal). The changes in the expression levels of *hog1, gpd1, p38, tlr2, tlr4* genes were assessed by qPCR as described previously [30]. The GAPDH primers were used as an internal control. The list of gene-specific primers used in this study was provided in Supplementary Table 1. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression.

2.11. Enzyme-linked immunosorbent assay (ELISA)

THP1 cells were grown in RPMI medium. The grown cells were incubated with 1 μg/mL of LPS at 37 °C with 5% CO_2 for 24 h. The cell culture supernatants from leptospiral LPS treated THP1 cells were analyzed by ELISA for levels of secreted TNF-α. Any non-adherent cells were removed by centrifugation and the remaining supernatants were analyzed using a human TNF-α ELISA kit (Invitrogen) according to manufacturers' instructions.

2.12. Statistical analyses

Data from multiple experiments (≥3) were quantified and expressed as Mean ± SE, and differences between groups were analyzed by using a two-tailed paired Student's T-test or, when not normally distributed, a nonparametric Mann-Whitney *U* test was used. Differences in means among multiple datasets were analyzed using one-way ANOVA with the Kruskal-Wallis test, followed by pairwise comparison using the Dunn test. A $P \leq 0.05$ was considered significant in all analyses. The data were computed with GraphPad Prism v8.0 and SigmaPlot v11.0 software.

3. Results

3.1. Identification and comparison of genes involved in LPS biosynthesis

KEGG pathway database showed 17 genes from *L. interrogans* and 19 genes from *L. biflexa* responsible for lipid A of LPS biosynthesis pathway. Some genes are common among both the species such as lpxA, lpxC, lpxD, kdsA, and gmhA. The number of genes responsible for core and O-antigen was varied based on the serovars. Also, no data are available to list all the genes and describe their functions in the LPS biosynthesis of *Leptospira* species. Based on the previous reports, the genes responsible for core and O-antigen regions were analyzed and identified. Further, the resulted genes were used for the BLAST search to identify and compare the protein similarity of pathogenic *(L. interrogans, L. borgpetersenii, L. kirschneri, L. noguchii, L. santarosai, L.weilii, L.alexanderi, L.alstonii, L.kmetyi)*, intermediate (*L. broomii, L. fainei, L. inadai, L. licerasiae, L. wolffii)* and nonpathogenic (*L. biflexa, L. meyeri, L. terpstrae, L.wolbachii, L. yanagawae, L. idonii)* strains and the proteins Gmtase, kdtA, OrfE, lpxK, lpxB, RfbF, rfaC2, Wzx, RmlB, 1, 3-glucosyltransferase, Heptosyltransferase IV present only in pathogenic species.The high similarity genes and their putative function were listed and compared along with the ID of the proteins (Supplementary Table 2). The supplementary fig. 2 represent the shared and distinct proteins involving in Leptospiral LPS biosynthesis classified based on pathogenic, intermediate and non-pathogenic species.

3.2. LPS profile, KDO and endotoxicity

We were able to successfully extract leptospiral LPS with a concentration in the range of ~575–625 μg/ml. The purified LPS was free of protein contaminations. The electrophoretic mobility patterns of extracted LPS revealed apparent molecular weights between 14 and 34 kDa (Fig. 1a). The total/inorganic phosphorous content of the pathogenic serovars was observed to be greater than that of the non-pathogenic serovars (Supplementary Table 3). The amount of KDO present in the leptospiral serovars was measured to be in the range of 2.13 pM–4.12 pM with significantly higher levels of KDO in pathogenic serovars than that of the nonpathogenic serovar (Fig. 1b). Because we observed increased phosphorous and KDO content in pathogenic serovars, we performed LAL assay to measure the endotoxicity of the extracted leptospiral LPS. LPS obtained from *E. coli* served as a positive control. The EU/ml for leptospiral LPS from pathogenic serovars was less than *E. coli* LPS (Fig. 1c and d), but the endotoxicity of non-pathogenic LPS was ~5 fold less than that of pathogenic serovars (Fig. 1c and d).

3.3. Fatty acid methyl ester analysis of leptospiral LPS

Next, we assessed the fatty acid (FA) composition of both the pathogenic and non-pathogenic LPS by GC-MS analysis (Fig. 2a- 2b). Palmitic acid (C16:0), Hydroxy lauric acid (3-OH C12:0), Oleic acid (C18:0), 3-hydroxy palmitate (3-OH C16:0) constituted the major FA of leptospiral LPS. The other FAs including Stearic acid (C18:0), Palmitoleic acid (C16:1), Margaric acid (C17:0), Myristic acid (C14:0), Myristoleic acid (C14:1) and Lauric acid (C12:0) constituted a minor proportion of the leptospiral LPS (Supplementary Table 4). Although many fatty acids were found in both pathogenic and non-pathogenic serovars, the significant difference was observed in their quantity. Pathogenic serovars had significant levels of Hydroxy lauric acid (3-OH

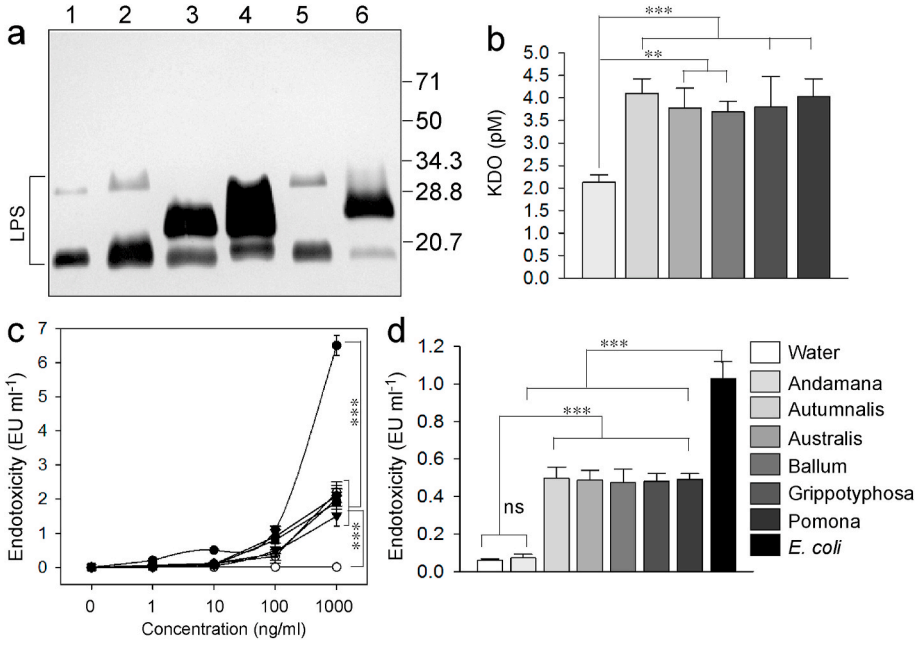

Fig. 1. Comparative LPS profile, KDO and endotoxicity of *Leptospira* serovars. (**a**) LPS profile. Lane 1- *L. interrogans* serovar Autumnalis (N2), Lane 2- *L. interrogans* serovar Australis (AHF651), Lane 3- *L. borgpetersenii* serovar Ballum (BDU51), Lane 4- *L. krischneri* serovar Grippotyposha (D22), Lane 5- *L. interrogans* serovar Pomona (H3), Lane 6- *L. biflexa* serovar Andamana (CH11). (**b**) Determination of KDO by Thiobarbituric acid assay. Y axis-KDO (pM); X axis – LPS of different serovars. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns: not significant. (**c and d**) Endotoxicity determination by Limulus amebocyte lysate (LAL) assay: (**c**) A dose reponse endotoxicity curve for the LPS from *E. coli* (●), *L. interrogans* serovar Autumnalis (▼), *L. interrogans* serovar Australis (■), *L. borgpetersenii* serovar Ballum (◆), *L. kirschneri* serovar Grippotyphosa (◊), *L. interrogans* serovar Pomona (□) and nonpathogenic *L. biflexa* serovar Andamana (○). Y axis-Endotoxicity ($EUml^{-1}$); X axis – concentration of LPS (ng/ml). (**d**) Comparison of endotoxicity of different leptospiral LPS at a concentration of 100 ng/ml. Endotoxin-free water served as control. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$, ns: not significant. n = 3–6 independent experiments.

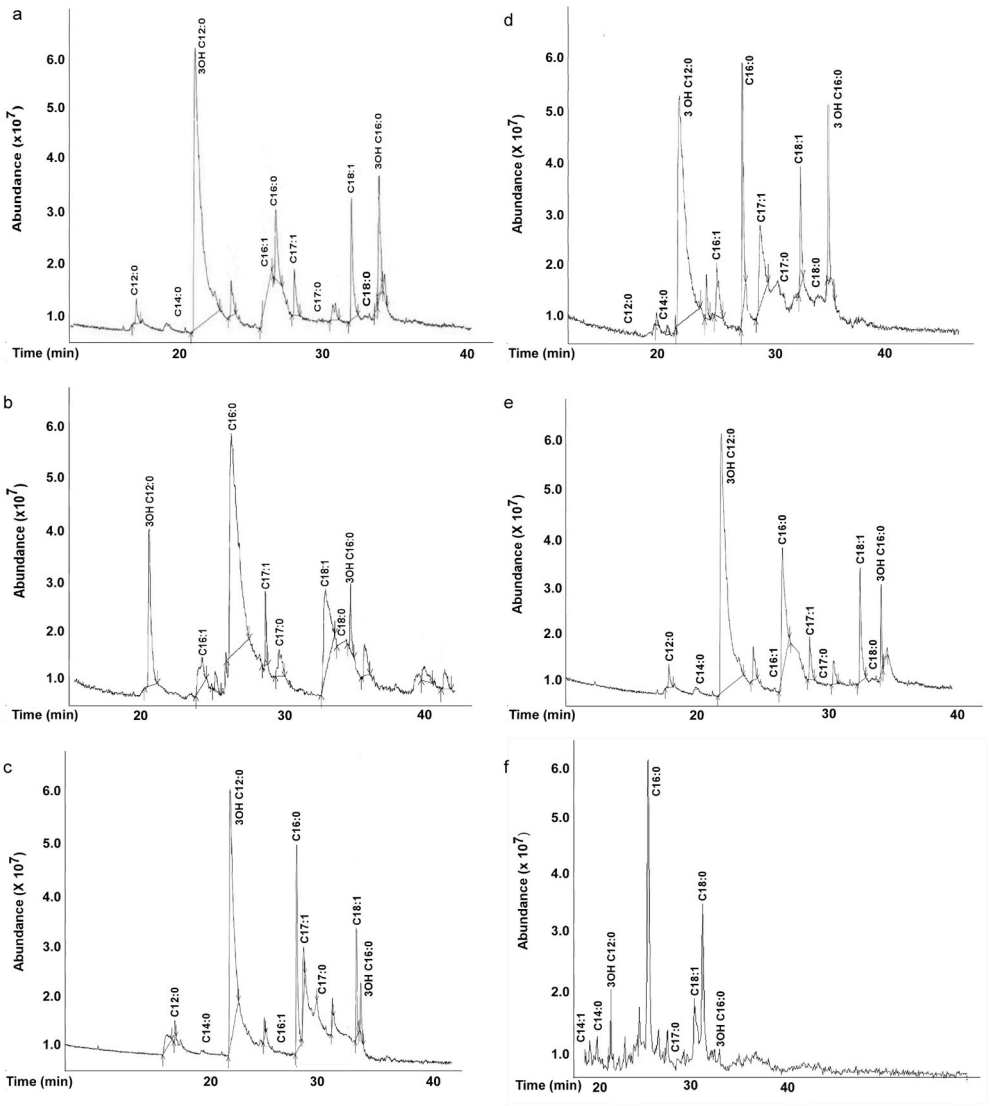

Fig. 2. Fatty acid methyl ester analysis of pathogenic and nonpathogenic leptospiral serovars LPS. (**a**) *L. interrogans* serovar Autumnalis, (**b**) *L. interrogans* serovar Australis, (**c**) *L. borgpetersenii* serovar Ballum, (**d**) *L. kirschneri* serovar Grippotyphosa, (**e**) *L. interrogans* serovar Pomona and nonpathogenic (**f**) *L. biflexa* serovar Andamana FAMES were subjected for GC-MS analysis and the fatty compound detected in the spectrum were shown in the figures. C12:0- Lauric acid, C14:0- Myristic acid, 3-OH-C12:0- Hydroxylauric acid, 3-OH-C14:0- 3-Hydroxy myristate, C16:0- Palmitic acid, C16:1- Palmitoleic acid, C17:1- Heptadecanoic acid, C17:0- Margaric acid, C18:1- Oleic acid, C18:0- Stearic acid, 3-OH-C16:0- 3-Hydroxy palmitate.

C12:0) and 3-hydroxy palmitate (3-OH C16:0) compared to the non-pathogenic serovar Andamana. Conversely, palmitic acid (C16:0) was significantly higher in Andamana LPS. 3-hydroxy myristate (3-OH-C14:0) was found only in the *L. krischneri* serovar Grippotyphosa and nonpathogenic *L. biflexa* serovar Andamana at a lower percentage. The major fatty acids constituents of leptospiral LPS were C16, C12, C18 and C16 but it also contains C14, C16:1, C17 and C18 in a minor proportion. A trace quantity of unidentified fatty acid signals was observed (data not shown). The uncharacterized fatty acids were not listed.

3.4. Functional group analysis of leptospiral LPS

FTIR spectral regions were assigned as described previously [31]. FT-IR spectra show absorbance corresponding to vibrational modes of 5 distinct LPS moieties (Fig. 3a–f). The lipid A is represented by stretching vibrations of C–H (2820-2940 cm^{-1}; 1460-1470 cm^{-1}) and phosphate (1200-1265 cm^{-1}, 1106 cm^{-1}, and 960-983 cm^{-1}). The C–O–C stretch of esters was observed around 1295 cm^{-1} (Supplementary Table 5). The distinct sharp peak at ~1035 cm^{-1} indicates a greater relative contribution of polysaccharides (O-antigen) in pathogenic leptospires compared to non-pathogenic serovar. The small peaks observed at ~1035 cm^{-1} evidence relatively diminished contribution of O-antigen in non-pathogenic LPS. Conversely, non-pathogenic serovar shows a relatively increased phosphate (PO_2^-) absorbance compared to pathogenic serovar. Asymmetric, symmetric, and bending CH_2 vibrations were observed in all LPS fractions except that Ballum and Pomona do not display symmetric stretch.

3.5. NMR analysis of leptospiral LPS

The anomeric configuration, the number of carbon and hydrogen, type of methyl groups and the purity of the carbohydrate content of the LPS were determined by single dimension ^1H NMR (Supplementary Fig. 2a–2f) and ^{13}C NMR (Supplementary Fig. 3a–3f). The signals from the methyl groups were detected between δ_H 1.2–2.3 ppm, which indicates the occurrence of 6-deoxy sugars, O and N acetyl groups and substituents such as 1- carboxyethyl esters. The anomeric region for ^1H NMR of all the serovars showed near δ_H 4.7 ppm. Oxygen or nitrogen bearing saccharide ring regions occurred between δ_C 50 and 85 ppm and protons occurred between δ_H 3.2 and 4.5 ppm. Signals from nitrogen-bearing carbons occurred between δ_C 50 and 60 ppm in all the serovars. The free ring carbons have the chemical shifts between δ_C 65 and 75 ppm. *O*-Acetylation in the ring region occurred between δ_H 0.6 and 1.2 ppm. The carbon signals raised from methyl groups were found in the region between δ_C 15 and 30 for LPS fractions of Autumnalis, Ballum, Grippotyphosa and Andamana. Australis and Pomona showed mild signals from the methyl group. There was no signal detection in the N-acetyl region (δ_H 1.97 and 2.08 ppm) for all the serovars. Methyl protons

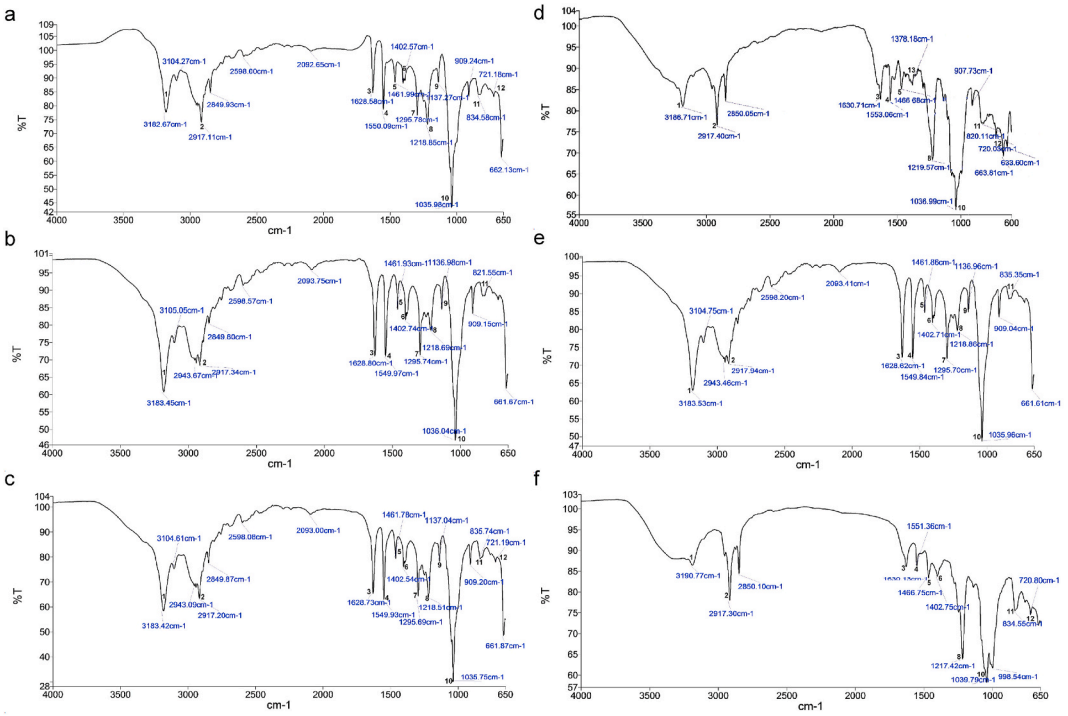

Fig. 3. Functional group analysis of pathogenic and nonpathogenic leptospiral serovars LPS. (**a**) *L. interrogans* serovar Autumnalis, (**b**) *L. interrogans* serovar Australis, (**c**) *L. borgpetersenii* serovar Ballum, (**d**) *L. kirschneri* serovar Grippotyphosa, (**e**) *L. interrogans* serovar Pomona and nonpathogenic (**f**) *L. biflexa* serovar Andamana LPS were subjected for FT-IR analysis and the functional groups detected in the spectrum were shown in Fig. 1) O–H stretch 2) C–H stretch 3) Amide I 4) Amide II, N–H bending, C–N stretch 5) C–H bend 6) C═O stretch 7) C–O–C stretch of esters 8) P═O stretch 9) 3°ROH 10) 1°ROH 11) Anomeric region 12) C–H rocking 13) COO^- symmetric stretch. n = 3–4 independent experiments.

of 6-deoxy sugars occurred near 1.2 ppm of ^{1}H NMR. The coupling constant ranged about 4Hz, which implies an α- anomeric configuration. Acetamido group present in the LPS gave rise to the signals from amino sugar residues near the 22 ppm range. The unsubstituted hydroxymethylene (C-6) occurred in samples with signals near the 60 ppm region. Detectable signals from Autumnalis, Ballum, Grippotyphosa and Andamana were observed near 70 ppm which indicates the presence of glycosylated C-6 resonates. All the serovars showed unsubstituted hydroxymethylene (C-6) signal between 57 and 62 ppm and glycosylated C-6 resonates near 70 ppm region, respectively. Signals from D-glucose was observed between δ_H 3.46–3.75 ppm in all the serovars. Signals from D- galactose were observed between δ_H 3.83–4.26 ppm for LPS fractions of Autumnalis, Ballum, Grippotyphosa, and Pomona. The ^{1}H NMR spectra of LPS fractions of leptospiral strains contained signals with different integral intensities that indicated nonstoichiometric acetylation, and the polysaccharide portion was in *O*-deacetylated form. The monosaccharide present at the reducing end occurred in different forms with the chemical shifts occurring differently.

3.6. Effect leptospiral LPS on Saccharomyces cerevisiae

To evaluate the involvement of Hog1 in LPS-mediated endotoxicity, we performed serial dilutions of mid-exponential-phase cultures of wild-type *S. cerevisiae* and *hog1*Δ mutant and spotted onto YNB agar plates containing 1 μg/ml of pathogenic and nonpathogenic leptospiral LPS. Growth of wild-type *S. cerevisiae* was not affected either in the presence of leptospiral pathogenic LPS or 0.8 M NaCl. Because Hog1 play a key role in osmoadaptation, we confirmed the growth of *hog1*Δ mutant in the presence of 0.8 M NaCl incorporated YNB agar plates. The growth of *hog1*Δ mutant was inhibited both in the presence of leptospiral LPS and NaCl, confirming Hog1's role in osmoadaptation and endotoxicity. We also performed a viability assay, pre-grown yeast cells, wild-type and *hog1*Δ mutant were incubated in YNB medium with or without leptospiral LPS. Although the viability of the wild-type strain was not affected during the 12 h incubation period, the *hog1*Δ mutant growth was inhibited compared to the WT strains (Fig. 4a and b). Because we observed the involvement of Hog1 in leptospiral LPS induced endotoxicity in yeast, we then asked whether the expression of GPD1, an enzyme involved in glycerol production is altered. Measurement of *hog1* and *gpd1* mRNA levels showed a significantly increased expression of both *hog1* and *gpd1* upon LPS exposure. After 90 min of exposure to 1 μg/ml pathogenic LPS we observed a 9-fold and 6-fold increase in *hog1* and *gpd1* mRNA respectively in WT strain. Conversely, both genes were down-regulated in *hog1*Δ mutant (Fig. 4c and d). Interestingly, LPS from nonpathogenic leptospires did not show any significant changes in the expression levels of *hog1* and *gpd1* mRNA.

3.7. Effect of leptospiral LPS on THP1 cells in comparison with E. Coli LPS

To evaluate the response of THP-1 cells to leptospiral LPS, we measured the viability and plasma membrane permeability in THP-1 cells after LPS exposure (Fig. 5a and b). *E. coli* LPS and leptospiral LPS decreased cell viability and increased membrane permeability with 1 μg of pathogenic Autumnalis LPS (Fig. 5f). On the other hand, nonpathogenic LPS did not have any effect on either the THP-1 cell viability or membrane permeability.

To further validate our results, *tlr2, tlr4* and *p38* expression levels were measured in THP-1 cells after exposure to pathogenic and nonpathogenic LPS. The mRNA levels of *tlr2, tlr4* and *p38* were significantly increased in THP-1 cells treated with pathogenic LPS, with ~1.2, 7.8 and 2.3-fold increase respectively compared to nonpathogenic LPS treated cells (Fig. 5b–e). This result implies that leptospiral LPS is recognized by the human TLR4 receptor, which in turn initiates the cell-mediated immune response through LPS-TLR4 complex immune signaling pathways. Because we anticipated a cell-mediated immune

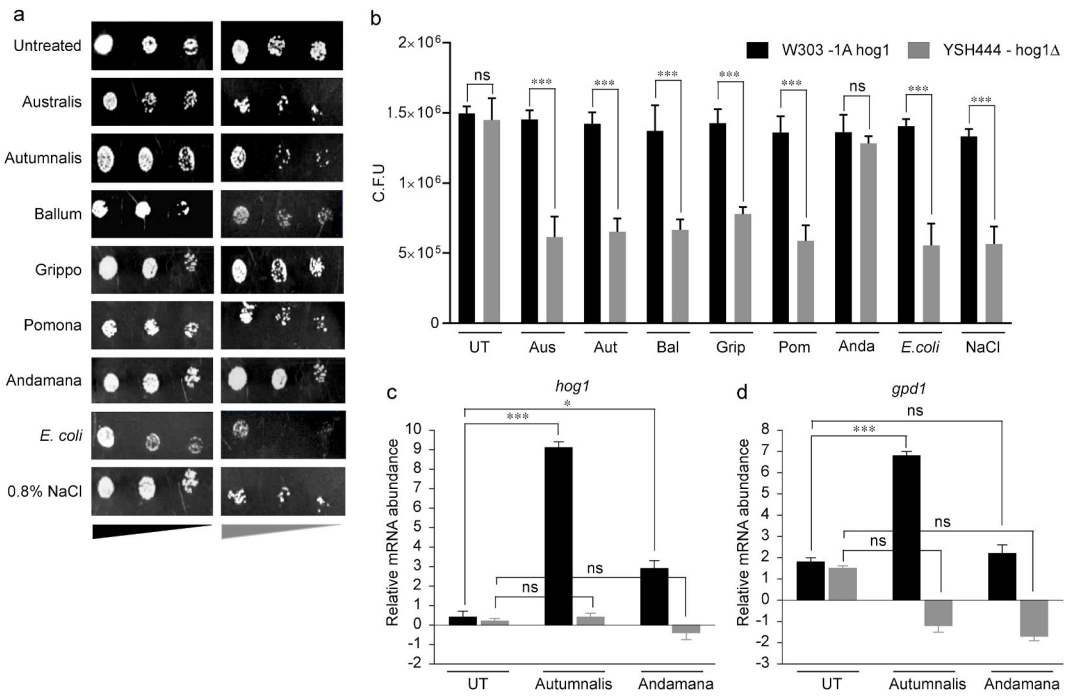

Fig. 4. Effect of pathogenic and nonpathogenic leptospiral LPS on *Saccharomyces cerevisiae* strains. (**a**) wild-type W303-1A (Mata *ade 2-1 can 1–100 his 2-11,15 leu 2–3,112 trp1-1 ura3-1*) and its derivative YSH 444 (*hog1*Δ: TRP1) mutant). Equal OD of cells was serially diluted (3 fold) and spotted on YPD agar plates with LPS. (**b**) The colony forming units measured. Y-axis C.F.U and X-axis different treatment conditions. Cells treated with 1 μg of *L. interrogans* serovar Australis LPS, *L. interrogans* serovar Autumnalis LPS, *L. borgpetersenii* serovar Ballum LPS, *L. kirschneri* serovar Grippotyphosa LPS, *L. interrogans* serovar Pomona LPS, *L. biflexa* serovar Andamana LPS, *E. coli*-LPS, and 0.8% NaCl. Untreated cells served as control. (**c and d**) Relative mRNA abundance of A) *hog1* and *gpd1* of cells treated with 1 μg of Leptospiral serovars *L.interrogans* serovar Autumnalis LPS and *L.biflexa* serovar Andamana LPS for 12 h ***p < 0.001, **p < 0.01, *p < 0.05, ns: not significant. Untreated cells served as control. n = 3–6 independent experiments.

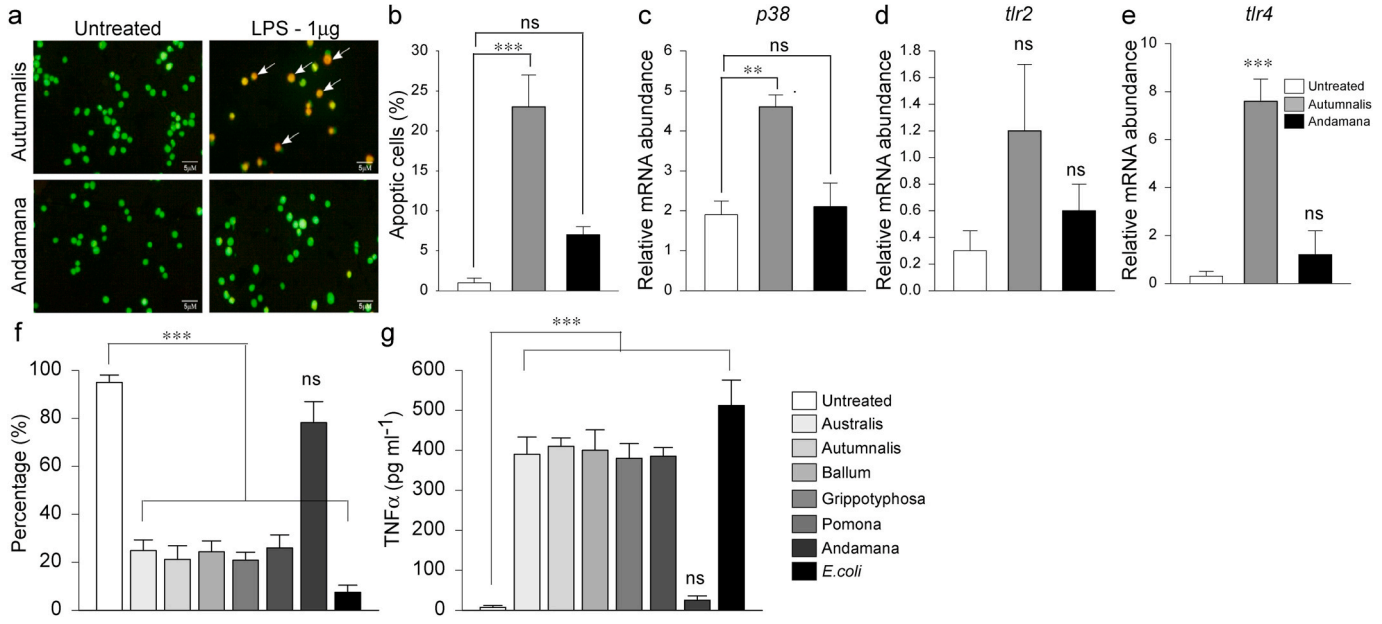

Fig. 5. Effect of pathogenic and nonpathogenic leptospiral LPS on THP1 cells in comparison with *E. coli* LPS. Cells treated with 1 μg of (**a**) *L.interrogans* serovar Autumnalis LPS and *L.biflexa* serovar Andamana LPS and assessed for plasma membrane permeability by AO/EO staining, (**b**) Percentage of apoptotic cells. Y-axis % of cells and x-axis LPS concentration (μg ml^{-1}). Relative mRNA abundance of (**c**) *p38*, (**d**) *tlr2*, and (**e**) *tlr4*. (**f**) Cell viability assay for pathogenic and non-pathogenic leptospiral LPS. Untreated cells served as controls. (**g**) TNFα (pg ml^{-1}) against leptospiral LPS. ***p < 0.001, **p < 0.01, *p < 0.05, ns: not significant. n = 3–6 independent experiments.

response to be initiated during leptospiral LPS treatment, we measure TNFα, a cytokine widely studied in immune-mediated diseases. TNFα levels were measured in cell supernatant and significantly increased during pathogenic LPS treatment compared to non-pathogenic LPS. (Fig. 5g).

4. Discussion

Leptospirosis is a zoonotic disease caused by the spirochete *Leptospira*. Humans become infected through contact with contaminated water or soil. The risk of infection depends on the ability of bacteria to survive, persist, and infect a new host. Though the factors that favor cycling and transmission of *Leptospira* are not well understood, it is believed that pathogenic *Leptospira* forms biofilms that would maintain bacteria at a sufficient concentration in large bodies to achieved infection.

LPS present in the outer membrane of Leptospira is one of the major essential immunodominant antigens. It has long been known that leptospiral LPS defines serovar and serogroups, and anti-leptospiral LPS antibodies are responsible for serovar specific immunity [32–34]. It is a known fact that *Leptospira* LPS plays a major role in immunity and may be potential candidates for vaccine formulations and used as a potential antigen in a variety of diagnostic formats [35–38]. In contrast, the other human spirochetes like *Borrelia* spp. and *Treponema* spp. do not have LPS or an outer membrane [39–41]. These LPS based biochemical components and structures do not have a role in the biology of these pathogens, and therefore they are distinguished from *Leptospira*. On contrary to spirochetes, LPS is a critical virulence determinant for many other pathogenic bacteria and plays a key role in the colonization of the vertebrate host [42].

Even though leptospiral LPS were reported as the first antigen to be recognized by the host immune cells [11], they are not a potent endotoxin [32]. In support of various studies, our present investigation also confirmed that though leptospiral LPS displayed endotoxicity, their endotoxic activity is significantly less when compared to *E. coli* LPS. Considering the use of LPS in serogroup specific diagnosis, we recently devised a serogroup-specific diagnostic strategy where we observed *L. borgpetersenii* serovar Ballum LPS to show 2–4% cross-reactivity with sera specific for *L. interrogans* serovars Autumnalis and Australis [14]. To present an in-depth understanding of the heterologous cross-reactivity, in the present study we performed a detailed comparative analysis of the chemical composition of different *Leptospira*. Recently, bioinformatics tools are playing a key role in providing insight and analyzing genes. Among them, KEGG is a knowledge database for the assignment of specific pathways functional information of higher order. The stored pathway database was supplemented with the ortholog table and conserved subpathways. The gene with chromosome position is useful to predict gene and function that is involving in the specific pathway [43]. *L. interrogans* and *L. biflexa* LPS biosynthesis pathway were shown as a network of gene products (enzyme) along with enzyme commission (EC) numbers; the gene information was retrieved from the KEGG database and the similar proteins from other *Leptospira* species were identified using BLAST protein-protein similarity search [44]. The functional groups present in the carbohydrate region and anomeric configuration showed significant similarity among the LPS profile of serovar Autumnalis, Australis and Ballum and this explains the moderate heterologous cross-reactivity of the Ballum LPS with other serovars. Interestingly, we found that LPS from pathogenic serovars has significant levels of hydroxyl palmitate, margaric acid, and lauric acid compared to nonpathogenic serovar.

The biological and pathological properties of LPS mainly depends on the lipid A, phosphate group, methyl group, degree of acylation pattern of fatty acyl chains and length of the fatty acids [5]. Tetra acylated lipid A with 16–18 carbons with ethanolamine substitution was observed in *H. pylori* [45] and bisphosphorylated disaccharide with the only amide linked acyl groups were observed in *L. pneumophila* [46]. Similar to other pathogenic bacteria, leptospiral lipid A also contained 12 to 18 carbon fatty acids with phosphate groups. Additionally, 3-hydroxy palmitic acid (3-OH C16:0), a very powerful autoregulator compound that regulates the virulence in the pathogenic bacteria [47], was present only in the pathogenic leptospires.

The negative charge of the core saccharide region was contributed by the phosphoryl substituents and carboxy acids like KDO. The complete core provides rigidity to the cell wall through intermolecular cationic cross-links. Our finding reveals that KDO and phosphorous were found in all the pathogenic leptospiral serovars and nonpathogenic serovar. The complete core is responsible for the resistance to bactericidal mechanisms during phagocytosis at some limited extends. Thus, pathogenic leptospires are seepage from the complement mediated eradication from the host system. A long oligosaccharide side chain of Gram-negative bacteria LPS can restrict complement activation [48]. In this study, FT-IR findings reveal similar kinds of frequencies in the anomeric region, amide linkage and carbohydrate vibration mode in *L. interrogans* and *L. borgpetersenii*.

The NMR analysis can provide vast structural information includes the number and types of monosaccharides, intersaccharide linkages, anomeric configuration, and position of substituents [49–51]. The one-dimensional NMR was mainly used to determine the chemical shift of a nucleus by the distribution of electron density and interaction with its neighboring nuclei. The biochemical composition and structural confirmation of LPS play an important role in determining host specificity, virulence, and pathogenicity [3,52–54]. In this study, we reported the possible structural similarities and assignment of chemical shifts between different pathogenic serovars and variations from the non-pathogenic serovar as well. Moreover, the basic structural characterization of LPS would greatly contribute to our understanding of the biochemical and potential virulent properties of LPS. The overall ratio observed in the number of hydrogens and carbons showed the difference between pathogens and non-pathogen strains. Poor signals from non-pathogenic carbon NMR revealed the absence of many sugars and fatty acids. The reduced number of sugars and fatty acid composition were observed in intermediately pathogenic *L. interrogans* than pathogenic strain [52]. The similar arrangement of 6-deoxy sugars and methyl group were found in *L. interrogans* and *L. borgpertersenii* serogroups. Based on 1D NMR results, we hypothesized the possible rationales behind the mild cross reactivity and virulence of *L. interrogans* and *L. borgpetersenni*. Nonetheless, the one-dimensional information overlaps and certain molecule structures are hard to be determined for complicated molecules. Also, the 1D NMR used in this study deeply limits the knowledge about carbohydrate residues and complete structural elucidation of LPS. The further analyses include mass spectrometry, homo- and heteronuclear two-dimensional NMR spectroscopy (DQF-COSY, TOCSY, NOESY and HSQC) are needed to correlate the assignment of all the proton and carbon shifts.

We adopted a surrogate model system (*S. cerevisiae)* to emphasize the pathogenic differences of the leptospiral LPS and the activation of the LPS-mediated pathogenic pathway. In this study, we showed that pathogenic LPS induce the HOG- mediated stress response in yeast similar to that observed in mammalian cells (p38-mediated). Even though this study provides distinct insights on LPS mediated osmotic stress pathway activation, further for their association with pathogenesis by detailed functional analysis is needed to establish the complete pathophysiology during leptospirosis.

It was described that the leptospiral LPS was recognized by the TLR2-dependent pathway but not the TLR4 pathway in the human cells. Also, it was highlighted that the avirulent strain of *L. interrogans* showed roughly tenfold higher stimulation than the virulent strain [10]. Viriyakosol et al. explained the role of TRL4 against a lethal infection of *L. interrogans* serovar Icterohaemorrahgiae [55]. Though we have observed responses from both TLR2 and TLR4, significantly higher responses were obtained in TLR4 from *L. interrogans* serovar Autumnalis strain N2 LPS treated cells. The present study provides a basic clear insight into the uncertainty of TLR2 and TLR4 activation by leptospiral LPS due to biochemical and structural variations of LPS components. Further exploration of LPS components and structure is likely to be great assistance to understand the pathophysiology of leptospirosis.

4.1. Contribution to the field statement

Leptospirosis is an important zoonosis of tropical countries and generally under-recognized public health problem worldwide. In this study, the chemical composition analysis of leptospiral lipopolysaccharides (LPS) extracted from pathogenic and nonpathogenic serovars elucidated the pathogenesis. Therefore, it must create awareness of the leptospiral chemical composition of LPS and its associated pathogenesis. This is the understudied aspect of leptospirosis and this present work uncovers the LPS associated pathogenesis. Limulus amebocyte lysate assay (LAL) showed significant endotoxicity among pathogenic serovars. This is because of the presence of palmitic acid and 3-hydroxy palmitate in pathogenic leptospires. C–O–C stretch of esters, 3°ROH functional groups and carbohydrate vibration range were detected similarly among pathogenic serovars. NMR analysis revealed similarity for 6 deoxy sugars and methyl groups of Autumnalis, Australis and Ballum of pathogenic serovars. The presence of palmitoleic acid and 3-hydroxy palmitate may be the significant pathogen-associated predisposing factor for leptospirosis.

Author Statement

SV, CSAM, MK, KN: Conceptualization, Methodology, Software. **SV, CSAM, KS, PB, PS, MP:** Data curation, Writing- Original draft preparation. **KP, GM, NAA, AV:** Visualization, Investigation. **KN, GM, AV, PV:** Supervision. **MK, AV, CSAM:** Software, Validation. **KN, NAA, PV, AV:** Writing- Reviewing and Editing.

Declaration of competing interest

The authors declare no conflicts of interest associated with this manuscript.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2021.104738.

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Leptospiral protein LIC11334 display an immunogenic peptide KNSMP01

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ABSTRACT

Leptospirosis is considered as a neglected tropical disease which is caused by pathogenic *Leptospira* spp. The precise mechanisms of leptospirosis pathogenesis are unclear and hence, the progress in development of treatment modalities has been dismal. The present study aimed to identify novel virulent factors of leptospires to understand the disease pathogenesis and to develop treatment modalities. *Leptospira interrogans* contains two chromosomes and encodes for ~3703 genes, but the functions of several open reading frames have not yet been explored. Among them, novel virulent associated leptospiral proteins (LIC11334, LIC11542, LIC11436, LIC11120 and LIC12539) were identified using VirulentPredict and the antigenicity of these targets was explored by VaxiJen server. Domain architecture of the pathogen specific proteins revealed that LIC11334 had potential to evoke significant immune response against leptospiral infection and LIC11436 contains four folds of immunoglobulin-like domain and plays a vital role in pathogenesis. Therefore, B-cell epitopes were predicted and the epitope of high virulence (and VaxiJen score from LIC11334) was chemically synthesized as peptide (KNSMP01) and labeled with Biotin (Biotin-SGSGEVENPDPKVAQEC). Binding affinity of KNSMP01 with MHC molecules was predicted and the molecule was discovered to have potential to elicit both humoral and cell mediated immune responses and found to interact with host components via hydrophobic interaction, hydrogen bonding and salt bridges. Rabbit antisera was raised against KNSMP01 and found to elicit antigenicity using Western, ELISA and dot blot assays. *In silico* and *in vitro* experiments show KNSMP01 to be a promising immunogen and may be a better vaccine candidate for leptospirosis.

1. Introduction

Leptospirosis is a re-emerging zoonotic infectious disease, which causes major public health problems worldwide. Recent outbreaks and reports have surveyed the global burden of this disease. Around 60,000 deaths and 1.03 million leptospirosis cases are registered annually [1]. Leptospirosis is an environment dependent tropical disease, caused by pathogenic *Leptospira* spp. It infects humans by direct contact with infected animal urine or with contaminated environments and disseminates haematogenously causing diverse clinical symptoms [2]. Leptospirosis is diagnosed by immunological, molecular and immunochromatographic methods. The disease is confirmed by the gold standard microscopic agglutination test (MAT). Due to non-specific early symptoms, early diagnosis and treatment of the disease has not been entirely successful [3]. Hence, elucidation of the molecular mechanisms of infection is crucial for developing methods to successfully diagnose and treat the disease.

The cell adhesion molecules of virulent *Leptospira* interact with host surface-exposed proteins to establish the infection. The surface associated proteins such as outer membrane proteins (OMPs), *Leptospira* OmpA-like lipoprotein (Loa22), LipL32, immunoglobulin-like proteins (LigA and LigB), *Leptospira* endostatin-like proteins (LenA-LenE) and lipopolysaccharides (LPS) are potentially involved in virulence mechanism and studied extensively to find vaccine targets [4]. Especially, the OMPs are being explored deeper insights to understand the pathogenesis of leptospirosis [5] The immune response elicited by OMPs has been evaluated using animal models and aid in the vaccine development. Recombinant proteins of pathogenic *Leptospira* spp. were shown to bind with host extracellular matrix components such as fibronectin, plasminogen, collagens, laminin and elastin [6]. But the molecular

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mechanism of these interactions has not yet been well established.

Due to enormous heterogeneity of lipopolysaccharide (LPS) moieties, ≥ 250 clinical serovars of pathogenic *Leptospira* spp. exist and this fact has perplexed the research community as lacunae in knowledge pertaining to mechanism of pathogenesis impedes the development of an efficient vaccine for leptospirosis. Many research findings had revealed the limited efficacy of subunit vaccines, recombinant protein and DNA vaccines as well as attenuated cells since they do not provide either long term or cross protection [7]. Several approaches have been initiated thus far towards understanding the pathogenesis process. However, the molecular mechanism of leptospiral infection is still obscure and hence effective strategies are required to develop protective vaccines or formulate therapeutics. The genome of *Leptospira* spp. comprises of two circular chromosomes containing DNA in the range between 39 and 46 MB. It encodes for 4768 genes but the function of most of the genes have not yet been deciphered. In this study, we hypothesised that determining the function of unknown open reading frames (ORFs) of leptospires would enable us to uncover the pathogenesis and to characterize the immunogenic epitopes to use as a candidate vaccine for leptospirosis. This study also aimed at elucidating the factors which are involved in host-pathogen interactions.

2. Material and methods

2.1. Samples and ethical statement

Serum samples (n = 29) collected during the early phase of illness (0–10 days after the onset of disease) were selected from a bank of 261 laboratory-confirmed cases of leptospirosis (a positive IgM ELISA or MAT titer of ≥1:160 or isolation of leptospires from the blood). A total of 22 seronegative healthy controls matched with respect to age (±5 years) and sex, and 31 patients who were hospitalized with a clinical suspicion of leptospirosis and subsequently diagnosed as having other illness based on laboratory evidences were also included. Informed written consent was obtained from both cases and controls before sampling, and the study protocol was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University (DM/2016/101/66 dated 06-09-2016; BDU/IAEC/2017/NE/26 dated 21-03-2017) as well as approved by the Institutional biosafety (Ref. No. BT/BS/17/29/2000-PID dated 24-05-2017) committee. The procedures were followed in accordance with the relevant guidelines and regulations.

2.2. Selection of open reading frames

The Spirochetes Genome Browser (SGB) was used to cluster the genes based on the functional orthologous groups and conserved hypothetical genes were selected. The intra-family specific OMPs were predicted from the genome of *L. interrogans* serovars Copenhageni, Lai 56601, Lai IPAV and Linhai. All the predicted genes were categorized based on the information available in the KEGG database. Genetic diversity and evolutionary analysis of the OMPs encoding genes were identified using MEGA6 software [8].

2.3. Subcellular localization, topology and domain architecture

The subcellular localization of the hypothetical genes was predicted using the most precise bacterial localization prediction tool, pSORTb v3.0.2 [9] and topology was identified using transmembrane topology predictor, Phobius [10]. Association networks of the hypothetical proteins were predicted using STRING 10.5 [11]. Conserved domains were identified using NCBI conserved domain search service [12] and Pfam database [13]. The Pfam database entries are exploited to identify the functional domains by multiple sequence alignment and hidden Markov models.

2.4. Identification of virulent factors

The hypothetical genes were screened using VirulentPredict [14] to identify the virulent proteins and they are segregated from the avirulent proteins. All the virulence genes were further identified for their vaccination potential using VaxiJen server [15] with the threshold level score of 0.4. This server is known for identification of protective antigens and subunit vaccines. Before proceeding further, the selected genes were subjected to basic local alignment search tool (BLAST) analysis [16] for identification of mammalian homologues.

2.5. Identification of B-cell and T-cell epitopes

The linear B-cell epitopes were predicted for the selected hypothetical genes using BCPreds [17] tool with more than 90% of specificity. BCPreds is used to identify and characterize the B-cell epitopes using the subsequence kernel and is applied for vaccine design, immunodiagnosis and production of antibodies. VaxiJen server was exploited to identify the potential of the predicted epitopes as vaccine antigens (threshold score >0.7). T cell epitopes were predicted indirectly by identifying the binding affinity of peptide to the major histocompatibility complex (MHC) molecules using MHCPred 2.0 [18]. The binding affinity of B-cell epitopes with known MHC molecules was estimated at a set threshold level of 500 nM (<500 nM is non-binder) and represented as IC_{50} value. The B-cell specific epitopes were compared with the MHCPred outputs to identify the combined epitopes that induce both B and T cell immune responses.

2.6. Homology modeling and molecular docking

Three dimensional protein structures were modeled for the selected hypothetical proteins using Phyre^2 [19] and the structures were validated with Ramachandran plot using RAMPAGE [20]. The homology models were visualized and analyzed using UCSF-Chimera [21]. The proteins and peptides were subjected to hydrogen adjustment and energy minimization using Amber force field available in the InsightII software package. All the selected proteins were docked to their respective peptides using GRAMMX protein-peptide docking server [22] and the best protein-peptide complex was subjected to flexible docking using FlexPepDock server [23]. The residue-wise interactions were analyzed using PDBsum database [24]. The structure of plasminogen (PDB ID: 1DDJ) and fibronectin (PDB ID: 3M7P) were retrieved from protein data bank (PDB) and used to find their affinity as well as interaction with selected proteins (and their peptides) using ZDOCK server 3.0.2 [25]. Throughout the study, fibrinogen and plasminogen were always kept as chain 'A' and protein of interests as chain 'B' and the interactions were calculated using PDBsum server.

2.7. Peptide synthesis and characterization

KNSMP01 peptide (Biotin-SGSG*EVENPDPKVAQE*C) was synthesized at Priveel Peptides, India and purified using reverse-phase preparative high-performance liquid chromatography (HPLC) on a C18 column (Phenomenex Luna 3μ C18 (2)). The molecular mass of the peptides was estimated using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The peptide was synthesized with biotin at the N-terminus and linked with a spacer sequence of SGSG.

2.8. Rabbit polyclonal antisera

Polyclonal antisera were raised in New Zealand white rabbits by subcutaneous administration of KNSMP01 peptide (200 μg/mL) along with the alhydrogel (Sigma Aldrich, USA). Three booster injections (14 days interval) were administered. After the booster injection, the blood was collected, and serum was separated by centrifugation at 4000 rpm for 30 min at 4 °C. The separated antisera were aliquoted and stored at

−80 °C until use.

2.9. Western analysis

Leptospiral whole cell lysate of different serovars were prepared as followed by Kanagavel et al. [26], and analyzed by 12% polyacrylamide gel electrophoresis using discontinuous buffer system [27]. The whole cell lysate was mixed with 2x loading buffer (125 mM Tris-Cl, 4% SDS, 2% Glycerol, 1% β-mercaptoethanol, 0.5% bromophenol blue) and boiled for 5 min before loading the sample on the gel. Electrophoresis was carried out in a Mini-PROTEAN Tetra Cell apparatus (Bio-Rad, USA) for 2 h at 60 V in Tris-Glycine running buffer (25 mM Tris-HCl pH 8.3, 192 mM Glycine, 0.1% SDS). The gel was transferred to nitrocellulose membrane (0.2 μm; Bio-Rad, USA) and blocked with 4% non-fat dry milk in Tris-buffered Saline (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20). The membrane was incubated with hyperimmune sera, followed by incubation with an anti-rabbit IgG conjugated with horseradish peroxidase (Sigma Aldrich, USA). The membrane was developed with SuperSignal™ West Pico PLUS (Thermo Scientific, USA) and documented in Fusion Solo S™ personal blot and Chemidoc gel imaging system (Vilber Lourmat, France).

2.10. Enzyme-linked immunosorbent assay (ELISA)

Whole-cell lysates were prepared by heat extraction [28] and ELISA was performed as described by Kanagavel et al., [26]. Briefly, whole-cell lysate (100 μg) or KNSMP01 peptide (0.5 μg) was coated on the flat-bottom polystyrene microtiter plate at 4 °C for overnight using carbonate coating buffer (pH 9.6). After blocking with 4% non-fat dry milk, the hyperimmune sera of rabbit or human samples of positive cases or healthy controls (1:200) were added in triplicate and incubated at 37 °C for 1 h. Bound antibody was detected using HRP-conjugated anti--rabbit IgG/rabbit anti-human IgM (1:8000; Sigma-Aldrich, USA) by developing with o–phenylenediamine (Sigma-Aldrich, USA). The reaction was stopped with 1 N H_2SO_4 (50 μL) and the optical density was measured at 490 nm using iMark™ Microplate Absorbance Reader (Bio-Rad, USA).

2.11. Dot blot assay

Dot blot assay was performed to validate the specificity of the leptospiral heat extracted proteins and KNSMP01 peptide. The heat killed antigen prepared from MACS [29] of different leptospiral serovars and KNSMP01 peptide concentration was optimized using rabbit hyperimmune sera. Briefly, heat killed antigen (2 μg) or KNSMP01 peptide (0.2 μg) were dotted on the nitrocellulose membrane and after air drying, the membrane was blocked with 5% non-fat milk and incubated at 37 °C for 1 h with the hyperimmune sera (1: 200 in PBS-T) and washed four times with PBS-T. The membrane was incubated with HRP conjugated anti-human IgM or anti-rabbit IgG (1:4000; Sigma Aldrich, USA) at 37 °C for 1 h and washed with PBS-T. The membrane was developed with SuperSignal™ West Pico PLUS Chemiluminescent Substrate (Thermo Scientific, USA).

2.12. Measurement of ROS and mitochondrial membrane potential ($\Delta\Psi_m$)

The COS-7 cells (NCCS, Pune, India) were grown (5×10^5 cells) in complete DMEM medium containing FBS (10%), Penicillin (100 U/mL) and Streptomycin (100 μg/mL) and plated on a cover glass. After 24 h of growth, the cells were treated with KNSMP01 peptide (1 μg/mL) for overnight at 37 °C in a humidified 5% CO_2 atmosphere. Then the cells were stained with dihyroethidium (DHE; 10 μM) at 37 °C for 30 min or stained with Rhodamine 123 (1 mM) at 37 °C for 20 min. The ROS production was analyzed using confocal microscope (Zeiss LSM710, Germany) at 518 nm and 605 nm of excitation and emission, respectively and the tert-Butyl HydroPeroxide (tBH; 200 μM) was used as positive control. Mitochondrial Membrane Potential ($\Delta\Psi_m$) acquired at 488 nm and 561 nm of excitation and emission, respectively. The protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP; 100 μM) was used as positive control and PBS served as the negative control.

2.13. Caspase and cytochrome C release

The protein content of the cytosolic and mitochondrial fractions of KNSMP01 peptide (1 μg/mL) treated COS-7 cells was quantified by BCA method (Sigma Aldrich, USA) and resolved in 12% SDS-PAGE using Mini-PROTEAN Tetra System (BioRad, USA). Proteins were transferred to the nitrocellulose membrane using V20 semi-dry blotter (Scie-Plas, UK). Membranes were blocked with 5% non-fat milk in Tris buffered saline containing 0.1% Tween-20 (TBST) for 1 h before being probed with antibodies specific for Caspase 8 (1:1000; Cell Signaling Technology, USA) and GAPDH (1:1000; Santa Cruz, USA) or antibodies specific for cytochrome-*c* (1:1000; Santa Cruz, USA) and Tom20 (1:1000; Santa Cruz, USA) at 4 °C for overnight. Then the membrane was incubated with anti-rabbit IgG-HRP conjugate (Sigma Aldrich, USA) at 30 °C for 1 h and the blots were developed with SuperSignal™ West-Pico Chemiluminescence-developing kit (ThermoFisher Scientific, USA) and documented in Fusion Solo S™ personal blot and gel imaging system (Vilber Lourmat, France).

2.14. Cell viability assay

COS-7 (2×10^3 cells) which were cultured in 96-well tissue culture plates in DMEM medium were treated with different concentrations of KNSMP01 peptide (0–150 μg/mL) for 24 h. The treated cells were then incubated with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 3 h. Later, the media were aspirated, and the formazan crystals were dissolved using Dimethyl sulfoxide (DMSO, 100 μL). The optical density (OD) was determined at 490 nm using iMark™ Microplate Absorbance Reader (Bio-Rad, USA). The cells were treated with 1 μg/mL of KNSMP01 peptide and stained with Propidium Iodide (PI; 0.5 μg/mL) (Invitrogen, USA) for 5 min and imaged at 533/617 nm of excitation/emission using confocal microscope (Zeiss LSM710, Germany). The tBH (tert-Butyl HydroPeroxide; 200 μM) was used as positive control and PBS was used as a negative control.

2.15. Statistic analysis

Data from multiple experiments ($n \geq 3$) were quantified and expressed as Mean ± SEM, and differences in means among multiple datasets were analyzed using one-way ANOVA with the Tukey test. $P \leq 0.05$ was considered significant in all analyses. The data were computed either with GraphPad Prism version 8.0 or SigmaPlot 11.0 software. The Image J software (NIH) was used to calculate the band intensity of blots and expressed as arbitrary units (AU).

3. Results

3.1. Identification of leptospiral outer exposed adhesions

Whole genome sequence of *L. interrogans* serovars were explored to find out the genetic and nucleotide diversity, segregating sites, and relative rate of the OMPs using MEGA6 software (Table 1). The analysis involved amino acid sequences from four different serovars and all positions containing gaps and missing data were eliminated and the final dataset was created. Descriptive information of OMP-encoding genes existing in different serovars of *L. interrogans* and their evolutionary analyses were conducted (Supplementary Table S1). Intra-family specific hypothetical genes encoding OMPs were predicted and among 60 families, only 32 families had a VaxiJen score ≥0.5 (Supplementary Table S2). These genes were scrutinized further based on localization (in

Table 1
Genetic diversity of OMPs of *Leptospira interrogans*.

Parameters	Serovars			
	Copenhageni	Lai 56601	Lai IPAV	Linhai
Overall Mean Distance	2.445 ± 0.194	2.397 ± 0.206	2.422 ± 0.205	2.481 ± 0.065
m	67	65	60	59
S	48	39	43	23
Ps	0.979592	0.975000	0.977273	1
θ	0.205175	0.205527	0.209571	0.215227
π	0.904670	0.899135	0.902478	0.916931
D	11.306708	11.072281	11.036780	10.311395

Abbreviations: m = number of sequences; S = Number of segregating sites; p_s = S/n; $\Theta = p_s/a_1$; π = nucleotide diversity and D is the Tajima test statistic.

the cell membrane), high VaxiJen and virulence scores, diverse phylogenic origin, and organism source – in particular, non-mammalian homologues. Five proteins like LIC11334, LIC11542, LIC11436, LIC11120 and LIC12539 from five distinct protein superfamilies' were selected for the study and their properties were analyzed (Table 2). All the genes used for this study are hypothetical but possess specific domains. Phylogenetic diversity among the selected proteins of interest was evaluated and it was found that they originated from same ancestor but possessed distinct characteristic features.

3.2. Occurrence of homologous genes

All the predicted OMPs were analyzed for the homologous genes in animals such as *Mus musculus* (Mouse), *Oryctolagus cuniculus* (Rabbit), *Canis familiaris* (Dog), *Bos taurus* (Cow) and *Homo sapiens* (Human) (Supplementary Table S3). The selected genes were scrutinized and care was taken to ensure that none of them are mammalian homologues. To identify the homologous genes, which are present in other pathogenic and non-pathogenic *Leptospira* sp., these genes were analyzed using NCBI-BLAST against *Leptospira* spp. (Table 2). Among the five genes, two of them (LIC11334 and LIC11120) do not have homologous genes in non-pathogenic *L. biflexa* strain Patoc I. Hence, these pathogenic specific genes were further characterized to discover potential vaccine candidates. Even though other genes possess homologous genes in non-pathogenic leptospires, their identity scores are very less (insignificant).

3.3. Properties of the hypothetical proteins

The virulence efficiency and vaccine potential of the selected hypothetical genes were analyzed (Table 2) and identified (as they are virulence associated genes). Also, these proteins belong to the functional and gene ontology category of cell envelope and immune response,. Interpro-protein sequence analysis and classification confirmed that LIC11436 consists of four immunoglobulin like (Ig-like) folds. LIC11334 is a pathogen specific protein and has an immunodominant 'SIMPL' domain. It is also found to interact with the chaperonin, GroEL. LIC11542 is an ABC transporter ATP binding protein and is also linked to phosphocarrier protein. LIC12539 is part of the preprotein translocase subunits SecD, SecF and YajC and found to interact with outer membrane lipoprotein-sorting protein.

3.4. Homology modeling and validation

Three dimensional structures for the selected proteins were modeled using multi-thread templates and validated using Ramachandran plot (Supplementary Fig. S1). The templates used for constructing the three-dimensional models were evaluated for their properties such as query coverage, identity, and functional descriptions. All the templates were utilized to model the proteins found to have role in virulence, immunogenicity and/or membrane-associated transport mechanism (Supplementary Table S4). As per the protein model of LIC11334, the molecule comprises of three α and three β-strands, but LIC11120 contains three helices with loops. However, LIC11542 possesses only β-strands but LIC12539 contains only helices (six transmembrane helices) with coiled coils and loops. LIC11436 is a larger protein with ~38% of extended strands. All the protein models which were found to be valid were used for further study. All finalized models possessed >90% of residues in the favored regions and only about 10% of residues in the disallowed region.

3.5. Identification and characterization of antigenic determinants

B-cell epitopes were identified from the proteins of interest and efficiency as well as vaccine potential of these candidates was examined. The candidates were found to be virulent and have good vaccination potential. The characteristics of the identified antigenic determinants were predicted using various bioinformatics tools (Table 3). Among them, the potent antigenic determinant, "*EVENPDPKVAQE*" (LIC11334 origin) was selected based on high virulence score, vaccine potential and uniqueness and named as 'KNSMP01'. The B-cell specific epitopes were studied further using MHCPred to find if they elicited cell mediated immune response. Most of them were found to be compatible with T-cell alleles and the binding affinity is expressed as IC_{50} value. All the B-cell epitopes were found to have appreciable binding affinity with the twenty selected T-Cell alleles and none of the epitopes was found to bind to H2Db, A0101, IEg, IEk and IEd alleles. All the epitopes bound strongly to H2Kb, A0203, A1101, DRB0101 and IAs alleles (Fig. 1). KNSMP01 peptide possess strong binding affinity to most of the alleles than other B-cell epitopes.

3.6. Interactions of virulent factors with host surface molecules

The protein and peptide models were studied for their interaction with human extracellular matrix components and the interacting features were determined. The proteins interacted with their respective peptides via non-bonded interactions and via hydrogen bonding, except LIC11334 (Supplementary Table S5a). Only LIC11542 formed a salt bridge with its peptide. The schematic representations of the

Table 3
Prediction of antigenic determinants and their properties.

Locus Tag	Position	Sequence	VaxiJen/Virulence Score
LIC11334	49–60	EVENPDPKVAQE	1.4785/1.0611
LIC11542	153–164	ADGTTIRGIGLR	1.9965/1.0630
LIC11436	169–180	LSAKYKDKTGIQ	2.2823/1.0606
LIC11120	64–75	KEVSYVETKYTP	1.3089/1.0606
LIC12539	58–69	KKKEKKRSKKKR	1.4307/1.0769

Table 2
Properties of the Virulence Genes of *Leptospira* sp.

Locus Tag	Pathogenic homologues	Non-pathogenic homologue	Virulence Score	VaxiJen Score	Fold Type	Assigned function
LIC11334	LA2656; LIFA2173; LIL11460	–	1.0753	0.6696	SIMPL	Immunogenic protein
LIC11542	LA2407; LIFA1969; LIL11662	LEPBII1647	1.1260	0.7094	ABC transporter	LPS export system protein
LIC11436	LIL11557; LA2537	LEPBII1827 LEPBII0545	1.1260	0.6787	FecR	LipL45 related protein
LIC11120	LA2941; LIL12510	–	1.0513	0.8156	λ-Bor	Virulence of some pathogenic bacteria
LIC12539	LA1141a; LIL11078	LEPBII1400	1.0297	1.0196	YajC	Transport protein

B Cell Epitope	T Cell Alleles																							
	A0201	H2Db	H2Kb	H2Kk	A0101	A0202	A0203	A0206	A0301	A1101	A3101	A6801	A6802	B3501	DRB0101	DRB0401	DRB0701	IAb	IAk	IEg	IEk	IAd	IAs	IEd	TAP
EVENPDPKVAQE	-	-	+++	+++	-	+	+++	++	+	+++	-	++	+++	+	+++	++	++	+	+	-	-	-	++	-	-
ADGTTIRGIGLR	++	-	+++	+++	-	+	+++	++	+++	+++	++	+++	+++	+	+++	+	+	-	+++	-	-	+++	+++	-	+++
LSAKYKDKTGIQ	++	-	+++	-	-	++	++	+++	-	+++	-	+++	+	-	+++	+	-	+	++	-	-	-	+++	-	+++
KEVSYVETKYTP	+++	-	+++	-	-	+	+++	+++	+++	+++	+	++	-	+	+++	++	++	+	++	-	-	-	+++	-	+++
KKKEKKRSKKKR	-	-	+++	-	-	++	+++	+++	+++	+++	-	+	++	+	++	++	-	+++	-	-	-	-	+	-	+++

+ Presence of epitope on the allele | + IC_{50} = 300-500 nM | ++ IC_{50} = 100-300 nM | +++ IC_{50} = 1-100 nM | - Absence of epitope on the allele

Fig. 1. Interaction of selected epitopes with the T-cell alleles.

interactions between the proteins and peptides have been elucidated using illustrations (Supplementary Fig. S2). All the residues in the peptides interact with the proteins, but not with the same residues. Lysine formed a hydrogen bond in protein LIC11120 and others interacted via non-bonded interactions. N-terminal regions of the proteins, LIC11542 and LIC12539 are likely to interact with their respective peptides. The interaction of proteins with plasminogen and fibrinogen was determined and the selected proteins were found to interact with the host surface molecules via hydrophobic interactions, hydrogen bonding and salt bridges (Supplementary Figs. S3–S4 & Supplementary Tables S5b–c). These results suggest that the proteins which are interacting with fibrinogen may participate in the coagulation cascade during the infection. LIC11334 exhibited a total docking score of 65.460 and was found to interact with the human extracellular matrix component, plasminogen (Supplementary Fig. S3a). At least twenty residues were found to participate in the interaction with plasminogen, except LIC11542. Aliphatic amino acids and/or aliphatic side chains are the major players in most of the interactions. At least a couple of hydrogen bonds were observed in interaction of peptide with plasminogen in the proteins, LIC11334, LIC11542 and LIC12539 (Supplementary Fig. S5 & Supplementary Table S6a). LIC11120 peptide makes hydrogen bonding with fibrinogen via threonine and serine residues (Supplementary Fig. S6 & Supplementary Table S6b).

3.7. KNSMP01 peptide synthesis and characterization

KNSMP01 peptide (*EVENPDPKVAQE*C) was identified as a unique peptide with ability to act as a virulent epitope and a potential vaccine candidate. It was synthesized to the purity level of ≥95% and confirmed by HPLC (Fig. 2 a, b) with the retention time of ~15.5 using the 214 nm detector. The molecular mass was estimated by MALDI-MS (Fig. 2c) and the mass spectra revealed the theoretical molecular weight of 1972.15 Da, but the observed molecular weight was 1971.90 Da. The KNSMP01 peptide was synthesized with the linker (SGSG) to improve the solubility and facilitate for better outputs in ELISAs and Biotin at the N-terminal is attached for detection in assays.

3.8. Antigenic potential and specificity of KNSMP01 peptide

The rabbit polyclonal antibody of KNSMP01 was tested by dot blot assay against the proteins extracted from different serovars (Australis, Autumnalis, Ballum, Bataviae, Canicola, Grippotyphosa, Hebdomadis, Javanica, Sejroe and Pyrogenes) along with the vehicle control PBS. The rabbit hyperimmune sera raised against the KNSMP01 peptide is reactive to all the serovars significantly except for *Ballum* (Fig. 3a). Immunoblot analysis of pathogenic serovars (Australis and Grippotyphosa) showed heterologous reactivity against rabbit hyperimmune sera of KNSMP01 and found that the same serovars which are showed reactivity in dot blot reflected in immunoblot as well. These reactive proteins could be the leptospiral adhesion LIC11334 in the range of 25 kDa and specifically interacted with rabbit hyperimmune sera of KNSMP01 (Fig. 3c). In the same way the homologous and heterologous reactivity of the KNSMP01 peptide was assessed using ELISA. The optimum antigen concentration of the KNSMP01 peptide was determined by checkerboard titration (0.2 μg/well) and this was utilized in ELISA assay. Interestingly rabbit hyperimmune sera of KNSMP01 (G1) and the sera of confirmed leptospirosis cases (G2) showed >0.2 OD @ 490 nm (Fig. 3b). Comparatively the sera of healthy controls (G3) and sera from other diseases (G4) showed less reactivity. G1 and G2 were significantly different from G3 and G4. This is evidence for the pathogenicity associated reactivity of the KNSMP01 peptide.

3.9. Mitochondrial membrane potential ($\Delta\Psi_m$) and ROS

Mitochondrial dysfunction is the ultimate target of any foreign particle inside the cell. Changes in the $\Delta\Psi_m$ are the foremost indication in all forms of metabolic dysregulation. To evaluate the $\Delta\Psi_m$ changes by KNSMP01 peptide, the cells exposed with different concentration of peptide along with untreated controls were stained with Rhodamine123 and images were acquired. KNSMP01 peptide was not observed to cause depolarization of mitochondrial membrane (Fig. 4a). KNSMP01 peptide was also observed not to cause oxidative stress to the cells but at the same time, the exogenous oxidative stress inducer, tBH escalated ROS production (Fig. 4b).

3.10. Cytotoxicity analysis

COS-7 cells were treated with KNSMP01 peptide (0.5–150 μg/mL) for 24 h and dose-dependent inhibition of the cell growth was observed. Significant cytotoxicity was observed only at a higher concentration (~100 μg/mL) when compared to Triton X-100 treated positive control (Supplementary Fig. S7). KNSMP01 peptide dependent cell death was estimated by PI staining and the confocal images showed that peptide did not cause cell death (Fig. 4c) but the t-BH induced cell death was observed in the control cells.

3.11. Caspase-8 activation and cytochrome c release

Cytochrome *c* is an initiator of mitochondria-dependent apoptotic cell death. The KNSMP01 peptide treated cells were analyzed for cytochrome *c* release from mitochondria to cytosol by membrane fractionation analysis. The Western analysis confirmed that the cytochrome *c* was not released in the cytosolic fraction (Fig. 5 a, b). Hence, the KNSMP01 peptide was not found to promote mitochondria dependent apoptotic cell death. Caspase 8 is an initiator of extrinsic pathway of mitochondria independent apoptotic cell death. The KNSMP01 peptide

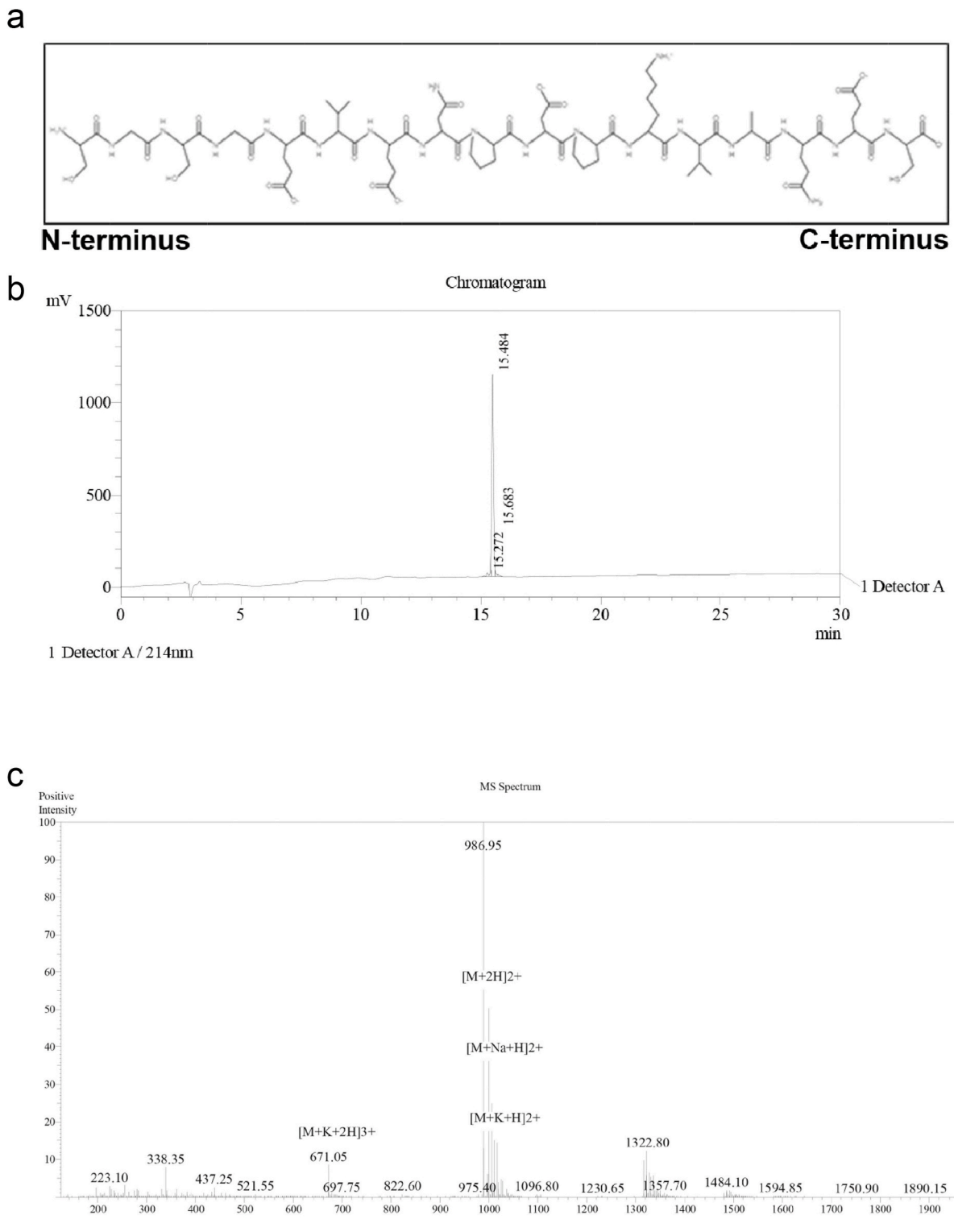

Fig. 2. Characterization of KNSMP01 peptide. (a) Structure of the peptide, (b) HPLC analysis and (c) MALDI-MS spectra of KNSMP01 peptide.

treated cells were not shown to cleave caspase 8 (Fig. 5 c, d), which is an active form of caspase which activates the downstream caspase in a cascade-like manner. Therefore, the KNSMP01 peptide was not found to cause either mitochondrial dependent or independent apoptotic cell death.

4. Discussion

The mechanism of disease establishment, clinical features, diagnostic measures and prevention of leptospirosis in humans was critically analyzed [30]. Several research groups are working on prevalence, diagnosis and treatment methods, but very few have focused on understanding the molecular mechanisms of leptospiral infection. Although more than twenty subunit vaccines were identified for

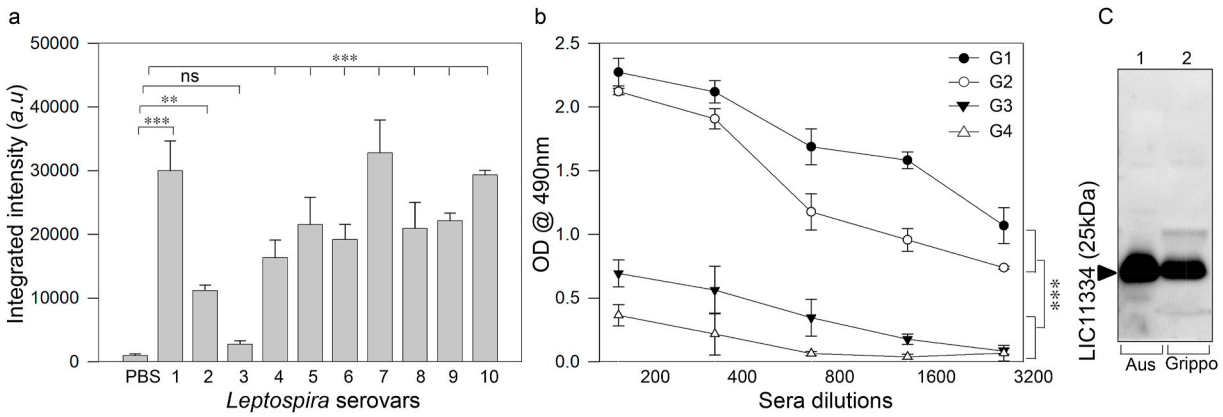

Fig. 3. Reactivity and Specificity of KNSMP01 peptide. (a) Dot-blot analysis. Reactivity of antibody raised in rabbit against the peptide was evaluated against different leptospiral serovars. Y-axis: integrated intensity in a.u, X-axis: PBS as vehicle control; 1) Australis; 2) Autumnalis; 3) Ballum; 4) Bataviae; 5) Canicola; 6) Grippotyphosa; 7) Hebdomadis; 8) Javanica; 9) Sejroe; 10) Pyrogenes. (b) ELISA. Specificity of KNSMP01 peptide against different sera. Rabbit polyclonal antibodyofKNSMP01 (G1); Sera of confirmed leptospirosis cases (G2); Sera from healthy seronegative individuals (G3) and sera from other diseases (G4). (c) Western analysis. Reactivity of rabbit polyclonal antibody of KNSMP01 peptide against pathogenic serovars. Lanes 1) Australis; 2) Grippotyphosa. Data represents Mean ± SEM and ***P < 0.001, **P < 0.005, *P < 0.01. ns - nonsignificant.

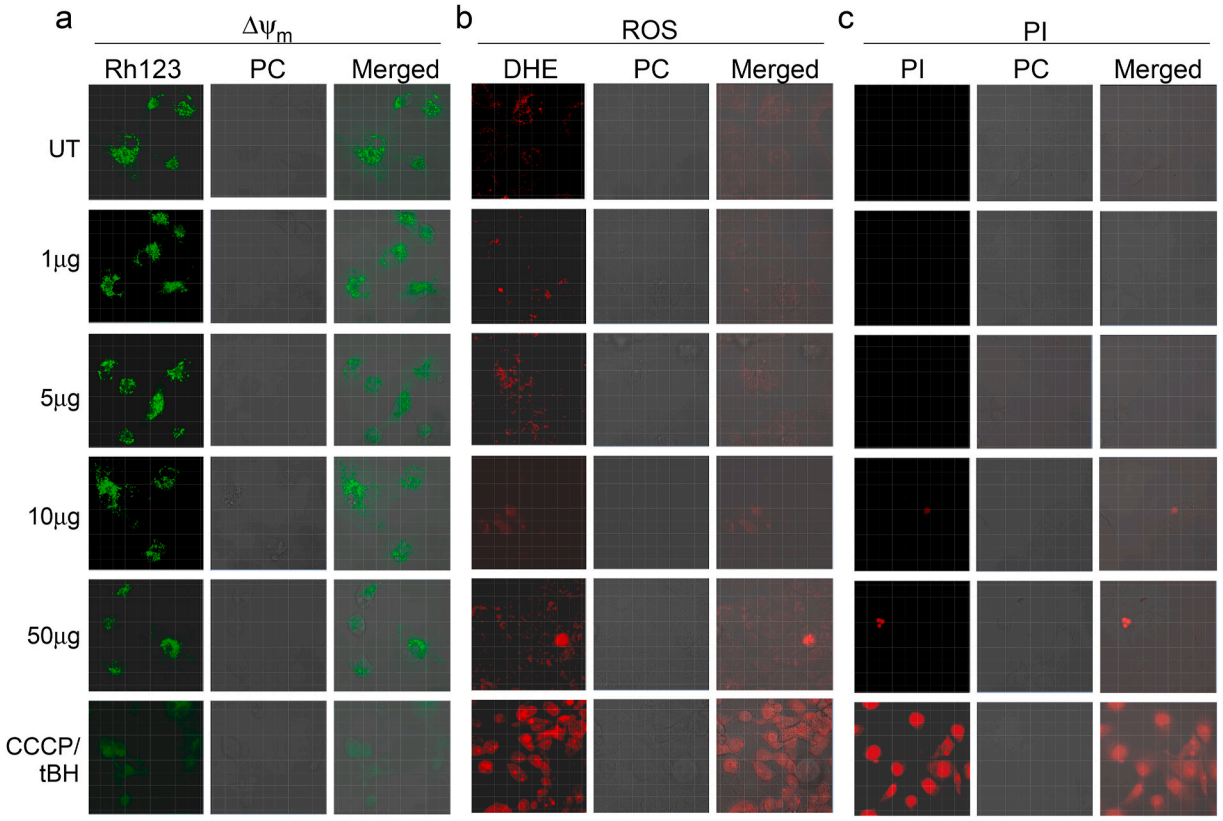

Fig. 4. Effect of KNSMP01 peptide in COS-7 cells. Different concentrations of KNSMP01 peptide (Untreated (UT), 1, 5, 10 and 50 μg) were exposed to COS-7 cells and examined using confocal microscopy. (a) Mitochondrial Membrane Potential ($\Delta\Psi_m$). Rh123 is Rhodamine 123 staining; CCCP is carbonyl cyanide m-chlorophenylhydrazone. (b) ROS Production. DHE is dihyroethidium; tBH is tert-Butyl HydroPeroxide (c) PI staining. tBH is tert-Butyl HydroPeroxide and PI is Propidium Iodide. PC is phase contrast image.

leptospirosis, none of them are yet to clear clinical trials. The success of these molecules is limited to laboratory experimental confirmation but they do not provide heterologous protection *in vivo* [31]. Therefore, we attempted to understand the molecular pathogenic mechanisms and identified a novel subunit vaccine candidate for leptospirosis.

SGB contains the genomic data of spirochetes and facilitates comparative genomic analysis. ~10 leptospiral whole genome sequences were published so far (2 strains of *L. interrogans* serovar Lai, 56601 and IPAV; *L. interrogans* serovar Copenhageni strain Fiocruz L1-130; 2 strains of *L. borgpetersenii* serovar Hardjo, L550 and JB197; *L. santarosai* serovar Shermani strain LT821; 2 strains of *L. licerasiae*, VAR010 and MMD0835 and 2 strains of the saprophyte *L. biflexa*, Paris and Ames). We have chosen the genome of four serovars of *L. interrogans* and for the understanding, the homologous genes of other serovars present in serovar Copenhageni strain Fiocruz L1-130 is designated throughout the study. The genome of *L. interrogans* was functionally

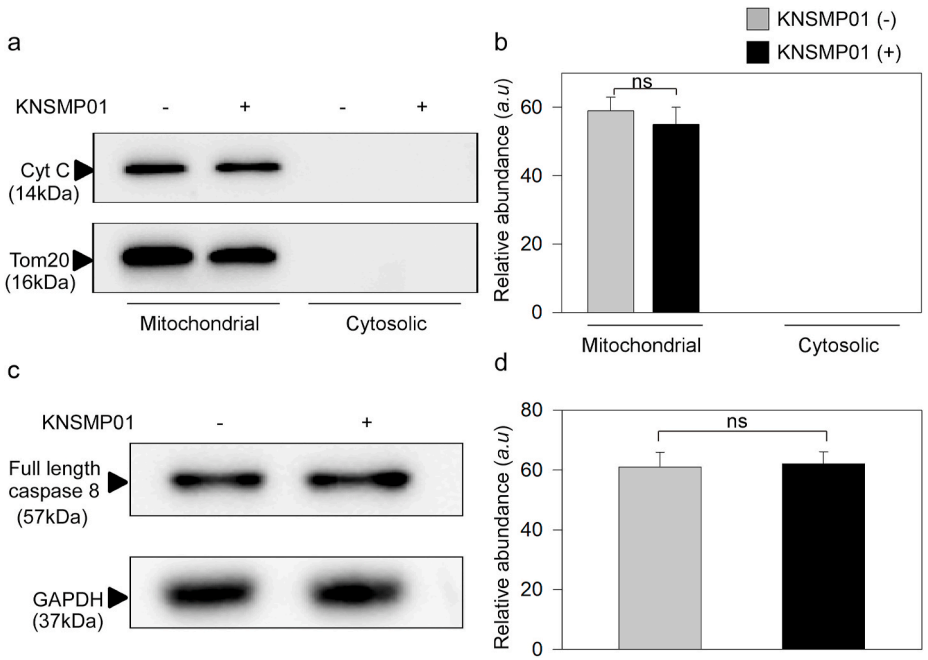

Fig. 5. *In vitro* analysis of KNSMP01 peptide in COS-7 cells for cytochrome C (a) and caspase (c) analysis. Cytochrome C and caspase 8 were normalized with Tom20 and GAPDH respectively and the relative intensity (a.u) was expressed (b, d). Data represents Mean ± SEM and ***P < 0.001, **P < 0.005, *P < 0.01. ns - nonsignificant.

classified and reportedly, about 48% of the genome is yet unknown. SGB categorized that 210 genes are unknown, and 459 genes are under the category of 'general function predicted only'. No significant similarity was found in 1952 protein coding sequences with other organisms. Moreover, 1105 genes are not classified under any category [32]. This study targets the hypothetical genes with unknown function; these genes were earlier predicted to have generic functions. OMPs are one of the main mediators of pathogenesis because the pathogen utilizes these proteins to interact with the host to establish infection. Their surface-exposed loops are considered as bacterial surface antigens which are potential targets to elicit protective immune response. Pathogenic serovars possess conserved OMPs that could be protective immunogens for vaccine development [33]. Approximately 4% of proteomes of *L. interrogans* are OMPs [34] but still many can be identified from the hypothetical genes. The genome search presented 21 hits for OMPs and except one (LIC20087), all other proteins are in chromosome I. These hypothetical genes which encode for the novel OMP family specific proteins were inspected further for identifying novel virulent factors to design the vaccine candidates. The selected genes, LIC11436, LIC11542 and LIC12539 belong to unclassified genes; LIC11334 is categorized under 'function unknown' and LIC11120 is listed in 'general function predicted only'. Genome-wide analysis of *L. interrogans* serovar Lai genome aided in identification of 177 novel membrane associated proteins [35], but the functions have not been explored. But in the present study, the role of these proteins was explored to find effective vaccine targets against leptospirosis. Subunit vaccines are the most promising targets than recombinant proteins or attenuated cells. Very few subunit vaccines have been found to elicit immune response against leptospiral infection. Felix et al. [36], identified 13 antigens which elicited immune response, but only LIC10325 and LIC13059 showed significant protection. LigB (131–645) confers protection against leptospirosis in hamsters [37]. Recently, immunogenic OMPs and their epitopes were predicted and it was found that most of them bind poorly to T-cell alleles [38]. However, KNSMP01 was identified to possess strong affinity with most of the alleles and it could elicit immune response against pathogenic serovars of *Leptospira* spp. Perhaps, the selected antigenic determinants will be effective and novel vaccine targets against leptospirosis. The KNSMP01 peptide which is detected to have a high VaxiJen score has been experimentally validated for its ability to induce immune responses and judging from the results, it can be considered as a potential vaccine candidate for leptospirosis.

The gene, LIC11334 contains SIMPL (signaling molecule that associates with mouse pelle-like kinase) motif that regulates NF-kB activity [39]. It is structurally similar to a major immunodominant antigen, BP26 of *Brucella abortus* [40] and used as a diagnostic marker and as vaccine against brucellosis [41]. So, the novel target, LIC11334 is probably involved in pathogenesis and could also be used as a vaccine candidate. LIC11542 belongs to LptC family and is involved in LPS transport and is therefore essential for envelope biogenesis. LPS is a well-known antigen responsible for pathogenesis and establishment of infection and LIC11542 could transport LPS to elicit immune response. LIC11436 is a LipL45-related protein and LipL45 DNA vaccine was reported to induce high folds of Interleukin-12 and IFN-γ cytokines expression [42]. Hence, LIC11436 was assumed to induce cytokines majorly, interleukins and interferons. LIC12539 is a Sec region protein and occurs only in the genus *Leptospira*. This protein is always encoded between the genes for the YajC and SecD components of the holo-translocon. The interactive partners (YajC and SecD) of the gene of interest required for protein secretion, insertion into membranes and assembly of membrane protein complexes [43]. These proteins have been shown to stimulate Th1 cell-mediated immune response and confer protection against brucellosis [44]. LIC11120 is a putative lipoprotein and belongs to the λ-Bor family. Expression of bor-protein significantly increases the survival of the *E. coli* cells in animal serum. This property is a well-known bacterial virulence determinant. Indeed, it confers virulence in animals [45] and is suggested to have a role in bacterial survival in animal hosts and perhaps in pathogenesis. All the selected proteins closely associated immune responses and/or pathogenesis.

Five virulence associated proteins identified to involve in adhesion of the bacteria to host ECM components and aid in bacterial colonization. Then the pathogen could also react with the host fibrinolytic system and/or with the coagulation cascade components, such as, plasminogen (PLG) and fibrinogen (Fg), respectively. The binding with the PLG generates plasmin (PLA), increasing the proteolytic power of the

bacteria, while the second interferes with clotting in a thrombin-catalyzed reaction, which may promote hemorrhage foci and increase bacterial dissemination. Leptospires penetrate the host tissue by chopping laminin and fibronectin and thereby, the bacteria disseminate and colonize the host. Several cell adhesion proteins were identified in leptospires and their interaction with the host components has been established, but their binding affinity and capability to produce activators differs with respect to the interacting partners [46]. Interaction of leptospires with the host is mostly dependent on the presence of specific domain/motif in the cell adhesive proteins. The protein, LIC11334 possesses typical secondary structure pattern in the SIMPL motif and is established as a major immunodominant antigen [40]. LIC11542 is a transporter protein consisting of a twisted boat structure with thirteen β-sheets as present in LptC, a conserved LPS transport membrane protein [47]. LIC11436 has four Ig-like domains and also possesses Serine, Proline/Lysine rich low complexity regions. Ig and fibronectin domains are common features of most of the surface encoding proteins involved in ligand recognition and cell adhesion [48]. Bacterial proteins containing Ig-like domains (Lig) containing proteins (LigA, LigB, and LigC), GroEL, DnaK and LipL41 were found to be associated with immunogenicity [49]. LIC11436 could elicit immune response as it contains four patches of Ig-like domains and may interact with Fg like other Ig-like domain containing proteins [50]. Although LIC11436 contains Ig-like domains and have better VaxiJen score, it has identical genes in non-pathogenic serovar, *L. biflexa* strain Patoc. LIC 11120 is a pathogen specific protein, which ensures survival of bacteria in animal hosts and is involved in pathogenesis [44]. This protein may exploit the host surface polysaccharide, glucuronate, for bacterial survival and in the process, establish infection. LIC12539 contains twelve transmembrane helices (similar to SecDF structure) and the superimposed structure showed a match with the P1 head (substrate binding site), hinge region and P4 of SecDF [51]; more over it was found to alter the proton gradient in the host. Preprotein translocase subunits such as SecD, SecF and SecY are the major players of protein transport and bacterial secretion system and they are reported as a common drug target for leptospirosis [52]. The LIC11834 [53] and LIC10821 [54] were demonstrated as the first leptospiral proteins interacting with human plasma components (PLG and Fg). Then the present study showed that the five proteins efficiently bind with them and in particular, LIC11334 exhibits efficient binding to PLG with the total docking score of 65.46. This study showed the interaction of putative OMPs with human extracellular components, plasminogen and fibrinogen and was found to mimic the interaction, as reported earlier.

The selected peptide, KNSMP01 was studied *in vitro* to identify its interference on the host cellular metabolism and cellular toxicity. To evaluate the non-toxic effect of KNSMP01 peptide on mammalian cells, MMP, cell viability, cytochrome C release and ROS measurement were performed [55,56]. These findings supported KNSMP01 peptide pertinency for the vaccine formulations due to its non-toxic effect on the mammalian cells. MMP is the ultimate factor responsible for maintaining the physiological function of the cell. The factors which cause changes in the permeability of the mitochondrial membrane result in the release of cytochrome *c* into the cytosol and trigger apoptotic cascade. Caspases are activated by the intrinsic or extrinsic factors that cause necrotic cell death. Measurement of Cytochrome *c* release and caspase activation helps to identify programmed cell death (PCD) [57]. The KNSMP01 peptide does not alter the mitochondrial membrane potential as also does not activate the caspases to cause any PCD. As this peptide does not cause any mitochondrial dysfunction and does alter any cellular metabolism, it is not envisaged to cause cytotoxicity. So, this KNSMP01 peptide is feasible for use as an effective vaccine candidate for leptospirosis.

5. Conclusion

The present study identified unique virulent markers associated with leptospiral infection which can serve as promising targets for the prevention of leptospirosis. They are pathogen specific, non-mammalian homologues and were found to provoke immune response against different pathogenic serovars of leptospires. They were found to interact with host components and thus, could potentially serve as an efficient subunit vaccine. This study has opened new avenues to identify the role of several hypothetical ORFs of *L. interrogans* and will spark to better understanding of the pathogenesis process; thereby, we may to develop preventive measures against this pathogen.

CRediT authorship contribution statement

Muthu Prasad: Conceptualization, Methodology, Software, Data curation, Writing - original draft, Visualization, Investigation, Validation, Writing - review & editing. **Palanisamy Bothammal:** Data curation, Writing - original draft. **Charles Solomon Akino Mercy:** Data curation, Writing - original draft, Software, Validation. **Krishnamoorthi Sumaiya:** Data curation, Writing - original draft. **Perumal Saranya:** Data curation, Writing - original draft. **Gangatharan Muralitharan:** Visualization, Investigation, Supervision. **Kalimuthusamy Natarajaseenivasan:** Conceptualization, Methodology, Software, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2020.104407.

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Silver enhanced nano-gold dot blot immunoassay for leptospirosis

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ABSTRACT

Leptospirosis is a widespread zoonotic disease and lacks in efficient diagnostic tools. In the present study, a nanogold based dot blot immunoassay was developed and evaluated for the detection of leptospirosis in human urine samples. This method was found to be rapid (< 4 h) with higher sensitivity (> 4.2–14.6%) than horse radish peroxidase (HRP) conjugated dot blot assay.

Leptospirosis is a re-emerging public health problem that is generally under diagnosed. The early and accurate diagnosis is still challenging and often leads to a fatal outcome (Bharti et al., 2003; Pappas et al., 2008). Annually, 500,000 cases of severe leptospirosis are reported, with fatality rates < 5 to 30% (WHO, 2011). The misdiagnosis of the disease can damage multiple organs and hence the development of a sensitive and reliable diagnostics is important. The gold standard diagnosis of leptospirosis is the microscopic agglutination test (MAT) that is often used for detection of leptospires. The other serological techniques including enzyme linked immunosorbent assay (ELISA), lepto dipstick, leptodridot and latex agglutination test are used but their sensitivity is dubitable during the acute illness. Advanced diagnostic formats including quantitative PCR and recombinant protein based ELISA have been developed (Raja and Natarajaseenivasan, 2015) but they have several drawbacks, including restricted availability; requiring skilled technicians and lack of uniformity. Moreover, seroconversion must be demonstrated with paired sera to confirm diagnosis. To overcome these limitations, there is a stringent demand for the development of new diagnostic formats for a simple and rapid method to perform in any diagnostic laboratory. Recently, the nanogold based diagnostic format has been evolving and showed significant outputs to resolve the above routes. Chirathaworn et al., 2011 reported detection of *Leptospira* in urine using anti-*Leptospira*-coated gold nanoparticles with high sensitivity. Furthermore, highly sensitive markers can be identified for rapid and prompt diagnostics and these lacunas will be empowered by nanogold based dot blot assay. The present study demonstrated the detection of human leptospirosis using a nanogold conjugate based dot blot immunoassay.

A total of 112 human urine and blood samples was collected from patients with febrile illness and suspected for leptospirosis at Government Hospital of Tiruchirappalli, Tamil Nadu, India between November 2010 and April 2012. Patients fulfilling any of the following criteria were considered as clinically suspected and laboratory confirmed cases of leptospirosis: (1) positive isolation of leptospires from blood/urine, (2) seroconversion or a 4-fold rise in titre in paired serum samples (collected with a mean interval of 23 days) by MAT; and/or (3) a seropositivity (> 1:400) in a crude leptospiral antigen based IgM ELISA. Diagnosis of leptospirosis was confirmed in 48 samples (group I) and 64 did not meet the diagnostic criteria and hence considered as discarded cases of leptospirosis (group II). Similarly, 45 seronegative healthy individuals matching for age (± 5 years) were recruited from patients attending the same hospital for complaints other than febrile illness (group III). Individual urine samples were centrifuged at 12,000 ×*g* for 10 min at room temperature; the supernatant was discarded leaving an aliquot of 50 µl, boiled for 30 min and centrifuged at 1000 ×*g* for 10 min. The supernatant obtained was subjected for protein estimation by bicinchoninic acid (BCA) method (Sigma-Aldrich, St. Louis, MO) and desired concentration was dotted individually onto NC strips (Kanagavel et al., 2017). Individual informed written consent was obtained from both cases and controls. This study was approved by the Institutional Ethics Committee (IEC) of Bharathidasan University, India (Reference No.: DM/2010/101/14).

Several leptospiral proteins are reported to be used for diagnosis and LipL32 and LigA are being widely used for diagnostics. But the

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Table 1
Comparative analysis of Nanogold and HRP conjugated dot blot assay.

Antigen	LipL32		LigA		ArgC		RecA		LruC		LruD	
Conjugates	HRP	NG	HRP	NG	HRP	NG	HRP	NG	HRP	NG	HRP	NG
Sensitivity (%)	91.6	95.8	91.6	95.8	87.5	93.7	91.6	93.7	60.4	75	91.6	93.7
Specificity (%)	92.1	93.7	92.1	95.3	92.1	92.1	92.1	92.1	89	89	85.9	85.9
PPV[a] (%)	89.8	92	89.8	93.8	89.3	90	89.8	90	80.5	83.7	83	83.3
NPV[b] (%)	93.6	96.7	93.6	96.8	90.7	95.1	93.6	95.1	75	82.6	93.2	94.8

HRP: Horseradish Peroxidase; NG: Nanogold.
[a] Positive Predictive Value.
[b] Negative Predictive Value.

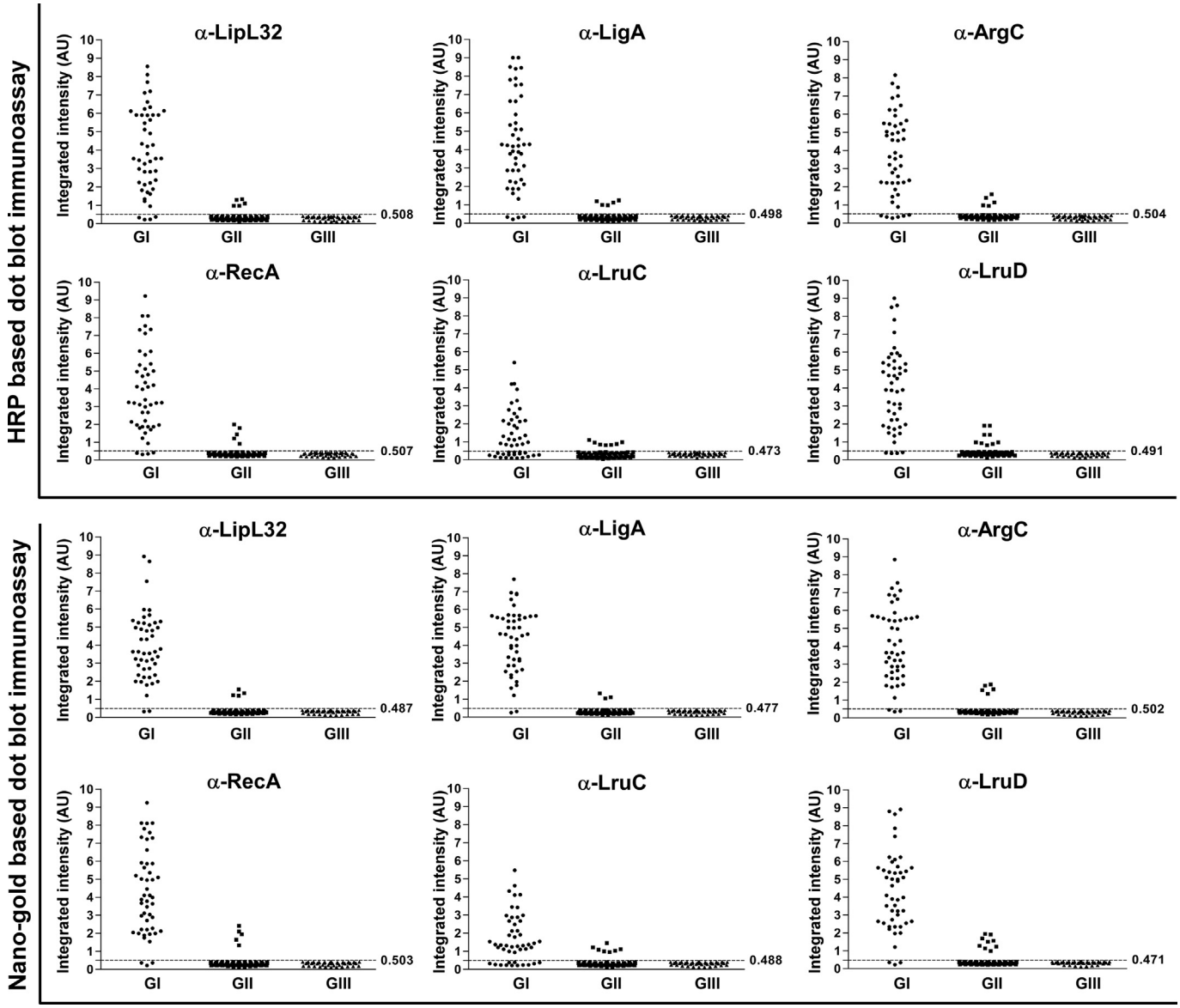

Fig. 1. HRP/nanogold conjugate based dot blot immunoassay graphs showing intensity of dots, GI- Laboratory confirmed cases (Group I), GII- Clinically suspected laboratory negative cases (Group II), GIII- Seronegative healthy controls (Group III). Recombinant proteins LipL32, LigA, ArgC, RecA, LruC, LruD were used for evaluation. A line at all graphs shows cut-off value for healthy control samples (Mean ± 2 SD).

present study has demonstrated yet other less studied proteins such as ArgC and RecA (Raja et al., 2015) along with the leptospiral recombinant proteins, LigA-C (Kanagavel et al., 2014), LipL32 (Vedhagiri et al., 2013), LruC and LruD (Verma et al., 2013) for the diagnostic evaluation. Polyclonal antisera were raised in ~2.5 kg 10–16 weeks of old female New Zealand White rabbits (National Centre for Laboratory Animal Sciences (NCLAS), National Institute of Nutrition, Hyderabad) by subcutaneous administration of 1 µl of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (Sigma, St. Louis, MO) and 100 µg of recombinant proteins adsorbed to aluminum hydroxide (Alhydrogel; Accurate Chemical & Scientific Corp., Westbury, NY) in a total volume of 200 µl. Booster injections containing 100 µg (subcutaneous) of the antigen were

administered 14 and 28 days after the primary immunization. 35 days after the primary immunization blood was taken by cardiac puncture and the clotted blood was centrifuged at 3000 ×*g* and serum was obtained (Raja et al., 2015). The study protocol was approved by the Institutional Animal Ethics Committee (IAEC) of Bharathidasan University (BDU/IAEC/2011/29/29.03.2011).

Urinary shedding of leptospires is common during the early infection (Bal et al., 1994) and hence the present study was done to evaluate the nanogold based dot blot immunoassay with the urine samples. Individual urine samples (40 ml) was centrifuged at 12,000 ×*g* for 10 min at 4 °C and the supernatant was discarded leaving an aliquot of 50 µl, boiled for 10 mins and centrifuged at 1000 ×*g* for 10 min. The supernatant obtained was subjected for protein estimation by bicinchoninic acid (BCA) method (Sigma-Aldrich, St. Louis, MO) and ~1 µg concentration was dotted individually onto NC strips. The supernatant fractions, in 5 µl volumes (1 µg protein), were spotted on nitrocellulose (NC) membrane (0.22 µm) (Merck Millipore, USA) in triplicates and air-dried. Nonspecific binding was blocked by 1% blotting grade blocker (Bio-Rad, USA) in PBS with 0.05% Tween 20 (PBS-T) for 1 h. After washing with PBS-T, NC membranes were incubated with polyclonal sera of specific recombinant proteins in 1:200 dilutions with PBS-T for 1 h followed by PBS-T wash. Protein A-20 nm colloidal gold labeled (1:2000; Sigma-Aldrich, USA) or anti-rabbit IgG HRP conjugate (1:2000; Sigma-Aldrich, USA) was added and incubated for 1 h and washed with PBS-T. The membranes were developed with 4-chloro-1-naphthol and silver enhancer solution (50 mM hydroquinone in citrate buffer pH 3.5; 7.5 µM silver nitrate solution) for HRP and nanogold conjugate, respectively. The reaction was stopped with deionized water and membrane documented using XR + imaging system (Bio-Rad, USA) and the density of each spot was determined by Image J software (National Institute of Health, Bethesda, MD, USA). Densitometric values were obtained as the integrated intensity of all the pixels in a spot excluding the background and were expressed as arbitrary units (AU).

The overall sensitivity and specificity of nanogold and HRP based dot blot is shown in Table 1 and Fig. 1. Representative dot blots of HRP and nanogold conjugate based dot blot immunoassay is shown in Fig. 2. The mean ± 2 standard deviation of the AU values of seronegative healthy individuals was defined as the cutoff values to achieve diagnostic sensitivity and specificity. The nanogold based dot blot for different proteins demonstrated increased sensitivity (75–95.8%) than that of the HRP based dot blots (60.4–92.1%). Interestingly, nanogold based dot blots of proteins ArgC and RecA showed increased sensitivity of 93.7%, over the ArgC and RecA, HRP based dot blots. The widely used markers of leptospirosis, LipL32 and LigA also exhibit high sensitivity with nanogold conjugate immunoassay.

In conclusion, the present study has opened up a new avenue of nano-material based accurate diagnosis method. The nanogold based dot blot immunoassay demonstrated as a rapid, reliable, sensitive method to diagnose the early detection of leptospirosis.

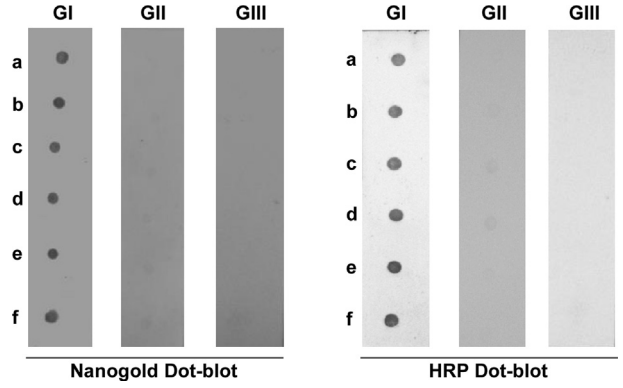

Fig. 2. HRP and nanogold conjugate based dot blot immunoassay.
GI: Laboratory confirmed cases (Group I); GII: Clinically suspected laboratory negative cases (Group II); GIII: Seronegative healthy controls (Group III). a) LipL32; b) LigA; c) ArgC; d) RecA; e) LruC and f) LruD.

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