
“IMMUNOMODULATION BY PARASITIC MACROPHAGE MIGRATION INHIBITORY FACTOR 2 IN TYPE I AND TYPE II DIABETES MELLITUS”

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In the Faculty of Interdisciplinary Science

By

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2025

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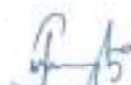
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Ishwar Singh

ABBREVIATIONS

AAM	Alternatively activated Macrophage
AD	Anno Domini
AGC	Automatic Gain Control
ANOVA	Analysis of Variance
APC	Antigen Presenting Cells
AUC	Area under the curve
BC	Before Christ
BMI	Body mass index
B-reg Cells	regulatory B cells
CAM/M1	Classical activation of macrophage
cDNA	Complementary DNA
CD74	Cluster of Differentiation 74
COX2	Cyclooxygenase 2
TLR4	Toll-like receptor 4
CURES	Chennai Urban Rural Epidemiology Study
CXCR2	C-X-C motif chemokine receptor 4.
DDA	Data-Dependent Acquisition
DHI	5,6-Dihydroxyindole
DHICA	5, 6-dihydroxyindole-2-carboxylic acid
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

DTT	dithiothreitol
ELISA	Enzyme Linked Immune Sorbent Assay
ES	Excretory-secretory
ERK1/2	extracellular signal-regulated kinase 1/2
FBG	Fasting blood glucose
FDA	Food and drug administration
FOXP3	forkhead box protein P3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase,
H&E	Hematoxylin and Eosin
HbA1c	Hemoglobin A1C
HCD	Higher-energy collision dissociation
HDL	High density lipid
HFD	High fat diet fed
hMIF	Macrophage Migration Inhibitory Factor
HOMA-IR	Homeostatic Model Assessment of Insulin Resistance
HT	Helminth therapy
IP	Intraperitoneal
IBD	Inflammatory Bowel Disease
IFN- γ	Interferon- γ
IL	Interleukin
IMAC	Immobilized Metal Affinity Chromatography
IMID	Immune-Mediated Inflammatory Diseases

ISO	(S, R)-3-(4-Hydroxyphenyl)-4, 5-dihydro-5-isoxazole acetic acid
ITT	Insulin tolerance test
JNK	Jun amino-terminal kinase
LB	Luria Bertani
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDL	Low density lipid
L-DOPA	l-3,4-dihydroxyphenylalanine
LF	Lymphatic filariasis
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
Max. IT	Maximum Ion Injection Time
Mf	Microfilariae
MHC-I	Major histocompatibility complex class I
MIF	Macrophage Migration Inhibitory Factor
MM-PBSA	Molecular Mechanics Poisson- Boltzmann Area
mRNA	Messenger ribonucleic acid
MS	Multiple Sclerosis
NCBI	National Centre for Biotechnology Information
Ni	Nickel
NO	Nitric oxide
NOD	Non obese diabetic
NPD	Noral pellet diet

OD	Optical Density
OGTT	Glucose Tolerance Test
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PANTHER	Protein ANalysisTHrough Evolutionary Relationships
PD	Proteome Discoverer
pH	Power of Hydrogen
PME	Particle-Mesh Ewald
PRODIGY	Protein binding energy prediction
PTM	Post-Translational Modification
RMSD	Root Mean Square Deviation
RMSF	Root Mean Square Fluctuation
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SD	Standard deviation
SDS	Sodium Do-decyl Sulfate
SEM	Standard Error Measurement
STH	Soil-transmitted helminth
STZ	Streptozotocin
T1DM	Type I diabetes mellitus
TEABC	Triethylammonium bicarbonate buffer
TG	Triglycerides

TGF- β	Transforming Growth Factor- β
Th	T helper
TLR	Toll like Receptors
TMT	Tandem Mass Tagging
TNF- α	Tumor Necrosis Factor- α
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
TRX	Thioredoxin
TSO	Trichuris Ova
Wba-MIF2	Wuchereria bancrofti- Macrophage Migration Inhibitory Factor 2
WHO	World Health Organization

LIST OF ABBREVIATION OF PARASITES AND THEIR
SCIENTIFIC NAME

<i>A. caninum</i>	<i>Ancylostomacanthum</i>
<i>A. ceylanicum</i>	<i>Ancylostomaceylanicum</i>
<i>A. duodenale</i>	<i>Ancylostomaduodenale</i>
<i>A. lumbricoides</i>	<i>Ascarislumbricoides</i>
<i>A. simplex</i>	<i>Anisakis simplex</i>
<i>A. viteae</i>	<i>Acanthocheilonema viteae</i>
<i>A. vitae</i>	<i>Acanthocheilonema vitae</i>
<i>A. ceylanicum</i>	<i>Ancylostomaceylanicum</i>
<i>A. lumbricoides</i>	<i>Ascarislumbricoides</i>
<i>C. elegans</i>	<i>Caenorhabditiselegans</i>
<i>D. immitis</i>	<i>Dirofilariaimmitis</i>
<i>E. vermicularis</i>	<i>Enterobiusvermicularis</i>
<i>F. hepatica</i>	<i>Fasciola hepatica</i>
<i>H. diminuta</i>	<i>Hymenolepsidiminuta</i>
<i>H. polygyrus</i>	<i>Heligmosomoides polygyrus</i>
<i>H. contortus</i>	<i>Haemonchuscontortus</i>
<i>H. polygyrus</i>	<i>Heligmosomoidespolygyrus</i>
<i>H. nana</i>	<i>Hymenolepis nana</i>
<i>L. sigmodontis</i>	<i>Litomosoidessigmodontis</i>
<i>N. americanus</i>	<i>Necatoramericanus</i>

<i>N. brasiliensis</i>	<i>Nippostrongylus brasiliensis</i>
<i>O. volvulus</i>	<i>Onchocerca volvulus</i>
<i>S. haematobium</i>	<i>Schistosoma haematobium</i>
<i>S. mansoni</i>	<i>Schistosoma mansoni</i>
<i>S. stercoralis</i>	<i>Strongyloides stercoralis</i>
<i>S. venezuelensis</i>	<i>Strongyloides venezuelensis</i>
<i>S. mansoni</i> ,	<i>Schistosoma mansoni</i> ,
<i>S. stercoralis</i>	<i>Strongyloides stercoralis</i>
<i>T. spiralis</i>	<i>Trichinella spiralis</i>
<i>T. trichura</i>	<i>Trichuris trichiura</i>
<i>T. crassiceps</i>	<i>Taenia crassiceps</i>
<i>T. suis</i>	<i>Trichuris suis</i>
<i>T. trichiura</i>	<i>Trichuris trichiura</i>
<i>W. bancrofti</i> .	<i>Wuchereria bancrofti</i>

SYMBOLS AND UNITS

Å	Angstrom
kDa	Kilo Dalton
Kg	Kilogram
mg	Milligram
ml	Milliliter
mM	Mili-Molar
nm	Nanometers
ppm	Parts per million
μl	Microliter
α	Alpha
β	Beta
γ	Gamma
°C	Degree centigrade

ABSTRACT

Background

Helminths parasites are considered as a master regulator for the immunomodulation. Macrophage migration inhibitory factor-2 is one among of the homologue's molecules of the host, secreted by the *W. bancrofti*. It is a pleotropic cytokine secreted by different immune cells and plays a significant role in immune mediated disorder. Interestingly Human MIF interacts with insulin and helps in its proper folding and release from the pancreatic β -cells. Immuno-metabolic diseases are the most demanding arena in the medical sciences is to develop new biological molecules. Parasitic Wba-MIF2 could be one promising molecule for the immune mediated diseases. Our aim of the study was to check the immunomodulatory potential of the Wba-MIF2 in diabetic animal model, phosphoproteomics signaling in macrophage cell line and Wba-MIF2-Insulin interacting peptide identification.

Objectives

Objectives of the study are a) Expression and purification of the Wba-MIF2. b) To investigate the immunomodulatory effect of Wba-MIF 2 in diabetic rats.c) To understand the role of Wba-MIF2 in downstream signaling of immune cells using LC-MS/MS based phospho-proteomics approach. d) To identify the Wba-MIF2 epitopes using bioinformatic tools and its in-vitro testing for insulin oxidoreductase activity.

Methodology

Recombinant Wba-MIF2 was expressed using *E.coli*. Wba-MIF2 was purified using Immobilized Metal Affinity Chromatography and Size Exclusion

Chromatography animal models were developed Recombinant Wba-MIF2-25µg, i.p. was used for the treatment. Gene expression, serum cytokines and blood biochemistry parameters and histopathology were assessed. For the downstream signaling studies RAW264.7 cells were used and phosphorylated proteins were analyzed using LC-MS/MS- Orbitrap Fusion Tribrid mass spectrometer at 5 and 15min, and discoverer (PD) version 2.2 software was used for data analysis. Epitope of Wba-MIF2 interacting with human insulin was identified using bioinformatics tools. Epitope was synthesized and tested for its in-vitro oxidoreductase activity.

Results and Discussion

Enzymatic active and endotoxins free recombinant Wba-MIF2 was successfully purified. In T1DM and T2DM animal model, Wba-MIF2 significantly reduces the pro-inflammatory cytokine (IL-1, IL2, IL6, TNF- α , IFN- γ) and enhance the anti-inflammatory cytokines (IL-4 and IL-10) at the gene expression level and serum level as well in vehicle vs Wba-MIF2 treated animal group respectively. Blood biochemistry was also significantly improved in Wba-MIF2 treated group. Histological findings showed a lower density of immune cells in the pancreas of Wba-MIF2-treated animals relative to those treated with vehicle. Recombinant Wba-MIF2 cytokine showed the immunomodulatory effects and improved the diabetes. LCMS-MS results found that 467 proteins were phosphorylated at 1201 sites. Among 1201 phosphorylated sites, 9, 117, 1075, were found on tyrosine (Y), threonine (T), and serine (S) residues respectively. Using bioinformatic tools we identified an epitope of ten amino acids. This peptide had been shown a similar oxidoreductase activity like as wild type Wba-MIF2. Parasitic Wba-MIF2 may be a new biological immunomodulator in immune-metabolic diabetes.

Conclusion

Present research concluded a totally new concept of immunomodulation activity specifically in diabetic animal models. We first time reported that Wba-MIF2 successfully alleviate the diabetes condition using immunomodulation approach.

Keywords: Macrophage migration Inhibitory Factor-2; diabetes; LCMS-MS; enzymatic activity; immunomodulation; *Wuchereria bancrofti*.

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1. INTRODUCTION

1.1 Background

Parasitic helminths, such as nematodes and flat worms (tapeworms and flukes), have beleaguered humans persistently from time immemorial [1]. Helminthic infections are highly prevalent globally [2] and continue to be a globally health concern of the community, especially in developing nations, *Trichuris* and *Ascaris* roundworms are the most common types of intestinal worms. These parasites impact human as well as animal health immensely [3]. Parasitic infections largely vary and interact in various ways with hosts and vectors, possess complex life cycle involving a range of hosts, vectors or intermediary hosts, forms of reproduction, and elicit varied responses [4]. Parasitic nematodes reside within the intestinal tract or specific host tissues, continuously encountering a variety of immune responses [4]. They migrate and undergo developmental changes within their definitive host and usually established a chronic infection [5]. The questions as to how parasites develop and survive, for long durations, and how they stimulate or modulate host immune responses for their advantage, have been the subject of research since long time [6]. They have evolved life-history strategies which facilitate them not only to transmit between spatially and temporally separated spaces of host resources, but also to survive within these environments, successfully. Further, some of the helminths have undergone evolution to systemically invade a host, and developed predilection for specific organs, tissues or host cell types as site to reside, mature and/or even proliferate. To achieve this strategy, they needed to develop complex and unique ways to evade host immune reactions [7]. Helminth parasites prolong their existence within mammalian hosts by altering the host's cellular immune responses [7]. Nematodes employed a survival strategy involving the secretion of specific molecules at the host-

parasite interface. These molecules, including those resembling host cytokines and receptors, possess immune-modulating properties that contribute to the parasite's long-term persistence within the host. These parasitic organisms produce multiple proteins resembling human immune system molecules. One such protein was reported MIF, that plays a lead role in regulation of inflammation [8].

In summary, these parasites adopt an intimate symbiotic and co-evolutionary relationship with hosts, dating back to more than two million years, since humans were hominids and therefore known as 'heirloom parasites' [9]. However, the molecular mechanism involved for the host immune evasion and long-term survival inside the tissue of host is not yet resolved satisfactorily [10], although proteins secreted at various stages of their development like as surface proteins, homologues protein/cytokines and Excretory-Secretory Products (ESPs) and small molecules can significantly influence immune system function [11].

There has been a fundamental shift in how we perceived the relationship between parasites and the health of humans and animals, particularly helminths, from disease-causing creatures to potential sources of biological therapeutics. This change is a result of the newly developed 'Hygiene hypotheses. In western countries, the incidence of autoimmune inflammatory disorders and allergies have increased alarmingly. This can be attributed to the lack of proper orientation of immune system caused by childhood parasitic worm infection [12]. The hygiene hypothesis (first proposed by Strachan) [13] states that reduced contact with infectious agents (particularly those that co-evolved with us) due to improved sanitation, lifestyle changes (including dietary habits) are attributed to the alarming rise in Immune-Mediated Inflammatory Diseases (IMIDs), including metabolic syndrome (e.g., obesity, insulin resistance, hypertension, and atherogenic dyslipidemia) [14, 15, 16].

As evidence for this, worm infection is inversely correlated with T-helper type-1/17 (Th1/17) dependent inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, inflammatory bowel diseases, and T1DM [17]. For the long-term existence, gastrointestinal parasites use Th2 regulation pathway to maintain the health of their host by preventing the formation of lesions due to pro-inflammatory and aggressive immune responses [18]. This shows that mechanism of immune modulation is a common feature of the helminth infection. Studies involving human and animal models have found that helminth infection reduced responsiveness towards standard allergens and auto-antigen development [19]. Research has demonstrated that people with heavy infections of schistosomiasis or filariasis have T-cells that don't react to the specific parasite they're infected with [20]. A recent study suggests filarial worms might protect against allergies and autoimmune diseases, because these parasites can trick the body's immune system, allowing them to live inside people for up to ten years [21]. People without chronic LF infections often have an immune system biased towards anti-inflammatory T-helper type 2 responses. Interestingly, research suggests that live worms are essential for this immune modulation. When people with LF are treated with medication, their immune response returns to normal [22]. Additionally, studies have shown that live worms can suppress the immune system in lab settings [23, 9]. This has led scientists to focus on identifying and studying the ESPS released by these parasites to evaluate anti-inflammatory properties, either individually or together.

Wuchereria bancrofti, a nematode parasite, is a major cause of long-term disabilities such as lymphoedema and hydrocele [24]. Adult filarial parasites are reported to live up to 15 years within the mammalian host [25] and excrete/secrete many different molecules [26]. One among them is a homologue of human host

cytokine, Macrophage migratory Inhibitory Factor (hMIF) [27]. The hMIF, a pleiotropic cytokine with two enzymatic activities viz., tautomerase and oxidoreductase activities, is produced by various cells of the human host. It plays an important role in the immunomodulation during infection and various other disease conditions, such as ulcerative colitis and type 1 diabetes (T1D) [8]. *W. bancrofti* produces two homologues of hMIF viz., Wba-MIF1 and Wba-MIF2 [28, 29]. Earlier, our lab member has cloned, expressed and functionally characterized the MIF-1 and -2 of *W. bancrofti*, and reported that MIF1 and -2 were enzymatically active [30, 31]. Evidence accumulated so far suggests that filarial nematodes modulate the immune system of their hosts for their advantage [30]. As stated above, the “hygiene hypothesis” [31, 32] suggests that lack of exposure to parasites during childhood suppresses the immature immune system, which leads to a higher frequency of allergic and immunological diseases such as asthma, allergic diseases, rheumatoid arthritis, and other diseases which include cardiovascular disease, multiple sclerosis, type 1 diabetes, and inflammatory bowel disease (IBD) [33, 34, 35, 36, 37]. Further, a recent study has shown that hMIF plays a role in the progression of autoimmune T1DM by controlling the functional activity of monocytes/macrophages and T-cells by modulating their secretory capacity of pro- and anti-inflammatory molecules [38], while another study reported that filarial worms can protect against the onset of T1DM in NOD mice mediated by Th2 shift [39]. These findings point towards a strong connection between the immunomodulatory activity of filarial parasite molecules, and protective or pathological effects in diabetes. In this study, we sought to understand the immunomodulatory role of Wba-MIF2 in the Streptozotocin-induced T1DM and T2DM mouse models with the following objectives:

Objectives

- ❖ Optimization of expression and purification of the recombinant Wba-MIF2.
- ❖ To investigate the immunomodulatory affects of Wba-MIF 2 in Type I diabetic animal model.
- ❖ To investigate the immunomodulatory effects of Wba-MIF2 in Type II diabetic animal model.
- ❖ To understand the role of Wba-MIF2 in downstream signaling of immune cells using LC-MS/MS based phospho-proteomics approach.
- ❖ In silico identification of Wba-MIF2 epitopes with insulin oxidoreductase activity.

1.2 Review of Literature

1.2.1 Helminths: Co-evolution as a master of immunomodulation

The earliest known records of parasitic infections come from ancient Egypt and Greece, with evidence dating back to 3000 BC [40]. Additionally, fossilized feces (coprolites) from prehistoric humans contain parasite eggs, suggesting infections were present even earlier, around 1250 AD [41]. However, our relationship with parasitic worms, known as helminths, goes back incredibly far. This connection stretches back hundreds of millions of years, all the way to when the immune system in mammals first started to develop complex defenses [42, 43]. Through millions of years of coevolution, helminths have developed complex strategies to influence the human immune system. These strategies reduce harm to the host by minimizing tissue damage and preventing an excessive immune response. At the same time, these strategies allow the parasites to stay inside the host for extended periods, ensuring their survival and reproduction [11, 44]. The success of this adaptation is evident in the widespread presence of chronic helminth infections around the world, affecting over 2 billion people [45]. Generally, when harmful bacteria or viruses invade the body, the immune system responds as pro-inflammatory and T-helper 1 (Th1) and Th17 (Th1/Th17) type responses. The innate immune system mainly composed of dendritic cells and CAM/M1 and as a defense these cells release pro-inflammatory cytokines e.g. TNFs, IL-1, IL-2, IL-6. This triggers the expansion of Th1 cells to excrete the pro-inflammatory response by mediating IFN- γ , which further activates the immune cells [46], in clearance of pathogen and resolution of innate immune system. Contrarily, when helminth parasite infects the host, it modulates the Th1 immune towards Th2/ Treg as an anti-inflammatory immune response [47, 48]. This stimulates the activation of B-regs, basophils, eosinophils, mast cells and resulting

activation of AAM/M2 and immature dendritic cells that deviates the immune system towards anti-inflammatory [49, 50, 51, 52]. Furthermore, AAM cells are more dominant in helminths infection and inhibit the pro-inflammatory activity against multicellular helminths infection, and, if fail to modulate, resulted in collateral damage of organ and tissue of the host. Additionally, helminth-induced Th2 and AAM cells triggers wound repair process for damaged tissue during parasite migration [44, 53, 54]. During chronic infection, differentiated Treg cells start secreting IL-10 and TGF- β , further characterized by inhibition of Th1 responses [55, 56]. This type of mechanism can interplay significant role in the improvement of wound healing and prevention of tissue fibrosis, mediated by AAM [53].

A pathogenesis of autoimmune diseases involved multifactorial and complex interplay of immune, environment and genetic factors that finally contributes varied clinical symptoms. Despite that, most of the autoimmune disorder showed commonalities of pro-inflammatory cytokine (IL-1 β , IL-2, IL-6, IFN- γ , TNF- α) production from CAMs and DCs cells [52]. In most of the autoimmune diseases first and foremost infiltration of CAM cells were reported [57]. Pro-inflammatory cytokine burst and Th1/Th17 activation and expansion play a major role in tissue damage [58]. Previous reports showed that elimination of predominant AAMs cells using silica or dichloromethylene diphosphonate, abolished the T1DM and improved the clinical symptoms of Multiple sclerosis (MS) in animal models [59, 60, 61]. This confirms that via Th1/Th17 differentiation, CAMs plays a central role in autoimmune disorders. Infiltrated neutrophils and CAM established a pro-inflammatory niche that induces Th1-cells for CD8⁺ T cell subpopulation, which damage auto-antigen expressing cells, such as auto-antigen expressing pancreatic β cells in T1DM [62]. A lineage of Th17/CD4⁺ T cells via production of IL-6, IL-17, and TGF- β cytokines

recognised as inducer and progression of autoimmune and inflammation disorders [17, 63, 65, 66]. Outcomes of the previous studies showed that an inverse correlation between sudden incident of autoimmune disorder and decrease in the helminths prevalence and immunological findings explains an antagonistic effect of the Th1/Th17 and Th2/Treg in helminths infected population as shown in Figure 1, [17, 46, 67]. Westernised populations of Europe and Australia are more prone to autoimmune disorders but incident remains low in under developed and developing countries in which exposure of concomitant helminth parasites due to low/moderate hygiene conditions [68, 69, 70, 71].

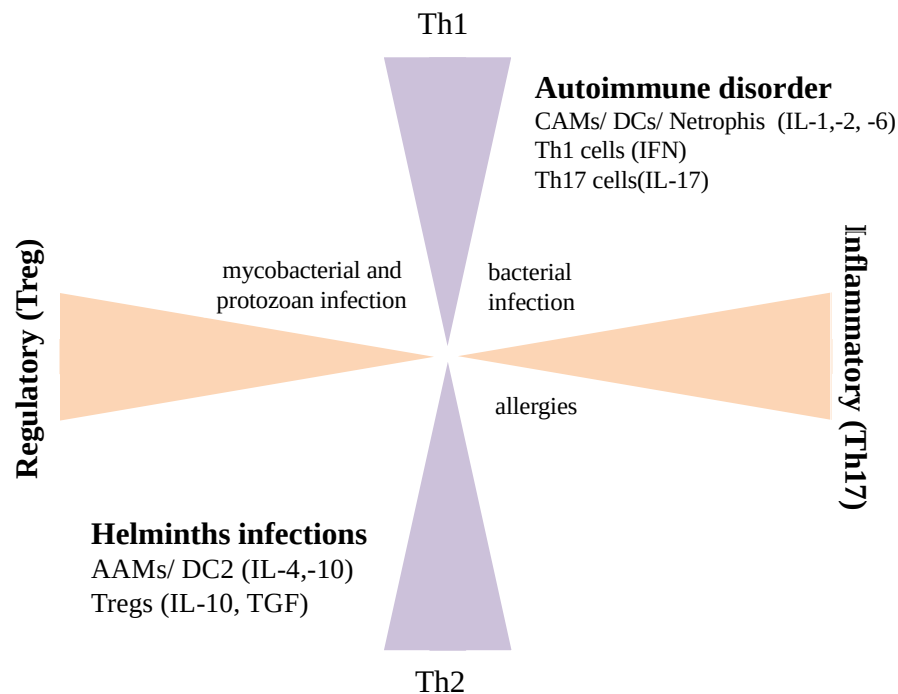


Figure 1: Immuno modulated axes of the activated CD4⁺ T cells. CD4⁺ T cell functionally categorized in four major subphenotypes: Thelper 1 (Th1), T-helper 17 (Th17), T-helper 2 (Th2), and T-regulatory (Treg). Each subpopulation mediates immune based pathology development against relevant pathogen (notably allergy and autoimmune disease) that accommodates between respective arms. Autoimmune disorders are mediating the Th1/Th17 CAMs/ DCs/ neutrophils axes and release of

pro-inflammatory cytokines. Indeed, Th1/Treg immune responses suppress the Th/Th17 responses and *vice-versa* characterised by anti-inflammatory cells (AAMs, Treg) and release of IL-10 and TGF beta cytokines [46].

Interestingly, a significant downfall of MS disease was observed when helminths infection rate exceeds more than 10% in any reported population [35, 72], and showed reported fewer neurological disorders. Th2/Treg skewed phenotype was more prominent in helminths infected areas, characterized with more secretion of TGF- β and IL-10, whilst level of IFN- γ and IL-12 was decreased [73, 74], shown in Figure 2 [30].

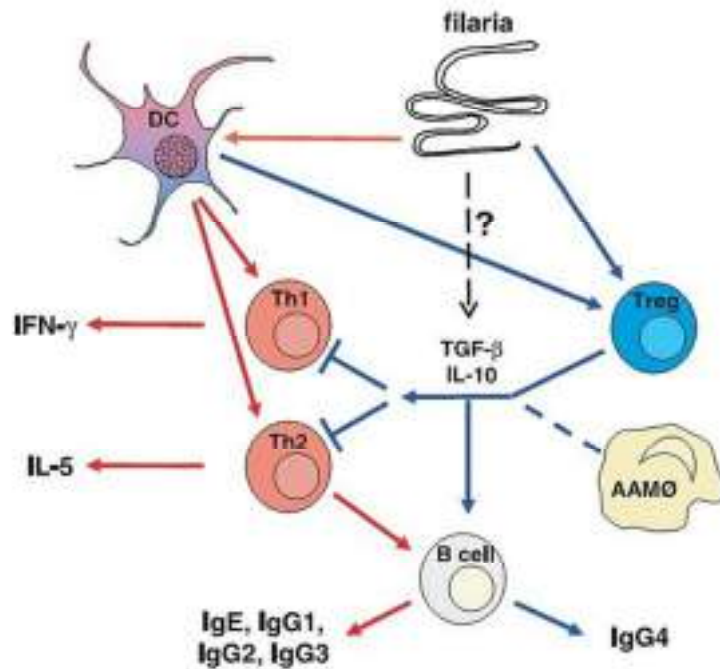


Figure 2: The Filarial parasite regulated host immune system and its modulation [30].

This supports that long-term association of helminths parasites with human host is strongly associated with its immune system development. During the analysis of 52 human populations of helminth infected endemic area, genes of 91 cytokines were showing highest polymorphism with single nucleotide in interleukin gene [75].

This indicates that polymorphism of immune-mediated genes was developed due to selective immune- modulation mechanism of the helminth. Furthermore, the population of helminths endemic area was immune compromised against the Th1 based vaccine due to existing helminths infection [20, 76]. Indeed, anti-helminths drug improves the vaccination responses; this mechanism further supports the idea that helminth parasites are master of immune modulation [77].

1.2.2 Live helminths therapy: Limitations and Challenges

While, earlier large-scale clinical trials haven't shown positive results, compared to smaller research studies, endemic populations and experimental animal testing, that provide compelling evidence to continue exploring 'worm therapy' as a potential preventative or therapeutic approach for immune metabolic and autoimmune diseases. However, option of the live parasite as a therapy is not optimal and patient compliance was reported very poor [78]. In live parasitic therapy, feeding and its migratory activities may cause some serious adverse effects such as subsequent infection and pro-inflammatory cytokine exacerbation [79, 80].

Additionally, a fear of co-infections, repeated dosage with high numbers of eggs or larva, problem of scale-up, finding pathogen free intermediate host, variability and stability of the parasites/ and its eggs, specificity with host are the limiting factor for helminth therapy as a whole [11, 81, 82]. As previously discussed, helminth endemic population is compromised with protective (Th1/ Th17) immune responses that hinder the innate immune responses against viral/bacterial and vaccination as well [79, 83]. Thus, risk benefits ratio of helminths therapy may be very high for the individuals (such as the elderly person, children, and pregnant women) those are already immune compromised [84, 85, 86, 87]. Hence, treatment based on live

helminths is no more realistic. A safer alternative therapy using ESPs/ homologues cytokines molecules of helminths could be novel approach for the new biological development. Individual molecules can be easily studied for its mechanism of action, toxicity, immunogenicity, and efficacy and that can be synthesized either recombinantly or chemically [88]. Though, previous research reported that individual molecules of the helminths have potent immunomodulatory activity and can be a promising as therapeutic molecule [21].

1.2.3 Helminth's ESPs: A proven modulators of immunity

In the mammalian host, ESPs and homologues molecules of the helminth parasite plays a main role for the invasion and its long-term survival. Throughout the growth and reproduction of helminths, ESPs performs various functions such as collagen tissue cleavage for migratory moments [89] and break down of red blood cells into nutrients [90]. Previous reports showed that ESPs of the helminths have the immunomodulatory capability, akin to live helminths [23, 91, 92]. ESPs ameliorated the symptoms of the autoimmune diseases in several animal models (Table 1).

Likewise, amelioration is mainly associated with an induction of Treg/Th2 type responses and inhibition of the Th1/ T17 immune responses that plays a central role in disease development. Shifting of the Th2 from Th1 type immune responses are governed by ESPs mainly via macrophage cells. For example, cystatin of *A. vitae* induce macrophage-based IL-10 production and improve the colon inflammation [93]. ES-62 of *A. vitae* inhibited pro-inflammatory cytokine release from CAM cells [94, 95]. This report concluded that ESPs differentiate CAM towards AAM and initiate the inhibition of Th1/Th17 responses, like infection of live helminths. Consequently, similar finding supports that pro-inflammatory cytokines are

diminished, and anti-inflammatory cytokines was enhanced in Wba-MIF2 treated T1DM animals [96]. Therefore, elucidation of mechanisms of action of Wba-MIF2 can be a highly advantageous for the development of novel therapeutics for immune metabolic and autoimmune disorders.

Table. 1. Use of helminth ESPs as therapeutic biologicals in animal models for immune mediated disorders [100-108].

Sr. No.	Diseases	Helminths/ Parasites	Name of ESPs	Study outcomes	Ref.
1	RA	<i>A. viteae</i>	ES-62	Severity of arthritis- reduced Th2 cytokines-increased	[23]
				Bregs cells restoration in joints	
				Th1 and Th17cytokine-decreases	
				collagen-mediated pro-inflammatory cytokine-reduction	
2	T1DM	<i>D. immitis</i>	DiAg	anti-inflammatory milieu- increased islet mediated antigen-reduced pancreatic insulitis-reduced	[91]
3	IBD	<i>A. caninum</i>	AcES	Th1 cytokine-reduced Th2 cytokine-increased	[92]
		<i>A. ceylanicum</i>	ESPs	Th1/Th2 cytokine-reduced	[90]
		<i>T. spiralis</i>	ESPs	Th1/Th2 cytokine-reduced Treg cytokine- reduced	[89]
			AV17/cystatin	Th1 cytokine-reduced Th2 cytokine-increased	[93]
		<i>H. polygyrus</i>	ESPs (55kDa)	Th1cytokine- reduced Treg cytokine- increased Pathology- reduced Weight loss- reduced	[91]
		<i>H. diminuta</i>	ESPs/ higher molecular wt.	Th1 cytokine-reduced Th2 cytokine-increased Treg cytokine- increased	[23]

Abbreviations used, RA: Rheumatoid arthritis; T1DM: Type 1 diabetes mellitus, IBD: Irritable Bowel Syndrome; ES-62: Excretory secretory-62; ESPs: Excretory–secretory products.

1.2.4 *Wuchereria bancrofti* life cycle

Filarial life cycle is digenetic and very complicated, involving human and mosquitoes as a final host and intermediate host respectively [97]. It resided in the lymphatic lumen of the host and average life span is 7 to 10 years [98, 99]. Lymphatic lumen is the mating place for male and female parasites, and after mating they produce millions of microfilariae and known as first stage larvae, those survived up to one year, until not fed by mosquitoes [100]. Microfilariae of *Wuchereria bancrofti* have sheathed structure. It sways from lymphatic system to peripheral blood vessels, in night times, and this schedule is matching with the mosquito's high biting activity/ time [101]. During female mosquito bite, microfilariae travels from the host peripheral blood to mosquitoes' digestive system. After reaching the mosquito's stomach microfilaria shed their sheath and travel to flight muscles of the thoracic region and developed as a first stage larvae called L1. After that it molts in sausage shaped; called second stage larvae (L2), and again molts in slender, highly active and ready to infective stage called third stage larvae (L3). From L1 to L3 stage it takes 10-12 days for molting. During a blood meal, mosquitoes transmit infective larvae (L3), via proboscis route to the host skin. Through bite wound, L3 larvae enters in the human host and in 2-4 weeks' time it molts into forth stage larvae (L4). It drifts towards lymphatic node and vessels for its final development to adult worm; it takes 3- 6 months to fully develop into reproductive adult worm (Figure 3), [97]. Female can actively reproduce more than five years [102].

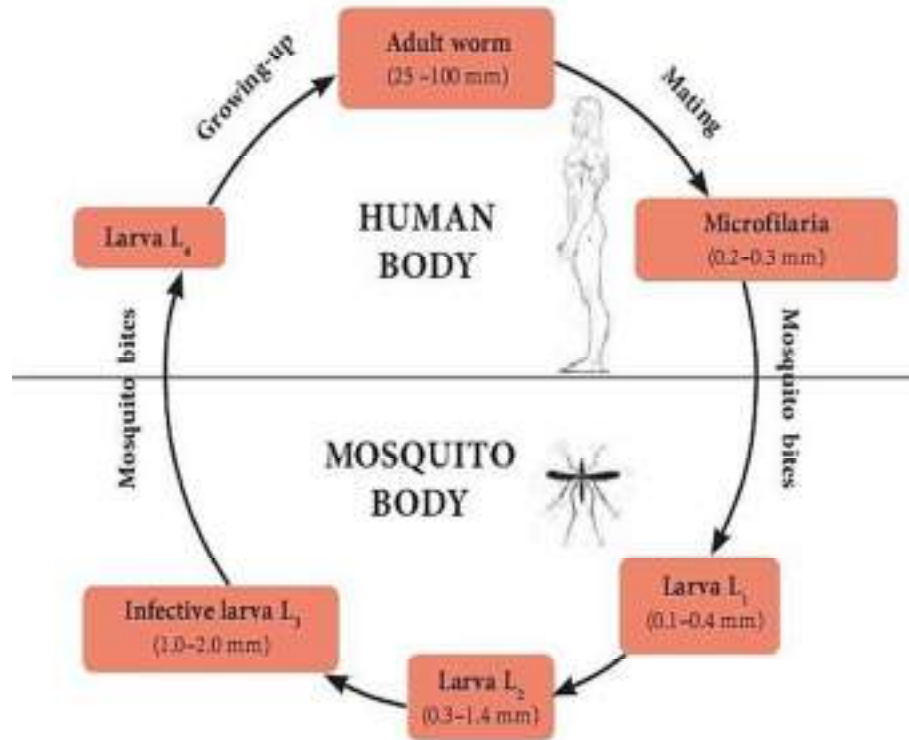


Figure 3:Life cycle of *Wuchereria bancrofti* parasite [97].

1.2.5 T1DM and T2DM and inflammation

Approximately 35 years ago, research revealed a link between inflammation, obesity and insulin resistance. This discovery involved finding of elevated levels of a molecule called TNF-alpha, a pro-inflammatory cytokine, in the fatty tissue of obese people [103]. Later studies have revealed adipose tissue to be a crucial endocrine organ. It secretes various signaling molecules, including pro and anti-inflammatory cytokines, and adipokines viz leptin and adiponectin. Later finding revealed that adipocytes secrete some cytokines, but tissue resident macrophage cells play major role in cytokine burst during inflammation [104].

In obese individuals necrosed adipocyte cells attract more “classically activated macrophage” (CAM) or “type 1 macrophage” that triggers the more pro-inflammatory cytokines releases. Consequently, this attributes the over nutrition and fast growth of adipose tissue, low blood flow and resulting hypoxia causes adipocytes death. Additionally, high concentrations of free fatty acids have the potential to directly trigger cell death [105]. Contrarily, in lean individuals, niche of adipose tissue is more prominent with the “alternatively activated macrophage” (AAM) or “the type 2 macrophage”. These AAMs are secreting more anti-inflammatory cytokines viz IL-4 [105]. Cytokine released by these CAMs or AAMs reveals a strong association with insulin function and glucose metabolism in adipose tissue. AAMs play a major role of energyhomeostasis in adipose tissue via IL-4 signaling. For sustaining the healthy and anti-inflammatoryenvironment of adipose tissue, AAMs down regulates the CAMs via release of IL-10 [105]. Furthermore, adaptive immune system plays a crucial role by increasing ratio of Treg over TH1 cells for maintaining of anti-inflammatory niche. Interestingly, infiltration of eosinophil in adipose tissue gives a strong stimulation for the AAMs production and longer sustainability in the tissue [105]. Similarly, in helminth infected mice, increased eosinophils sustained the AAMs and improved the insulin sensitivity of adipose tissue. Hence, parasite infection is more associated with AAMs and TH2 immunity that influenced the anti-inflammatory cytokine production IL-10 and IL-4. This may improve glucose tolerance and a decreased incidence of T2DM [105]. In the development of T1DM and T2DM role of pancreatic immune cells interplay has been shown in Figure 4 [106].

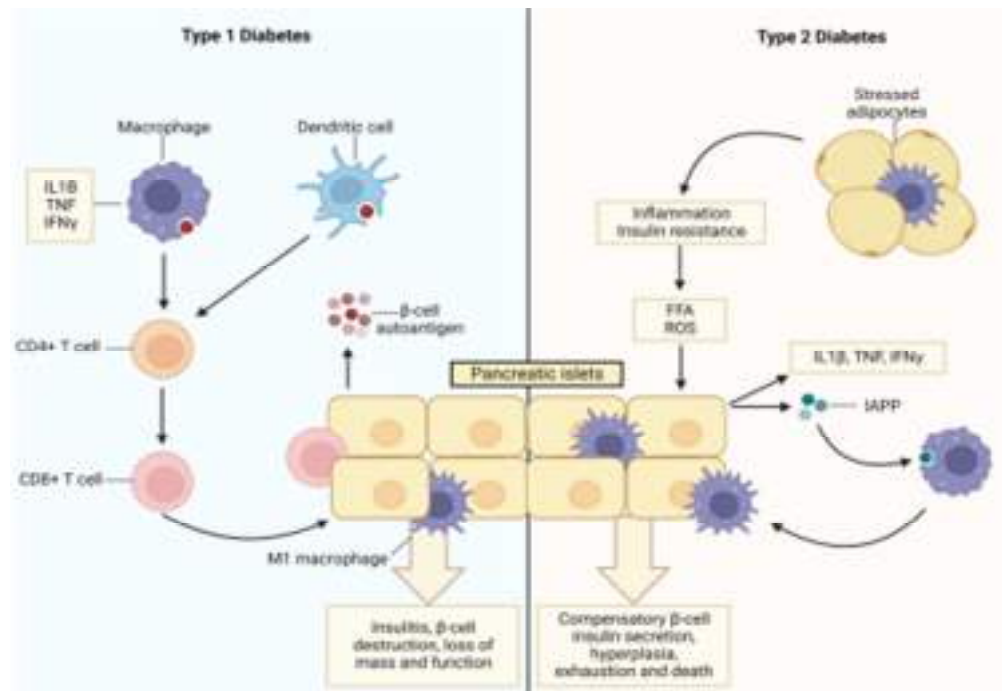


Figure 4: Inflammation of pancreatic islet and death of β -cells underlie type 1 and 2 diabetes. In T1DM conditions, apoptotic beta-cells generate naive and auto-antigens that activates antigen presenting cells dendritic cells and macrophages cells. This triggers the immune system to attack healthy tissues. Activation of macrophagemediated CD4+ T helper cells further activates cytotoxic CD8+T cells. These cytotoxic CD8+ cells migrate to the pancreatic islets (a process called insulinitis) and damage β -cells. During insulinitis CAM and T cells release pro-inflammatory cytokines, IL-1 β , IFN- γ and TNF- α , which accelerate the β -cells death. Additionally, β -cells under stress condition increases MHC-I and II, and co-inducing molecules enabled them act as an APC, which in turn leads to the activation and expansion of T cells that attack the body's own tissues. In T2DM condition, increased FFA and adipocytes inflammation increases β -cells stress and induces the secretion of pro-inflammatory cytokines. This process, along with the production of islet amyloid polypeptide, resembles the events happening within pancreatic islets in type 1 diabetes (T1D). This modulates the classical activation of macrophages in pancreatic islet and release of pro-inflammatory cytokines (IL-1 β , IFN- γ and TNF- α). Stressed adipocytes exacerbate the inflammation in peripheral tissue and resulting in insulin resistance. Consequently, in response to high blood glucose, β -cells overdrive to produce more insulin which leads to compensatory hyperplasia and death. This decline in β -cell number and a hallmark of T2DM [106].

1.2.6 Hygiene Hypothesis -Helminths v/s inflammatory diseases

Epidemiological data have reported that episode of allergic reactions (asthma, rhinitis, and eczema,) were higher in economically advanced countries as compared to economically low or under developed countries [10, 108, 109]. This concluded that higher attribute of living standard and hygienic condition of developed countries resulting in the less exposure of childhood infection, and more dysregulated immune system, collectively known as "hygiene hypothesis" [110, 111]. Research indicated that helminth infections play a crucial role in the reduction of allergic reaction in developing countries population [112, 113]. Several studies suggest that cohorts infected with helminths tend to have a lower likelihood of allergies, especially children infected with *S. mansoni* [114, 115, 116] or *S. haematobium* [117, 118]. Observational studies supported a link between parasitic infections and reduced allergic reactions, mass drug administration of anthelmintic leads to sensitivity of skin test in children [119, 120]. Studies have shown a link between reduced allergic reactions and the secretion of IL-10 by T-cells when exposed to parasite antigens [118]. Basophils are known to be important cells involved in allergic reactions. However, research suggests that people infected with parasitic worms (helminths), basophils become less responsive to triggers, regardless of whether those triggers involve immunoglobulin E (IgE) or not [121]. Similarly, research on mice suggested that unresponsiveness may be linked to IL-10 [122]. Some studies suggested that gastrointestinal nematode infections might reduce allergies [123]. A large meta-analysis found that exposure to certain types of roundworms (*Ascaris* and *Trichuris*) may be linked to a lower risk of skin allergies. However, another analysis focused specifically on asthma and found that only hookworm infections seemed to offer protection, while exposure to *Ascaris* might increase the risk [124]. Allergies are

generally more common than autoimmune diseases, and few epidemiological studies have been performed to find a link between prevalence of helminth infection and autoimmunity disorders. Studies have shown an inverse correlation among the prevalence of MS and the presence of helminth infections in various geographical regions. However, rised MS cases in various nations may be influenced with other factors those were beyond infectious diseases [35]. A study in Argentina provided stronger evidence between helminth infections and MS protection. The study involved patients diagnosed with MS who later became infected with *T. trichiura*, *H. nana*, *E. vermicularis*, *S. esstercolaris*, and *A. lumbricoides*. Interestingly, these patients experienced a higher rate of remission and fewer relapses compared to uninfected patients with similar disease severity [125]. Furthermore, treating a subgroup of infected individuals with anthelmintic drugs led to a remission of symptoms but, not observed in those who remained infected but weren't treated with the drugs [74]. An interesting finding was reported that patient of helminth infection and reduced disease activity had higher levels of B-reg, T-reg immune cells and TGF- β and IL-10 as compared to healthy controls [125, 73].

1.2.7 Trials of helminths therapy– an overview

The concept of employing helminths as a treatment for inflammatory conditions has been explored for several decades. This interests began after a case report of self-infection of hookworms (*N. americanus*) seemed to eliminate hay fever symptoms in an individual [126]. There are ongoing reports of people with conditions like colitis finding potential benefits from whipworm (*T. trichiura*). However, more research is needed for validation of these reports to understand the safety and efficacy of such treatments [127]. Studies investigated the effectiveness of live helminth therapy gained momentum in 2006 when ova of *T. suis* was used in controlled trials.

This parasite establishes a short-lived infection in humans and is considered potentially less harmful compared to helminth species that naturally infect humans. Notably, the administration of *T. suis* ova (TSO) successfully alleviated idiopathic bowel dysfunction in a colony of macaque monkeys [82]. Interestingly, neither study observed that elicit of FOXP3⁺ regulatory cells were in association of *Trichuris* spp. infection. Tregs are typically more abundant in inflamed tissues compared to healthy ones and control groups. Initial studies using TSO for ulcerative colitis and Crohn's disease showed encouraging results [128, 129]. These positive outcomes led to larger clinical trials investigating its effectiveness not just for inflammatory bowel disease (IBD), but also for conditions like multiple sclerosis (MS) and rhinitis [130, 88]. The potential applications of helminth therapy have even been explored in the context of autism disorder [72]. Few studies posted an inconclusive result of MS and inflammatory IBD diseases [21, 131, 132], while some research suggests a minimal [133] but statistically insignificant results. Interestingly, the studies found that treating patients of celiac disease using hookworm therapy led to a notable improvement in their gluten tolerance. This improvement coincided with a significant rise in FOXP3⁺ regulatory T cells of intraepithelial lymphocytes [134], however, no such increase was observed in the blood [135]. Experts are looking into why helminth therapy hasn't been as effective as initially hoped, whether due to age factors, high intensity or long-term infection may play a role [136], and most uncertainty in the parasite selection either pigworm or human parasite respond significantly to the immune system [137].

Till date, research related to immune response and experimental human helminth infections hasn't revealed a single, unifying pathway for immune regulation. Surprisingly, there's limited research that suggested a key role of regulatory T-cells.

Helminth therapy offers valuable insights for treating human inflammatory disorders, but further research is needed to ensure its safety and efficacy [137].

1.2.8 A pleiotropic cytokine: MIF

In late 1960s, independent research by David and Bloom reported MIF from the T lymphocytes supernatants showed inhibition of macrophage random migration [138, 139, 140]. After 30 years of its discovery, MIF structure was characterized using nuclear magnetic resonance (NMR) spectroscopy, X-ray crystallization, and different biochemical methods (Figure 5).

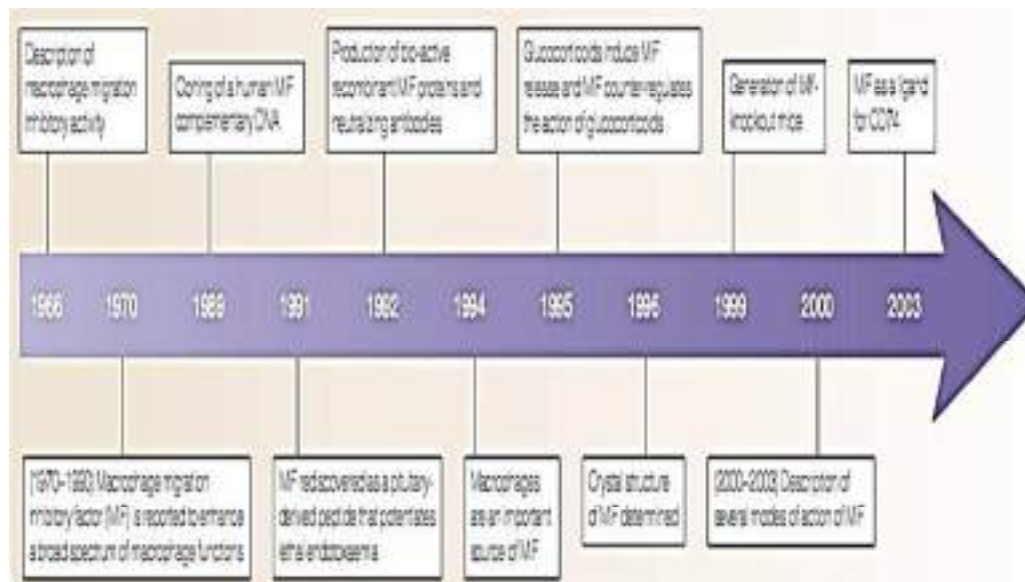


Figure 5: Timeline/The history of MIF [141].

Ambiguity in the MIF structure is reported such as crystallography revealed its trimeric structure but it's unclear whether MIF functions as a monomer, dimer, or trimer under physiological conditions [142]. MIF is a versatile signaling molecule ubiquitous released by various non-immune and immune cells such as keratinocytes, and eosinophils, cells lining internal organs (endodermal cells), macrophages and T-cells [143, 144]. Pituitary gland also secreted MIF and it acts like endocrine factor or

hormone molecules [145]. MIF exhibits two interesting catalytic activities: oxidoreductase and tautomerase [14, 147]. However, due to these enzymatic functions, MIF is also referred to as a redoxkine or cytozyme. MIF critically regulate pro-inflammatory function of lymphocytes, macrophage cell and other endocrine functions. It counter regulates the anti-inflammatory and immune-suppressive function of the glucocorticoid [148, 149]. MIF follows a non-canonical pathways and leader less expression due to absence of N- terminal sequences for endoplasmic reticulum internalization [149].

1.2.9 Mechanisms of action: hMIF and parasitic MIF

While scientists have a good grasp of MIF's general role in biology, but exactly how it functions remains unclear. Surprisingly, the structure homology of MIF is distinct from other cytokines [150, 151]. Beyond its roles in physiological processes, MIF also exhibits enzymatic activity. It catalyzes as both a phenylpyruvate tautomerase and a thiol-protein oxidoreductase. Research suggested that proline residue of N-terminal in MIF plays a significant role of tautomerase reaction, although the exact mechanism remains unclear. While researchers have investigated the potential link between MIF's tautomerase activity and its biological functions, a definitive connection hasn't been fully established [152]. A study in 1998 found that MIF can act as an oxidoreductase enzyme. This activity is carried out by a specific region of the protein called the cysteine-alanine-leucine-cysteine (CALC) active site. The CALC active site forms a reversible bond within the MIF molecule between two cysteine residues, specifically at positions 57 and 60 [147]. Literature reported that, biological functions of MIF are more connected to the oxidoreductase activity instead of tautomerase activity. Oxidoreductase activity significantly influences apoptosis inhibition, redox homeostasis, activation of macrophage/ monocytes and respective

MIF2 binding sites [153]. However, its catalytic activity and biological functions are associated with various signaling pathways [154].

MIF works through CD74-CD44 receptor complex of cell surfaces; this receptor complex also influences the induction of classical receptor-dependent MAPK pathways [155]. MIF-CD74/CD44 binding triggers the stimulations of ERK1 and ERK2, which are members of the MAP kinase family. Activation of the ERK1/ERK2 further phosphorylates the A2 (PLA2) and other cytosolic proteins those are responsible for pro-inflammatory cascade. This further triggers the arachidonic acid formation, which initiate the synthesis of prostaglandins and leukotrienes. This induced the activation of JNK, that in turn increases the production of proinflammatory mRNA and proteins [156]. Further research found that MIF interacts with CD74 and CXCR2 receptor induce a series of cellular responses including activation of MAPK signaling pathways, calcium influx and integrin activation. Subsequently, MIF activates the ETS transcription factors that increase the expression of TLR4. TLR4 helps in detection of harmful toxins (endotoxins) from invasive microbes and strengthens the immune system's ability to fight infections [157]. When nitric oxide (NO) is suppressed, it leads to the accumulation of the p53 protein this triggers apoptosis. Interestingly, the inhibition of p53 is dependent on the activation of several signaling pathways mediated by MIF, PLA2, including ERK1/2, and cyclooxygenase 2 (COX2) figure 6 [158]. Unexpectedly, in one mechanism of human MIF is essential for proper immune function, but in another it contributes to inflammation in diseases like RA, asthma, inflammatory bowel disease and psoriasis [159]. It modulated the pathways of immune cells viz. macrophage, NK-cell, T cell and neutrophils towards pro-inflammatory side and activates the synthesis of immunoglobulin [160, 161, 162]. Surprisingly, parasitic MIF cytokine [13] also

shared the same CD74-CD44 receptor complex for its signaling transduction [164]. On the contrary side, it enhanced the anti-inflammatory immune system via IL-10, TGF- β releases and T-reg activation. Parasitic MIF has shown a promising effect in immune mediated diseases such as colitis, T1DM, and other allergic inflammation diseases [159, 165].

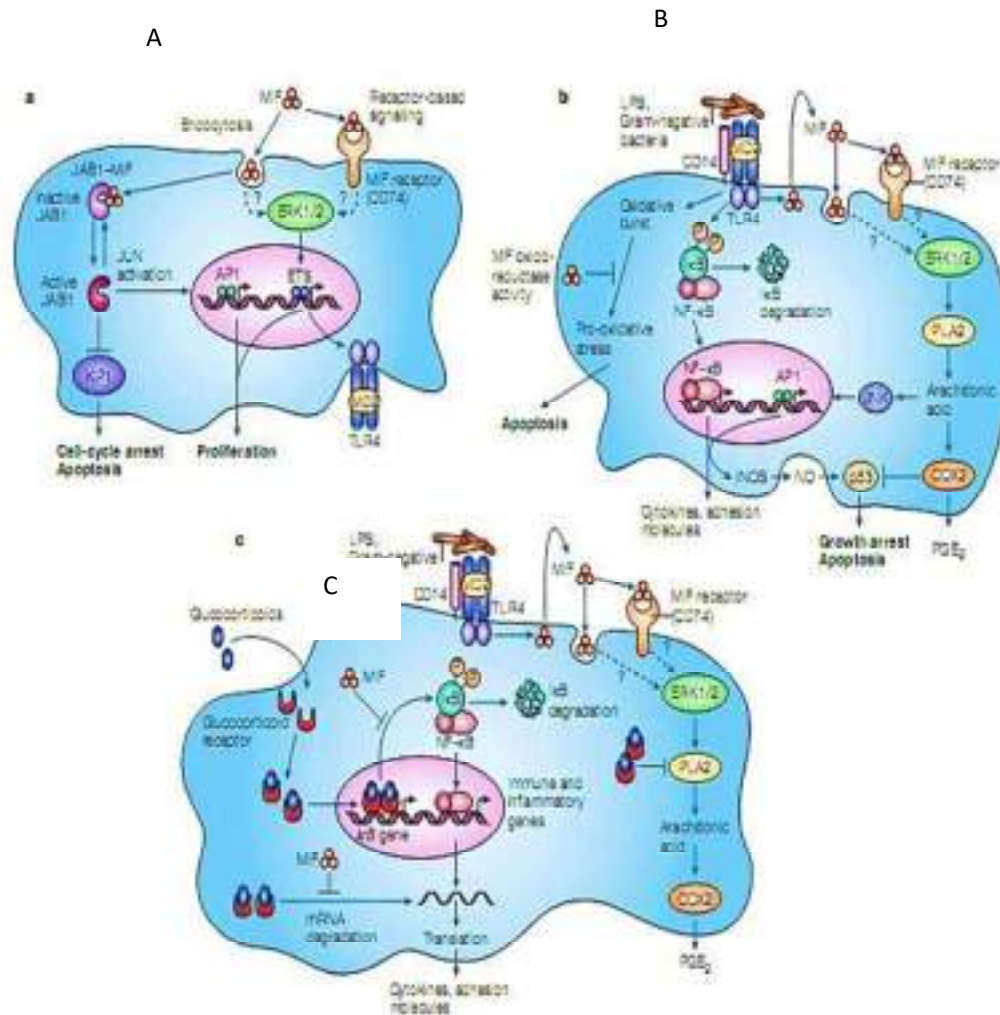


Figure 6: Human MIF regulated cell signaling pathways [141].

1.2.10 Biocatalytic activities of MIF: Tautomerase and Oxidoreductase

MIF cytokine plays a significant contribution in immunomodulatory reaction of adaptive and innate immune system of the host. As previously reported, it has two

biocatalytic activities viz., oxidoreductase activity dependent on Cys-Ala-Leu-Cys (CALC) thiol group and tautomerase activity due to Proline amino acid at second position of MIF [152]. Presently, for the elucidation of MIF mechanism of action oxidoreductase activity is in prime leads [166].

In a previous study, a protein with oxidoreductase activity, isolated from adult leukemia T-cells, was found to exhibit functions like chemokines [167]. While the link between MIF's enzymatic activity and its immune functions remains unclear, a study found that mutating a specific amino acid (Cys60 to Ser) in MIF eliminated its ability to counteract glucocorticoids and its other functions. Interestingly, mutating a different cysteine (Cys81) located far away from the enzyme's active site had no oxidoreductase activity link behaves same as wild type MIF [147, 168]. These results imply a direct role of oxidoreductase activity in certain immunological functions of MIF. However, even though Pro2 impart a significant action in the enzymatic reaction of MIF (tautomerase activity), but there is no direct link has been established in tautomerase activity and glucocorticoid function [152]. A family of thioredoxin (TRX) proteins viz., thioredoxinreductase, glutaredoxin, dihydrolipoamide dehydrogenase and glutaredoxin reductase have conserved CXXC motif sequence. While the cysteine residues in the CXXC motif are spaced apart and typically don't form a bond within the same molecule (intramolecular disulfide bond), they can react with cysteines on other molecules (intermolecular bonds) figure 7 [150].

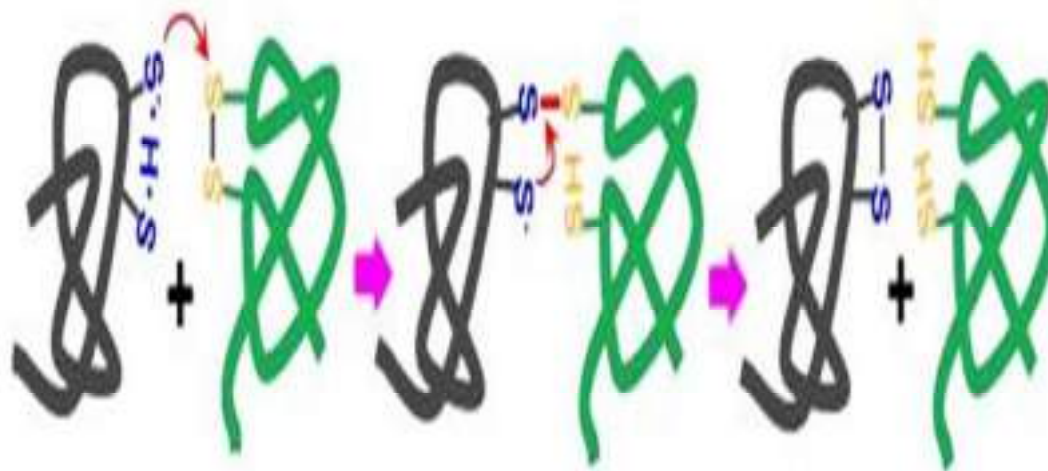


Figure 7:Thioredoxins and their catalytic mechanisms [169].

A small fragment (amino acids 50-65) of the MIF protein exhibited redox activity and mimicked the full-length MIF protein by initiating phosphorylation of ERK1/2 and displayed overriding of glucocorticoid action [170]. This finding indicates a close link between MIF's ability to act as an oxidoreductase and its function in regulating the immune system. This “proof of concept” could pave the way for the discovery of small molecules that can inhibit the process. Unfortunately, identified inhibitors of tautomerase activity were failed in stopping MIF mechanism of function [171, 172]. Developing novel inhibitors that target MIF's oxidoreductase activity holds greater promise. This is because a strong link has already been established between this specific enzymatic function and MIF's overall role in biological processes. Interestingly, to date, not a single molecule has been identified that can block the oxidoreductase function of MIF. This catalytic activity limits the inhibitors finding due to non-targeted, non-selectivity, limited screening of compound and low reproducibility. Till date, no promising inhibitor has been reported [166]. In contrast, thioredoxin and glutaredoxin systems are well-characterized and supported by experimental evidence. MIF oxidoreductase activity can provide valuable insight

of mechanism and function of various thiol based redox reaction in the host. Furthermore, MIF catalytic sites can elucidate a wide range of biological activity of signaling pathways and cytokine release [166].

1.2.11 MIF homologous in nematode parasites

Parasitic nematodes have evolved a complex arsenal of molecules. These molecules can be found on their outer surface (cuticle) or as ESP. ESPs implies a critical function to evade the host's immune system, allowing them to maintain viability for maximum period within the host [173, 174]. The vast arrays of function exhibited by parasite-derived molecules have been proved its ability to manipulate the host's immune system. This underscores the dynamic interplay of adaptation between parasites and hosts, characterized by reciprocal evolutionary pressures [16]. Scientists have discovered and described various molecules that can influence the host's immune response, including proteases, antioxidant proteins, protease inhibitors and mammalian cytokines homologs [19]. MIF is one among them. Research has shown that MIFs produced by parasitic nematodes can influence various immune cells. This influence the way of various geneexpression and ultimately affecting the production of important signaling molecules called cytokines [175, 176]. Eventhough, their native protein structure is not same but being homologs parasitic MIF showed enzymatic activity. A *C. elegans* (free-living nematode) contained four MIF genes viz. Cel-MIF-1, Cel-MIF-2, Cel-MIF-3, and Cel-MIF-4. These genes exhibited sequence similarity ranging from 15% to 32% among themselves and 22% to 35% compared to human MIF (hMIF).This study using genome and transcriptome sequencing reveals the presence of the MIF gene family in other parasitic nematodes. This finding expands our understanding of the MIF gene family across four clades within the nematode phylum. This included MIF homologs from Clade I: *Trichuris*

and *Trichinella* spp.; Clade II: *W. bancrofti*, *Ascarissuum*, *Brugia* spp., and *O. volvulus*; clade IV: *S. esstercoralis*, *Heterodera* and *Meloidogyne* spp.; and clade IV: *Ancylostoma* and *H. contortus* spp. [177]. A study on MIF homologs in parasitic nematodes identified two distinct types based on their similarity to Cel-MIF-1 or Cel-MIF-2. Cel-MIF-3 and Cel-MIF-4 stood out from other nematode MIF proteins due to their larger size (146 amino acids) and a unique amino acid change at position 2. MIF-2 type proteins exhibit a higher degree of sequence similarity among their homologs compared to MIF-1 type proteins. Interestingly, human MIF was found more similar to the MIF-1 than the MIF-2 in terms of sequence homology [177].

1.2.12 MIF homologous are potential candidates as therapeutic agent or drug target

Risk benefit ratio of the live parasite as a drug entity has been proved to have very low in potential; even some chronic adverse side effects were also reported such as disturbance of gastrointestinal motility, resulted in diarrhea and abdominal cramps [178, 179]. To be safe helminths derived ESPs may be more promising as therapeutic drug molecule [180, 181]. Previous studies reported that ESPs can differentiate T-reg cells and AAMs, to induce TGF- β and IL-10 both are potent anti-inflammatory cytokines. Other studies also reported that parasite-mediated immunomodulatory molecules like as homologs of mammalian ALT, TGF- β , MIF and different protease inhibitors has been showed promising results (Figure 8) [159]. Research evidence reported that recombinant MIF cytokine of *A. simplex* ameliorated hyper-reactivity in asthmatic animal model via inhibiting goblet cells hyperplasia and eosinophilia differentiation mechanism [159].

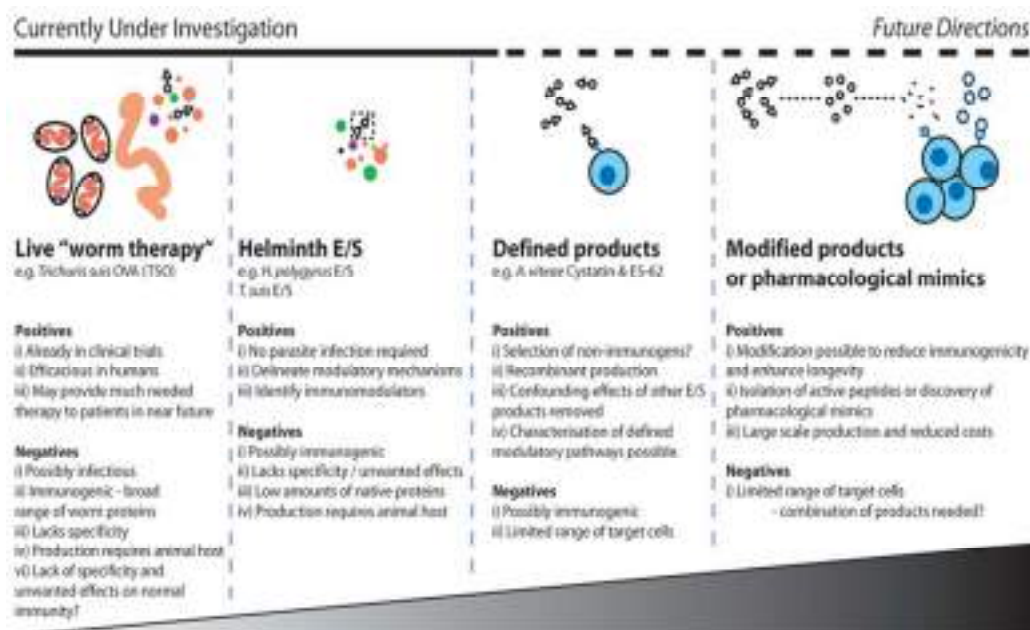


Figure8:The future directions for the development of parasite derived molecules as therapeutic agents [178].

With all information, further studies of Asi-MIF could be a promising anti-asthmatic drug molecule [182]. Research has also showed that Asi-MIF can directly reduce inflammation in the intestine via TLR2 binding of T-reg cells requirement and lowering the release of inflammatory molecules like TNF- α , IL-6, and IFN- γ . This research highlights the potential of nematode-derived MIF cytokines as a immune modulator. This study provided a strong evidence for MIF as a promising target molecule for developing therapeutic strategies to treat IBD, [142]. Similarly, *Leishmania* MIF modulated macrophage/ monocytes [183]; *Plasmodium falciparum* MIF are capable of inhibit monocytes migration and specifically decrease expression of the TLR2, CD86, and TLR4 [184]; *Strongyloides* MIF induce IL-10 secretion from the monocytes [185]. A promising new approach is emerging for screening FDA-approved drug molecules and natural compounds that could target the parasitic MIF. Previous study supported this, during the vaccination program against hook worm; a

partial protective effect was reported with the recombinant MIF of *A. ceylanicum*. This finding supports the novel idea of targeting nematode MIF could be a viable approach to preventing or treating inflammatory and infectious diseases [159].

1.2.13 Role of helminths mediated macrophage phenotypes in T1DM and T2DM

Studies using mice models showed that macrophages might be key players in the positive effects of helminths on diabetes. This was because removing of T cells or manipulating their signaling molecules didn't affect the protection against diabetes provided by infections with parasites like *S. mansoni*, *H. polygyrus*, and *L. sigmodontis* [186, 187]. In a closer look at immune cells of the infected animals that were not diabetic, research reported higher levels of markers associated with AAMs in the lymph nodes of the pancreas. This outcome proved that AAMs plays central in diabetes protection [188]. Other research using ESPs from *T. crassiceps* and *F. hepatica* further supported the link between protection induced by helminths and the AAM cells [189, 190].

A molecule identified from ESPs of the *F. hepatica* known as FhHDM-1. This protein has been found just as effective as the whole ESPs in preventing both inflammation of the insulin-producing cells and high blood sugar in mice with T1DM and non-obese diabetes (NOD) [191]. Helminths- mediated AAMs phenotype switch pro-inflammatory to anti-inflammatory cytokines release [191]. In T1DM helminth-mediated AAMs plays major role. Moreover, infection of various helminths, like as *S. venezuelensis*, *L. sigmodontis* [192], *S. mansoni* [193], *N. brasiliensis* [194], *H. polygyrus* [195, 196], and *T. spiralis* [197], have been ameliorated insulin sensitivity and glucose tolerance in T2DM animal models (Table 2).

Table 2:Therapeutic outcomes of helminths/ ESPs on macrophage phenotype of T2DM animal model.

Sr. No	Helminth/ ESPs	T2DM animal model	Macrophage phenotype/ targated tissue	Therapeutic outcome	Ref.
1	<i>S. mansoni</i>	HFD	M2, WAT	weight gain -reduced glucose tolerance -improved insulin sensitivity-improved	[193]
2	w1, <i>S. mansoni</i>	HFD	M2, WAT	weight gain- reduced glucose homeostasis- improved IL-33-increased	[189]
3	w1, <i>S. mansoni</i>	HFD	M2, WAT	weight gain -reduced glucose tolerance -improved insulin sensitivity-improved	[198]
4	<i>LNFP III</i>	HFD	M2, WAT	glucose tolerance- improved insulin signalling- increased IL-10- increased	[199]
5	<i>L. sigmodontis</i>	HFD	M2, epididymal adipose tissue	glucose tolerance- improved insulin signalling- increased	[200]
6	<i>N. brasiliensis</i>	HFD	M2 epididymal adipose tissue	weight gain -reduced glucose tolerance -improved	[201]
7	<i>T. spiralis</i>	Ob/ob and HFD	M2 adipose tissue	insulin sensitivity- improved glucose level -improved	[202]
8	<i>H. polygyrus</i>	HFD	M2 gonadal fat tissue	weight gain -reduced glucose tolerance -improved white adipose tissue- increased	[203]
9	<i>H. polygyrus</i>	HFD	M2, WAT	weight gain -reduced glucose tolerance -improved	[204]

1.2.14 Helminth- mediated macrophages and β -cells crosstalk

Previous research has reported that helminths mediated macrophages cells played a critical role for the improvement of diabetic symptoms. Helminths infection and their ESPs influenced macrophages in a way that communicates with β -cells and may exerting a protective effect. Indeed, helminths infection modulates AAMs and increase expression of IGF1 [205]. Macrophage-based ablation of IGF1 signaling has shown a challenge of IGF1 mediated endocrine signaling that may causes a significant IR in mice [205]. Even though, in these studies a direct link of β -cells was not explored, in pancreatic islet macrophage monitored the IGF1 secretion and this may enhanced the insulin secretion [206]. Along with IL-10, other signaling molecules like TGF-beta 1 (TGFb1) and epidermal growth factor (EGF) released by AAM may also promoted the survival and growth of B cells via induction of SMAD7 expression [207]. Supporting the helminth-mediated AAM activation and releases of anti-inflammatory cytokines may prevent the diabetes. In diabetes, when macrophages engulf apoptotic beta cells, it triggers the releases of ROS that further activates inflammasomes, which induced IL-1 β secretion, a hallmark for CAM phenotype [208]. In previous discussion, we highlighted that these CAM negatively impact β cells and impaired the differentiation of β -cells, glucose mediated insulin secretion, and further induction of apoptosis. The interaction between CAM and pathogenic β cells amplified the inflammatory responses and further responsible for disease development. Indeed, depletion of CAM cells prevented T1DM in NOD mice [209]. Typically, diminished phagocytic ability of CAM causes improper clearance of apoptotic β -cells which enhanced inflammation in the pancreatic islet of NOD mice [210] and human [211]. Studies supported that helminths dampen the harmful effects of CAM by either suppressing the inflammasome or modulating them towards AAM

state [212]. Helminths or its ESPs mediated approach may inhibit the destructive signaling communication of β -cells-macrophage, and able to promote anti-inflammatory pathways that would help maintain β -cells function, numbers, and ultimately contribute to disease prevention.

1.2.15 Helminth infection and T2DM

An observational study from Turkey investigated the link between diabetes and a type of parasitic worm infection called soil-transmitted helminth infection (STH) [213]. The researchers included people with both T1DM and T2DM in their analysis. Interestingly, they found that a smaller percentage of diabetic patients (47%) had the infection compared to the control group (55%) from 1024 controls ($p < 0.05$). However, the possibility of STH infection itself might offer some protection against diabetes was not explored in this study. The earliest research explored this potential link in 2010. However, like previous studies, this investigation focused on diabetes as a risk factor and filarial infection as a potential health consequence. In south India, Chennai Urban Rural Epidemiology Study (CURES) was conducted with the objective of diabetes and prevalence of LF [214]. An analysis of 1416 individuals revealed a significantly lower occurrence of LF sero-positivity in those with both existing and newly diagnosed diabetes (4.3% and 5.7%, respectively) compared to those with prediabetes (9.1%) and without diabetes (10.4%). The analysis showed a connection between the two variables even after accounting for age, gender, and socioeconomic background. BMI was not adjusted. Notably, the diabetic groups had a significantly higher BMI compared to the non-diabetic groups. Study reported lower level of pro-inflammatory cytokine (TNF- α , IL-6, GM-CSF), and filarial antigen in parasite infected diabetic's people. There were non significant releases of the anti-inflammatory cytokine reported. Outcome of the study showed that T2DM was

ameliorated due to lower level of inflammatory cytokine [214]. In 2013, researchers in China investigated the link between a history of schistosomiasis and T2DM in a rural population. Their study involved 3,913 people over 60 years old and examined for Insulin resistance and T2DM. Past history, metabolic factors and infection of schistosomiasis were cross-checked using government record. People who had previously been infected showed negative results in all the measured metabolic markers. This trend remained even after accounting for factors like BMI, sex, age and diet [215]. In another Chinese study researcher investigated a link of metabolic diseases associated factors viz. blood pressure, obesity, total cholesterol, HDL, LDL, triglyceride levels, blood sugar levels and historical prevalence of *Schistosoma japonicum* infection. Study involved 1132 participants, with 465 having a history of infection. Finding showed that connection with blood sugar levels became less statistically significant when considering other factors together [216].

A study conducted in 2015 by Wiria et al. [217] investigated the connection between insulin sensitivity and soil-transmitted helminth (STH) infection in Indonesia. The study involved 646 adults who were tested for five different STH parasites: *T. trichura*, *A. lumbricoides*, *A. duodenale*, *S. strongyloides*, and *N. americanus* and measured participants' insulin levels and Homeostasis Model Assessment of Insulin Resistance (HOMA-IR). The study found that individuals with STH infection had lower insulin levels and a significant improvement in HOMA-IR scores this concluded higher insulin sensitivity. Interestingly, the more STH infections a person had, the stronger the association with improved insulin sensitivity. Even after accounting for body mass index (BMI), the link between STH infection and better insulin sensitivity remained significant [217]. In 2016, researchers from Indonesia published the results of the first prospective trial investigating how deworming

treatment impacts insulin resistance. Researchers in Indonesia conducted a cluster-randomized, double-blind trial to investigate the effect of albendazole regimen for soil-transmitted helminth infections on insulin resistance. The study involved households on Flores Island, a region with a high prevalence of STH infections. A “SUGARSPIN” trial was carried out in the STH infected population of Indonesia. Study was designed as a double blind, cluster- randomized and household-based population for the effect of albendazole treatment and insulin resistance. The findings published in 2017, showed no overall change in insulin resistance after treatment [218]. Individuals infected with helminths revealed a significant impact on HOMA-IR. Moderate effect of treatment on insulin resistance may be due to short follow-ups periods and previous infection exerting its effect on IR. Prevalence of infection may also influence the protective effect.

1.2.16 Helminth infection and T1DM

Initial studies explored the link between helminth infections and diabetes prevention in NOD mice, the focus was on a potential immune response shift from Th1 to Th2 cells was considered as a main protective mechanism. Later research using NOD mice with live infections and helminth extracts investigated how these parasites and their molecules influenced the properties and functions of macrophages and dendritic cells [219, 220, 221]. A specific set of molecules or cellular processes associated with reduced inflammation in innate-immune cells were found to be accountable for promoting the growth of T-reg and Th2 cell populations. Diabetes protection in NOD mice has been connected to the penetration of Th2 immune or T-reg cells in pancreatic tissue [222, 223, 224, 225].

Many studies have shown that for helminth infection or its ESPs to be effective, they need to present before a significant amount of β -cell were damaged by the autoimmune attack. This suggested that helminth-derived therapies might be most effective in preventing diabetes in high-risk individuals (prophylaxis) or as complementary treatments (co-therapies) to induce tolerance alongside beta-cell replacement approaches. A report showed that *S. mansoni* larval antigens at its infective stage of human can prevent diabetes in NOD mice. This preventive effect was observed both at an early stage (4-5 weeks old) and later in disease progression (10 weeks old). The ways in which helminth infections safeguard against diabetes are multifaceted and not fully understood. The importance of Th2 immune response in preventing diabetes was investigated using IL-4 deficient NOD mice. These mice did not develop the typical Th2 shift after exposure to *L. sigmodontis* or, *H. polygyrus* yet they were still protected from developing diabetes [225, 226]. However, a study involved NOD mice (IL-4-competent) infected with *H. polygyrus* found that diabetes prevention mechanism involved the growth of T and B-reg cells via IL-10 secretion [227]. List of helminths infection and their mode of action on immune system is mentioned in the Table 3.

Table 3: Helminth infections and immune responses to prevention of T1DM in animal model.

Sr.No.	Infections of helminth	Diseases model	Mechanisms of protection	Ref.
1.	<i>S. mansoni</i>	T1DM	Th2 type immune mediated response	[125]
2.	<i>T. spiralis</i>	T1DM	Th2 type immune mediated response	[228]
3.	<i>L. sigmodontis</i>	T1DM	Th2 type immune mediated response	[225]
4.	<i>H. polygyrus</i>	T1DM	Th2 type immune response	[226]

1.3 Justification of the study

Recently, along with conventional treatment, a trend of alternative therapies is emerging mainly for the inflammation, autoimmune and immuno-metabolic disorders [114, 115]. In alternative therapies, a approach of parasitic whole worms or its derived products are used, to modulate the immune system or to reduce the hyperactivity of the immune cells. This concept of alternatives treatment is called as “helminth therapy” (HT) and it emerged in pre-1990s [116, 117]. Several laboratories have explored the helminths-based immunosuppression of allergies, inflammation and other immune reactions [118, 119]. To treat immune related diseases, earlier trails were performed using parasitic eggs, live or attenuated parasites [80]. Though, several factors are concerned while using live parasites and their eggs; a) risk of co-infection b) may elicit minimum pathology in the host tissue, c) sometime live parasite may be chronic, d) problem may occur due to aberrant migration, e) for use of live helminths ethical issues are more concerned, and f) non-compliance factors like hesitation for live parasites therapy [120]. These limitations created new research challenge for the immunologist to explore individual molecules secreted by the parasites for immunomodulation.

Unlike whole parasites and mixture of its secreta (biologicals), single molecule-based approach can be more ideal for novel biological development, being an individual molecule, it is easy to scale up, structural elucidation, mechanistic studies and easy to monitor its toxicity and immunogenicity [121]. Moreover, unlike other drug molecules these novel biologicals will be more effective and bioavailable as parenteral delivery and boosts patient compliance and satisfaction [122]. Previously, immunomodulatory proteins such as Bma-MIFs and ES62 (leucyl aminopeptidase) were identified, which are abundantly expressed in the ESPs of

adult females and microfilariae. Especially, Bma-MIF2 have been shown to promote IL-4 dependent alternatively activation of functionally suppressive macrophages *in vitro* [83, 123, 124]. There is always a need to identify an individual immunomodulatory molecule of filarial parasite which could be more specific and to be developed as therapeutic agent or drug target. As per the literature review an inverse relationship was observed between parasitic infection and diabetes [35]. Instead of whole parasite, single molecule can be a more promising therapeutic approach.

Finding of previous research justified our scientific approach, to elucidate the immunomodulatory role of the parasitic Macrophage migration Inhibitory Factor (MIF-2) in type I and type II diabetes in animal model.

2. MATERIALS& METHODS

2.1. Expression and purification of Wba-MIF2 cytokine

2.1.1. Expression of His-tagged Wba-MIF2 cytokine

A clone of DH5 α , containing the Wba-MIF2 gene insert, was grown in LB broth with 100 μ g/ml ampicillin, overnight. Recombinant plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen, Cat. No: 27104) following the manufacturer's instructions. *E. coli* (Rosetta DE3) cells were transformed with the isolated recombinant plasmid using heat shock method. Positive clones were screened on Amp⁺ agar LB plates and further cultured in LB broth on rotary shaker at 180 rpm, and 37°C for 16 hours. For protein expression, a small amount (0.1%) of the overnight culture was used to inoculate 1 liter of LB broth. The culture was then incubated until it reached a specific cell density (OD 600). To induce the expression, 0.6mM IPTG solution was added and incubated for 3 hours at 180rpm and 37°C. The cells were harvested by centrifugation at 6000rpm for 20 minutes, at 4°C. The resultant pellet was washed with PBS and stored at -80°C until further use [28, 29].

2.1.2 Purification of His-tagged Wba-MIF2 cytokine

Bacterial pellet was re-suspended in the lysis buffer (50mm Tris chloride, 300mm NaCl, pH 8.0) and sonicated for 15min (3sec on and 7sec off cycle) on ice bath using probe sonication (Vibra-Cell™ Ultrasonic Liquid Processors, VCX 130, Vibra Sonic& Materials Inc.). Lysed pellet was centrifuged at 15000rpm at 4°C for 50min, in a High Speed Refrigerated Centrifuge (Model 6500, Kubota, Laboratory Centrifuges, Japan), the supernatant was collected, and syringe filtered using Millex-LG, 0.20 μ m, (Cat. No.SLLG025SS, Sigma-Aldrich Chemicals Pvt. Ltd., India). The filtrate was passed through pre-equilibrated HisTrap HP 5 ml column (Cat. No. 17-

5248-02, GE Healthcare Bio-Sciences AB, Sweden), using equilibration buffer (50mM Tris, 300mM NaCl, pH 8.0). Unbound protein was washed using wash buffer (50mM Tris, 300mM NaCl, 20mM imidazole, pH 8.0). His-tagged Wba-MIF2 protein was eluted using a gradient imidazole (50-300mM) solution in elution buffer [28, 29].

2.1.3 SDS-PAGE

After elution of Wba-MIF-2 recombinant protein, purity of the eluted protein was checked by SDS-PAGE [29]. Samples were loaded on 12% SDS-PAGE gel and separated using Mini-PROTEN Tetra Cell (BioRad Laboratories Inc.) electrophoresis unit. Purified fractions were pooled and concentrated using 10kDa molecular weight cut-off filter (Vivaspin 2, GE Healthcare, Sweden) and quantified using the Pierce BCA Protein Assays kit (Thermo fisher), as per the manufacturer protocol [29].

2.1.4 Western blotting of His-tagged Wba-MIF2

Following separation by SDS-PAGE, the purified Wba-MIF2 proteins were transferred to a PVDF Membrane (0.2 μ m) (Cat. No. 88520, Thermo-Fisher, Scientific, USA). Membrane was activated using methanol and TBST buffer. The transfer was performed at 35 volts, 16 hours, 4°C, using a Mini-PROTEAN® Tetra Cell, Mini Trans-Blot® Module, and PowerPac™ system (Bio-Rad, Cat. No. 1658033). Following incubation, the membrane was rinsed three times with tris-buffered saline containing 0.1% Tween 20 to remove unbound molecules. Then, it was incubated with 5% non-fat milk for one hour at room temperature. After another wash with TBST, the membrane was incubated overnight at 4°C on a rocking shaker with a 1:1000 dilution of HRP conjugated-anti-His monoclonal antibody, (Cat. No. MA1-21315-HRP, Thermo-Fisher Scientific, USA). The membrane was then washed with TBS containing 0.1% Tween 20. To develop the western blot ECL clarity max (Cat. No. 1705060, BioRad) substrate was used [28, 29].

2.1.5 Purification of His-tagged Wba-MIF2 using Size Exclusion Chromatography

IMAC eluted samples were pooled and concentrated using Amicon 3kDa filter (Merk, India). This concentrated supernatant was passed through Size Exclusion column (Sephadex 16/300 column, BioRad, India) using AKTA System (BioRad Laboratories Inc.). Initially the column was equilibrated with running buffer (50mM Tris, 300mM NaCl, pH 8) and the sample was loaded using 1ml loop. Flow rate of the column was set 0.5ml/ min and fractions of 1.5ml were collected using fraction collector (BioRad, India). Column was optimized using standard molecular weight protein marker (BioRad).

2.1.6 Endotoxin removal from recombinant Wba-MIF2 protein

Endotoxins were removed using commercially available 0.5ml Pierce™ High-Capacity Endotoxin Removal Spin Columns (Cat. No: 88274, Thermo-Fisher Scientific, USA) using manufacturer's protocol. The resin in the 0.5 ml spin column was centrifuged at 500 x g , 1 minute to remove the storage buffer. Then, the resins were incubated with 0.2 N NaOH overnight. Washed three times with endotoxin-free water and followed by a washing with 2 M NaCl. Further, the resin in the column was prepared by washing it with endotoxin-free phosphate-buffered saline. Then, the sample was loaded onto the column and incubated for 2 hours at 22°C with gentle end-over-end mixing on a rocking platform. The column was centrifuged at 500 x g for 1 minute to isolate the sample. A LAL Chromogenic Endotoxin Quantitation Kit, (Cat no. 88282, Thermo- Scientific) was used to measure the amount of endotoxin in the samples. The Bradford method was employed to determine the concentration of the purified protein.

2.1.7 Enzymatic activity of recombinant His-tagged Wba-MIF2 protein

2.1.7.1 Oxidoreductase activity

To assess the oxidoreductase activity of Wba-MIF2, insulin disulphide reduction assay was performed [29, 151, 229]. The reaction mixture contained 100 mM potassium phosphate (pH 7.0), 1 mM EDTA (adjusted to pH 8.0), 240 μ M insulin, and 10 μ g of Wba-MIF2. A control containing only the buffer was also run. All solutions were freshly prepared and added to the 96 well plates (Tarsons India Ltd., India). The experiment was begun at a temperature of 25°C. One millimolar (mM) of dithiothreitol (DTT) was added to a total volume of 100 μ L. The absorbance of the solution was measured at a wavelength of 650 nm, every 5 min for a total duration of 60 mins, in a multimode reader (Infinite 200 PRO, Tecan Trading AG, Switzerland). The absorbance of each sample was determined by averaging the results of three separate experiments. The blank absorbance value was subtracted from each measurement before averaging.

2.1.7.2 Tautomerase activity

Tautomerase activity of purified Wba-MIF2 was determined as explained previously [153]. L-DOPA was used as the substrate for the tautomerase activity and the conversion of L-Dopachrome methyl ester into D- form was measured by monitoring the change in absorbance at 475 nm wavelength [152, 153].

2.2 Immunomodulatory effect of Wba-MIF2 in T1DM and T2DM

2.1.2 Animal study

C57BL/6 male mice (6-8 weeks old), were provided from Central Animal Facility, Indian Institute of Science, Bangalore. All animal studies for thesis were conducted after the ethical clearance acquired from the Institutional Animal Ethical Committee of the ICMR-National Institute of Traditional Medicine, Belagavi (Annexur-I). All animals were maintained at 24-26°C, RH 60 ± 10 %, 12 h light and dark cycle, and 100% fresh air exchange.

2.1.3 T1DM Induction in C57BL/6 mice

Following a one-week acclimatization period, mice were fasted overnight and subsequently induced with type 1 diabetes mellitus. Diabetes was initiated through five consecutive days of intraperitoneal injections of multiple low doses of streptozotocin (40 mg/kg) [230, 231]. Streptozotocin (STZ) was freshly dissolved in sodium citrate buffer (pH 4.5) and administered to mice intravenously within 20 minutes of solution preparation. Control mice received an intraperitoneal injection of citrate buffer as a vehicle control. To assess diabetic condition, FBG levels were quantified a week followed by STZ treatment. Mice exhibit sustained hyperglycemia, and FBG levels exceeding 350 mg/dl for a consecutive two-week period, were categorized as diabetic and utilized for subsequent experimentation [22]. The diabetic mice were evenly distributed across four experimental cohorts through a randomized process. Animal study groups were shown in Table 4. The vehicle and test protein was administered *intraperitoneally*.

Recombinant Wba-MIF2 treatment commenced once the STZ-induced condition of the animals stabilized, followed by an evaluation of immunological parameters. Following the experimental period, mice underwent a fasting regimen prior to being euthanized according to humane protocols. The Wba-MIF2 dose was chosen in alignment with dosing regimens employed in similar studies[232, 233].

Table 4.Animals grouping for T1DM

Sr. No.	Animals grouping	Animals/group
1	Group-I: NPD (Normal Control)	8
2	Group-II: NPD + STZ (40mg/kg body wt.)	9
3	Group-III: NPD + STZ +Vehicle (PBS)	9
4	Group-III: NPD +STZ+ Wba-MIF2 (25µg)	9

2.2.3 Induction of T2DM in Balb/cmice

4-6 weeks old male Balb/c mice were used for Type 2 diabetes mellitus model development. One-week post acclimatization animals were divided in to two groups: Group I: feed with normal pellet diet and Group II: was fed with high fat diet (Research diet-D12451, Annexure-II) for four weeks. High fat diet fed animals further subdivided into two groups; Group II. a.: Only fed with HFD (HFD control group), Group II. b: HFD with single dose 40mg/kg body wt. of STZ intraperitoneally. Animals were monitored for fasted blood glucose level every week using AccuCheck glucometer. Animals having blood glucose more that 250mg/dl for two consecutive weeks were considered as a diabetic. This group (Group II. b.) was further sub-divided into three groups: Group II. b. STZ control (STZ), Group II. c.: Vehicle treated (Vehicle; PBS) and Group II. d.: Wba-MIF2 treated group (Wba-

MIF2-25µg). Animal groups were given in (Table 5) STZ solution was freshly prepared in ice cold sodium citrate buffer (pH 4.5) and protected from direct light exposure. STZ solution was injected i.p. within 20min of preparation [230, 231, 234]. Control group animals were equally injected with citrate buffer only.

Table 5.Animals grouping for T2DM

Sr. No.	Animals grouping	Animals/group
1	Group-I: NPD (Normal Control)	6
2	Group-II: HFD	9
3	Group-III: HFD+ STZ	9
4	Group-III: HFD +STZ+ Vehicle (PBS)	9
5	Group-IV: HFD +STZ+ Wba-MIF2 (25µg)	9

2.2.4 Oral glucose tolerance test

To access the rate of glucose clearance, oral glucose tolerance test was performed in overnight fasted animals. Animals were kept devoid of food but freely access to water for overnight. Blood samples were collected using tail prick method. Before blood collection 70% ethanol cotton swabs were used to clean and sanitize the tail. A 26-gauge needle was used for animal's tail pricking. For blood glucose monitoring AccuCheck Performa glucometer was used. Baseline reading was recorded in fasted animals at 0th minutes and animals were immediately fed orally with glucose solution (2 g/kg body weight). Blood glucose level was further monitored at 30, 60, and up to 120 min time points. The results were presented as the area under the curve for glucose (AUC_{glucose}), and calculated using trapezoid rule [230, 234].

2.2.5 Samples collection

Following an overnight fast, animals were euthanized at the conclusion of the experimental period using humane methods. Blood samples were collected and processed to isolate serum for immunological analyses. These serum samples were subsequently stored at -80°C for future investigations. Spleens were harvested and preserved in TRIzol reagent for subsequent cytokine mRNA analysis. Finally, pancreatic tissue were collected and immersed in 10% formalin for histopathological examination.

2.2.6 Evaluation of blood biochemistry parameters

Blood biochemistry parameters were measured in diabetic animal groups using multiple biochemical assays kits for the estimating of cholesterol, triglycerides (TG), high density lipid (HDL), low density lipid (LDL), HbA1C, fasting blood glucose (FBG) in serum samples. All the biochemical assays were performed as per the manufacturer's recommendation using kits from ERBA Mannheim, UAE [234].

2.2.7 Cytokine measurement in animal sera samples

Serum levels of pro-inflammatory cytokines (IL-1, IL-2, IL-6, TGF- α , and IFN- γ) and anti-inflammatory cytokines (IL-4 and IL-10) were quantified using a sandwich enzyme-linked immunosorbent assay (ELISA) (RayBioTech, Norcross, GA, USA) according to the manufacturer's instructions [232].

2.2.8 Isolation of RNA and cDNA synthesis from spleen tissue

Total RNA was extracted from spleen tissue samples using TRIzol reagent according to the manufacturer's protocol (Invitrogen). cDNA was synthesized from 1 microgram of RNA employing the iScript Select cDNA Synthesis Kit (Cat. No.

1708897; BioRad). Quantitative real-time PCR (qPCR) was performed using a cDNA template, following a previously published method [233]. All primers were custom-designed and synthesized by Agri genome, India. The primer sequences utilized in the assays are listed in Table 6.

2.2.9 Real-time qPCR analysis of cytokine in spleen tissue

Real-time qPCR analysis was conducted using the protocol outlined in [232, 233]. In brief, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's guidelines. RNA quantification was performed using a Nanodrop (Thermo-Fisher Scientific, USA). Equal amounts of RNA (1 µg) were reverse transcribed into cDNA using a Prime Script RT Reagent Kit (Takara Bio, Inc., Dalian, China). Quantitative PCR was subsequently performed to measure the expression levels of pro-inflammatory (IL-1, IL-2, IL-6, TNF- α , IFN- γ) and anti-inflammatory (IL-4, IL-10) cytokines using SYBR Green-based detection and specific primers on a real-time PCR platform. GAPDH served as the endogenous control for gene expression analysis. Relative gene expression levels were determined using the $2^{(-\Delta\Delta Ct)}$ method. The $\Delta\Delta Ct$ value was calculated by subtracting the ΔCt of the calibrator sample from the ΔCt of the experimental sample. The ΔCt for each sample was obtained by subtracting the Ct value of GAPDH from the Ct value of the target gene.

Table 6.List of primers used for RT-PCR.

Cytokine gene	Direction	Nucleotide sequence
IL-1 β	Forward	5'-CCAGGGACAGGATATGGAGCA-3'
	Reverse	5'-TTCAACACGCAGGACAGGTACAG-3'
IL-2	Forward	5'-AGCTGTTGCTGGACTTACAGG-3'
	Reverse	5'-AATTCCACCACAGTTGCTGG-3'
IL-4	Forward	5'-TCTCGAATGTSACCAGGAGCCATATC-3'
	Reverse	5'-AGCACCTTGGAAGCCCTACAGA-3'
IL-6	Forward	5'-AGTTGCCTTCTTGGGACTGA-3'
	Reverse	5'-TCCACGATTTCCCAGAGAAC-3'
IL-10	Forward	5'-GAGATGCCTTCAGCAGAGTGAAGA-3'
	Reverse	5'-AGGCTTGGCAACCCAGGTAAC-3'
TNF- α	Forward	5'-CGTCAGCCGATTTGCTATCT-3'
	Reverse	5'-CGGACTCCGCAAAGTCTAAG-3'
IFN- γ	Forward	5'-CGGCACAGTCATTGAAAGCCTA-3'
	Reverse	5'-GTTGCTGATGGCCTGATTGTC-3'
GAPDH	Forward	5'-TATGTCGTGGAGTCTACTGGT-3'
	Reverse	5'-GAGTTGTCATATTTCTCGTGG-3'

2.2.10 Histopathological analysis

At the end of the study (5 weeks for T1DM and 6 weeks for T2DM treatment) animals were sacrificed and the pancreas was isolated from each animal, washed with PBS and preserved in 10% neutral buffered formalin solution and embedded in paraffin for sectioning of 5 μ m tissue on slides. Tissue sections were stained with hematoxylin and eosin (H&E) and imaged at 400x magnification for histopathological examination.

2.3 Phospho-proteomics analysis to study the signaling mechanism of Wba-MIF2

2.3.1 Cell culture conditions

We used RAW 264.7 cells (ATCC, Manassas, VA) in our experiment. The cells were grown and kept in DMEM + 10% FBS + 1% antibiotic solution (Thermo Fisher Scientific, USA). The cells were cultured in an incubator set at 37°C with controlled humidity and 5% carbon dioxide (CO₂). Investigate the impact of MIF on RAW 264.7 cells, these cells were treated with 100 ng/ml of either human MIF (h-MIF) or parasitic Wba-MIF2 over different time points.

2.3.2 Protein extraction from cell lines treated with hMIF and Wba-MIF

The proteins from the treated cells were extracted as described earlier [236, 237]. In brief, The treated cells were resuspended in lysis buffer containing 4% SDS, 2.5 mM sodium orthovanadate, 1 mM sodium pyrophosphate and 1 mM β -glycerophosphate in 50 mM TEABC buffer, and sonicated on ice for 2 minutes using Q-Sonica (Cole-Parmer, India), with 30% amplification for 30 seconds pulse on and 30 seconds pulse off cycles. The sonicated lysate was centrifuged at 12000 g for 30 min. Before centrifugation, the lysate was heated at 95°C for 10 min. to denature proteins. Further, the supernatant was collected, and protein estimation was carried out using the BCA method.

2.3.3 Sample preparation for proteomics

Proteins from each treatment underwent a reduction step using 100 mM dithiothreitol and subsequent alkylation with 200 mM iodoacetamide. Acetone was added to the sample and incubated at -20°C to precipitate the targeted proteins. The

precipitate was isolated through centrifugation at 25°C for 30 minutes and dried under ambient conditions. The protein pellet was resuspended in 200 microliters of a 50 mM TEABC buffer and digested with TPCK-trypsin overnight at 37 degrees Celsius. Digestion efficiency was assessed by separating the sample components on a 10% SDS-PAGE gel. The resulting peptide fragments were dehydrated using a SpeedVac and kept frozen at -20°C until they were prepared for TMT analysis [236].

2.3.4 Tandem Mass tag labeling

Peptide samples from each experimental group were reconstituted in 200 microliters of 0.1% formic acid and subsequently purified using the previously described C18 stage tip protocol [238, 239]. Subsequent to purification, the peptides were dried in a vacuum concentrator and subsequently redissolved in a 50 mM TEABC buffer solution. An equal amount of peptide from each experimental group was selected for labeling using the TMT-six plex reagent kit [239]. Six distinct peptide samples were labelled. Following the manufacturer's protocol, TMT labeling reagents were prepared by dissolving them in anhydrous acetonitrile and subsequently adding them to the respective samples. These mixtures were then incubated at ambient temperature for one hour. To stop the reaction, we added 8µL of a 5% hydroxylamine solution. To confirm that all samples had the same labeling efficiency and amount of labeled peptides, we extracted an equal volume (5 µL) of peptide samples from each condition for a subsequent analysis of the TMT labels. Next, we combined equal amounts of labeled peptides from all samples and removed the water overnight using a SpeedVac. The resulting peptide mixture was kept frozen at -20°C until fractionation of the peptides.

2.3.5 Basic pH reverse-phase liquid chromatography based fractionation

Fractionation was conducted in accordance with the previously described protocol [237]. The TMT six plex-labeled dried samples were reconstituted using solvent A for bRPLC (10 mM TEABC) and subjected to chromatographic separation using a Hitachi LaChrom Elite HPLC system. Peptides were separated using a Waters XBridge C18 column (Waters Corporation, Milford, MA; 130 Å, 5 µm, 250 mm × 4.6 mm) attached to the HPLC system. The solvent was run at a constant rate of 0.5 milliliters per minute. A linear gradient, spanning from 5% to 100% of solvent B (a 10 mM TEABC buffer solution containing 90% acetonitrile, pH approximately 8.5), was used to separate the components. This process took 130 minutes. Initially, we collected 96 separate fractions, which were subsequently consolidated into six combined fractions which were then dried using a SpeedVac concentrator.

Peptide desalting was accomplished using C18 Stage Tip method, as described previously [236]. C18 beads were filled into 200-microliter pipette tips. These were then treated with 100% acetonitrile to activate them, followed by equilibration with a 0.1% formic acid solution. The peptide samples were initially dissolved in a 0.1% formic acid solution and then applied to a C18 column. The liquid that passed through the column was collected and re-applied. Subsequently, the column was rinsed with a 0.1% formic acid solution before the peptides were extracted using a mixture containing 40% acetonitrile and 0.1% formic acid. The dried peptide samples were kept frozen at -20°C until they were analyzed using LC-MS/MS.

2.3.6 Phosphopeptides enrichment by Titanium dioxide:

Phosphopeptides were enriched utilizing Titanium dioxide (TiO₂) beads, following a protocol described in a previously published study [238]. In summary,

TiO₂ beads were incubated with DHB solution (mixture of 80% acetonitrile with 1% trifluoroacetic acid and 3% DHB) for a period of 2-4 hours, RT. Each fraction was dissolved in DHB and then mixed with the pre-treated TiO₂ beads. The TiO₂ beads containing the phosphopeptides were washed thrice with DHB solution and twice with a 40% acetonitrile solution. Subsequently, the peptides were extracted three times using 40 microliters of a solution composed of 2% ammonia in 2% trifluoroacetic acid. The enriched phosphopeptide samples were dried completely and then redissolved in a 30µL solution containing 0.1% formic acid. These samples were subsequently purified using C18 StageTips. The purified peptides were finally analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

2.3.7 LC-MS/MS analysis

Peptide analysis was performed using tandem mass spectrometry (LC-MS/MS) on an Orbitrap Fusion Tribrid mass spectrometer coupled to an Easy-nLC 1200 nanoflow UHPLC system (both Thermo Fisher Scientific, Bremen, Germany) [237]. The dried peptide fractions were redissolved in a 0.1% formic acid solution. This solution was then loaded onto a 2-centimeter long, 75-micrometer inner diameter nanoVipertrap column packed with 3-micrometer C18 particles (Thermo Fisher Scientific).

For separation of peptide, an EASY-Spray C18 column (75 µm × 15 cm, 100 Å) was utilized, at constant 40°C. Each sample underwent a 140-minute acquisition, with peptide resolution taking place over 120 minutes using a gradient starting from 30% solvent B (comprising 0.1% formic acid in 80% actonitrile). Subsequently, percentage of solvent B was increased rapidly to 60% at the 130-minute mark and further increased to 100% at 136 minutes, which was sustained for an additional 4

minutes (a total of 140 minutes). The solvent was delivered at a rate of 300 nanoliters per minute. Upon elution from the column, the peptide precursors were subjected to ionization through a nanoelectrospray ionization source (EASY-Spray™). This involved applying a spray voltage of 2.8kV and maintaining an ion transfer tube temperature of 275°C. Data was collected using a data-dependent acquisition (DDA) method, with each acquisition cycle taking 3 seconds.

Precursors falling within the 400-1600 m/z mass range were captured in the Orbitrap mass analyzer at a resolution of 120,000 (at 200 m/z). The Automatic Gain Control (AGC) and Maximum Ion Injection Time (Max. IT) settings were configured to 200,000 and 50 milliseconds, respectively. Following each precursor scan, precursor m/z values possessing a charge state of $z = 2$ to 7 and a minimum intensity of 50,000 were isolated using the quadrupole, with an isolation window of 1 m/z. Subsequently, they underwent sequential MS/MS fragmentation. For the MS/MS fragmentation, the Higher-energy collision dissociation (HCD) technique was employed, utilizing normalized collision energy of $35 \pm 3\%$. The resultant fragment ions (MS/MS) were captured in the Orbitrap mass analyzer at a resolution of 60,000 (at 200 m/z). The AGC and Max IT settings for the fragment scan were adjusted to 100,000 and 200 milliseconds, respectively. A dynamic exclusion feature was applied, with exclusion set for 30 seconds and a 10 parts per million (ppm) mass windows. It's important to note that all the fractions within the total and phosphoproteomic datasets were subjected to data acquisition in technical triplicates [237].

2.3.8 Database search for identification of peptide and protein

Raw data generated by mass spectrometry were analyzed using Proteome Discoverer (PD) software, version 2.2, obtained from Thermo Fisher Scientific (Bremen, Germany).

The raw data were subjected to searches against the mouse reference proteome database, which also included known contaminant protein sequences. This was achieved through the employment of the MASCOT and SequestHT search algorithms. Peptide sequences were searched with the following parameters: a minimum peptide length of seven amino acids, a maximum of two missed cleavages by trypsin, and mass tolerance settings of 10 parts per million for precursor ions and 0.05 Daltons for fragment ions. Static modification was used, which involved the addition of TMT to the peptide N-terminus and lysine residues and carbamidomethylating of cysteine. Methionine oxidation and protein N-terminal acetylation were considered dynamic modifications in this study.

To ensure high confidence, the false discovery rates were strictly controlled at the 1% for both peptide and peptide-spectrum match levels by using the percolator mode during the uses of Proteomics Discoverer (PD) software. In the case of phosphor proteomic raw datasets, a search with the inclusion of a phospho moiety (+79 Da) as a variable post-translational modification (PTM) on serine (S), threonine (T), and tyrosine (Y) residues was conducted. In this instance, the ptmRS algorithm was applied to determine the probability of phosphorylated site localization. Phosphopeptides with a site localization probability of 75% or greater were considered genuine modified sites and subsequently used for protein identification [238].

2.3.9 Data analysis

The raw protein and phospho-peptide (Phospho-proteomic analysis) abundance values were used for relative quantification analysis. Proteins/phosphopeptides with no abundance values across all channels were excluded from further analysis. Missing values were imputed by zero and batch correction was performed to remove the variation between replicates using “ComBat” algorithm from *sva* package (version 3.44.0). Later, the batch corrected data was normalized by smooth quantile normalization technique using *qsmooth* package (version 1.12.0) [239]. The p-value by Student T-test and fold change values by taking average of technical replicates was calculated. Differentially expressed proteins of the cells treated with human and Wba-MIF2 at 5 min and 15 min time points with respect to control (5min) and human MIF vs Wba-MIF2 treatment at 5 min and 15 min were calculated. Here, proteins/phosphoproteins with fold change value ≥ 1.25 and ≤ 0.8 were considered as upregulated and downregulated. All the statistical analysis was calculated using in-house R scripts (version 4.1).

2.4 Bioinformatic study to identify Wba-MIF2 epitops for insulin and its oxidoreductase activity

2.4.1 Homology Modelling of Wba-MIF2

Wba-MIF2 protein crystal structure was not available in the PDB, structural template search for homology modelling was performed vs. PDB using BlastP. The crystal structure of MIF2 from *A. ceylanicum* PDB ID: 2OS5 (1.6Å) showed 36.84% identity with 95% query coverage. Hence this protein structure was selected as a template to perform homology modelling. Modeller-9.23 software was used for homology modelling of Wba-MIF2 protein in homotrimeric form. To start with, 100

models were generated and the one with the lowest DOPE score energy was preceded for stereochemical check using Ramachandran Plot, ProSA [240] and ProQ [241] analysis. Work flow for homology modeling is shown in Figure 9.

2.4.2 Wba-MIF2-Insulin protein-protein docking

The crystal structure of insulin PDB ID: 5E7W (0.95Å) was downloaded from PDB, in which only A & B chains were retained for further studies. The protein-protein docking of MIF2 with insulin was performed using the HDOCK server, which implements global docking protocol [242, 243]. From the HDOCK output, the complex with the lowest dock score was preceded for binding energy calculations using PRODIGY [244] and for visualization of intermolecular interactions using DIMPLOT.

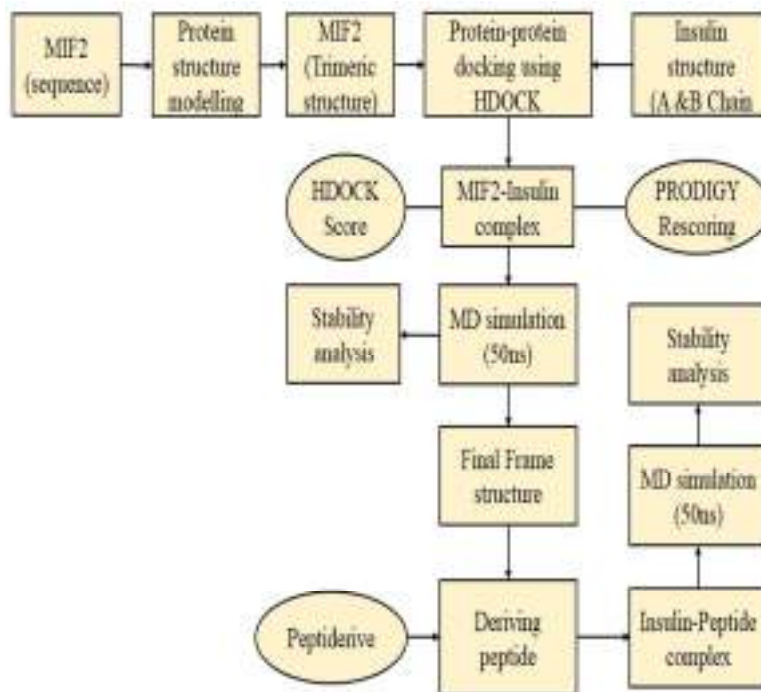


Figure 9: Flow chart showing the bioinformatics workflow.

2.4.3 Molecular Dynamics Simulation studies

Molecular dynamics simulation for the MIF2, Insulin (INS) and MIF2-Insulin complex were carried out using the Gromacs-5.1.4 [245, 246] with Gromos53a6 as forcefield. Initially, the proteins were solvated in a cubic box with the SPC216 water model, and the systems were neutralized by adding counter ions. The solvated systems were energy minimized for 50,000 steps using the steepest descendent method. The systems were equilibrated with NVT and NPT ensemble with V-rescale temperature coupling (300K) and Parrinello-Rahman pressure coupling(1bar) for 100ps. LINCS algorithm [247] was used for constraining the bonds and in case of long-range electrostatics, Particle-Mesh Ewald (PME) was implemented. Finally, the MD production run of 50ns were carried out, wherein, the positional restraints were removed, and the trajectories were sampled at an interval of every 10ps. The Root Mean Square Deviation (RMSD), Root Mean Square Fluctuation (RMSF), H-bond analysis was carried out using gromacsutility tools such as g_rms, g_rmsf and g_hbond.

The binding free energyof MIF-2-Insulin complex was calculated using the Molecular Mechanics Poisson- Boltzmann Area (MM-PBSA) method implemented using the g_mmpbsa [248].

The binding energy is calculated using the formula:

$$\Delta G_{\text{binding}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}})$$

Where, G_{complex} is the protein-ligand complex total free energy, G_{protein} and G_{ligand} is the protein and ligandfree energies in solvent condition.

The individual component free energy can be given as,

$$G_x = E_{\text{bonded}} + E_{\text{vdW}} + E_{\text{elec}} + G_{\text{polar}} + G_{\text{nonpolar}} - TS$$

Where, the first three terms are the Molecular Mechanics calculation energies, G_{polar} and G_{nonpolar} are the solvation free energy of electrostatic and non-electrostatic contributions, T is the absolute temperature and S is the entropy. The above equation can be applied for biological interactions such as protein-protein, protein-peptide and protein-DNA complexes. The implicit solvent model was used for calculating the solvation energy. Free energy decomposition analysis was also performed by *g_mmpbsa*, wherein it provides the types of energy terms that contribute to the stabilization of MIF2-insulin complex with respect to each residue in the interacting interface.

2.4.4 Peptide identification via Rosetta Peptiderive:

The region of MIF2 which contributes to major interactions with the Insulin was identified and utilized to derive a competitive peptide using Rosetta Peptiderive protocol implemented via ROSIE server [249, 250, 251]. The Rosetta Peptiderive protocol identifies the linear region of the proteins which contributes more towards the interface energy and binding score for the complex. The peptides of 10-mer in size were feed as input for deriving the peptide region from the MIF2-Insulin complex. The peptide solubility was calculated using the Peptide solubility calculator available in Innovogen server (<https://pepcalc.com/peptide-solubility-calculator.php>) [252]. The Allergenic properties of the peptides were predicted using AllerTop-v2.0 [253] and AllergenFp-v1.0 [254]. Cell-penetrating property was predicted using CellPPD [255, 256] and Haemolysis property was predicted using HemoPI server [257]. The derived

Insulin-peptide complex was further subjected to MD simulation of 50ns, in order to determine the stability of the complex throughout the simulation.

2.4.5 *In-vitro* oxidoreductase activity of the recombinant Wba-MIF2 peptide

Oxidoreductase activity was determined using an insulin reduction assay adapted from [142]. The precipitation of the insulin β -chain was quantified by measuring absorbance at 650 nm, in blank and positive control and test reaction using Wba-MIF-2 protein. For determining the oxidoreductase activity of the custom synthesized peptide **SNVPASRFPS** (Genscript, USA) insulin with recombinant protein Wba-MIF2 (whole molecule) was taken as standard, and insulin alone was taken as an internal control. Precipitation of the insulin β -chain was measured for 60 minutes, and a graphical spectrum was plotted between time and absorbance at 650nm.

2.4.6 Statistical analysis

Experimental data were expressed as mean values with standard deviation (SD). To compare differences among multiple groups, one-way ANOVA followed by Tukey's post-hoc test was performed. For pairwise comparisons, Student's t-test was utilized. Statistical significance was set at a p-value less than 0.05. All statistical analyses were conducted using GraphPad Prism software, version 6.

3. RESULTS

3.1. Expression and purification of Wba-MIF2 protein

Recombinant full-length Wba-MIF2 protein was expressed with an approximate molecular weight of 17 kilodaltons. In elution buffer, a 250mM imidazole concentration was found optimized for Wba-MIF2 elution from IMAC column. SDS-PAGE and western blot analysis, employed a mouse anti-His antibody, and verified the identity of the his-tagged Wba-MIF2 protein. A band corresponding to the expected molecular weight of 17 kDa (Figure 10) was observed [29]. Affinity chromatography was employed to eliminate endotoxins, and their levels in the purified Wba-MIF2 were found to be below the acceptable threshold of 0.5EU/ml[166].

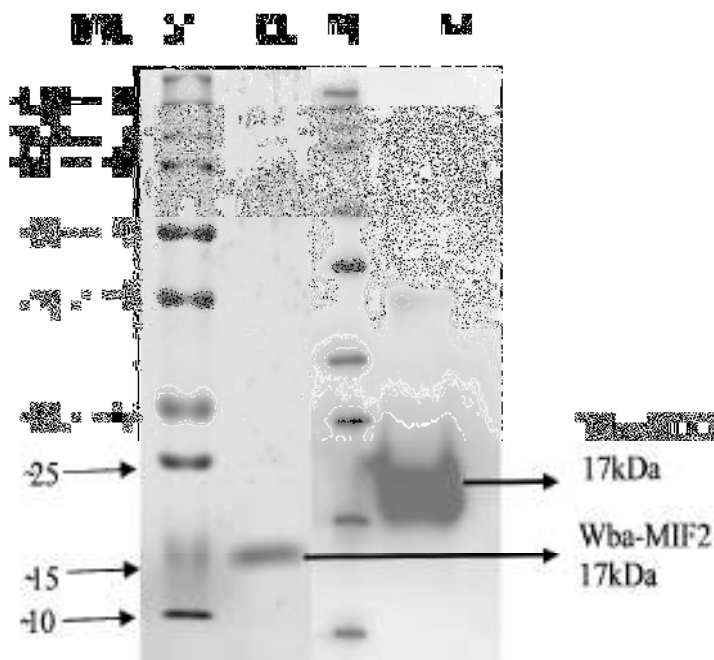


Figure 10. Results of Western blot using 12% SDS-PAGE; L1 and L3: prestained molecular weight marker (10-250kDa); L2: purified 17kDa recombinant Wba-MIF2 and L4: His-tagged recombinant Wba-MIF2.

3.2. Enzymatic activities

Recombinant Wba-MIF2 protein exhibited enhanced oxidoreductase activity relative to the control (Figure 11.A). Tautomerase activity was determined by two approaches. The first method monitored the spontaneous conversion of L-dopachrome methyl ester into DHI, quantified by absorbance at 308 nm (Figure 11.B). The second method involved measuring the formation of DHICA from L-dopachrome methyl ester in the presence of sodium periodate, with absorbance recorded at 474 nm (Figure 11.C). Recombinant Wba-MIF2 protein demonstrated a significant ability to convert L-dopachrome methyl ester in vitro. The enzyme's specific activity for this conversion was measured at $19.04 \pm 0.83 \mu\text{mol}/\text{min}/\text{mg}$. The observed enzymatic activities verified the identity and function of recombinant Wba-MIF2.

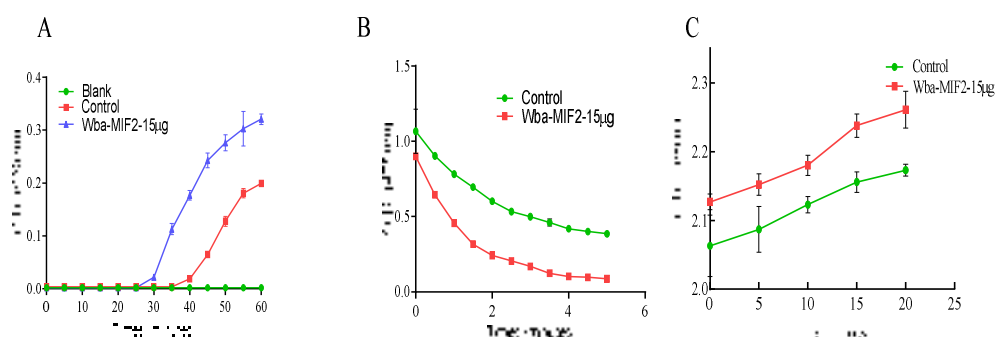


Figure 11. Enzymatic activities of recombinant Wba-MIF2; (A) Oxidoreductase activity was at 650 nm (blank containing only insulin, a control containing insulin and DTT, and a test sample containing 15 μg of Wba-MIF2, insulin, and DTT). (B) Tautomerase activity was assessed 475 nm for five minutes at room temperature (control reaction: L-Dopachrome methyl ester+sodium periodate+ elution buffer and test reaction: containing 15 μg Wba-MIF2 protein). (C) The spontaneous tautomerization rate of L-Dopachrome methyl ester was determined at 308nm (control sample: elution buffer, test reaction: L-Dopachrome methyl ester+sodium periodate+15 μg Wba-MIF2 protein).

3.3. T1DM animal model

A T1DM animal model was successfully generated by administering multiple low doses of STZ (Figure 12). Animals exhibiting blood glucose levels more than 350 mg/dl on two consecutive measurements were classified as diabetic [230].



Figure 12:Flow chart showing the development of T1DM animal model using multiple low doses of STZ.

3.3.1 Oral glucose tolerance test and area under the curve

Overnight fasted animals were used for conducting Oral Glucose Tolerance Test (OGTT). Glucose clearance rate was measured in normal and STZ-induced diabetic animals. The rate of clearance was significantly lower in the STZ-induced diabetic animals when compared to non-diabetic animals (Figure 13.A) with a significantly greater area under the curve (Figure 13.B), indicating that the multiple low dose STZ treatment induced severe T1DM among mice, successfully.

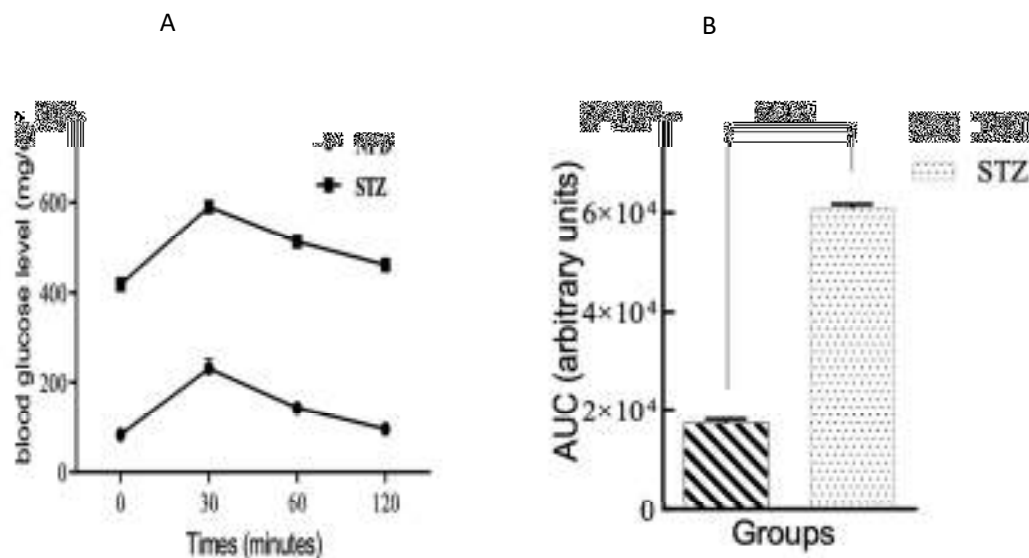


Figure 13. T1DM model development OGTT and AUC; A) Oral glucose tolerance test, B) area under the curve. NPD group: normal pellet diet fed animals group, STZ group: normal pellet diet fed animals group injected with STZ. Data points were presented as mean $\pm$ SD (n=6 animals) and compared with normal control vs Streptozotocin (STZ) treated groups were analyzed by using unpaired student-t test. (***) $p < 0.0001$. (All the data points were obtained from the single study).

3.3.2 WbaMIF-2 treatment improved the blood biochemistry of T1DM animals

Overall blood biochemistry parameters were improved in the STZ+WbaMIF-2 group compared to STZ group and NPD group. The cholesterol level in STZ+WbaMIF-2 group was significantly reduced (136.8 ± 5.081 , $p < 0.05$) compared to STZ+vehicle group (163.6 ± 10.77 , $p < 0.05$). Similarly, the level of TG and LDL were also significantly reduced in WbaMIF-2 treated group (118.1 ± 13.93 , $p < 0.001$), (37.21 ± 4.581 , $p < 0.01$) compared to vehicle (160.9 ± 11.80 , $p < 0.001$), (48.39 ± 3.668 , $p < 0.01$) respectively. In contrast, HDL was increased significantly in WbaMIF-2 treated group animals (36.52 ± 4.115 , $p < 0.05$) compared to vehicle group

(25.82 ± 3.308 , $p < 0.05$). Additionally, Hb1Ac and FBG were increased significantly in vehicle group animals (7.013 ± 0.7753 , $p < 0.01$) (136.0 ± 18.43 , $p < 0.0001$) compared to WbaMIF-2 treated group animals (9.310 ± 0.3396 , $p < 0.01$) (434.0 ± 9.849 , $p < 0.0001$) respectively (Figure 14).

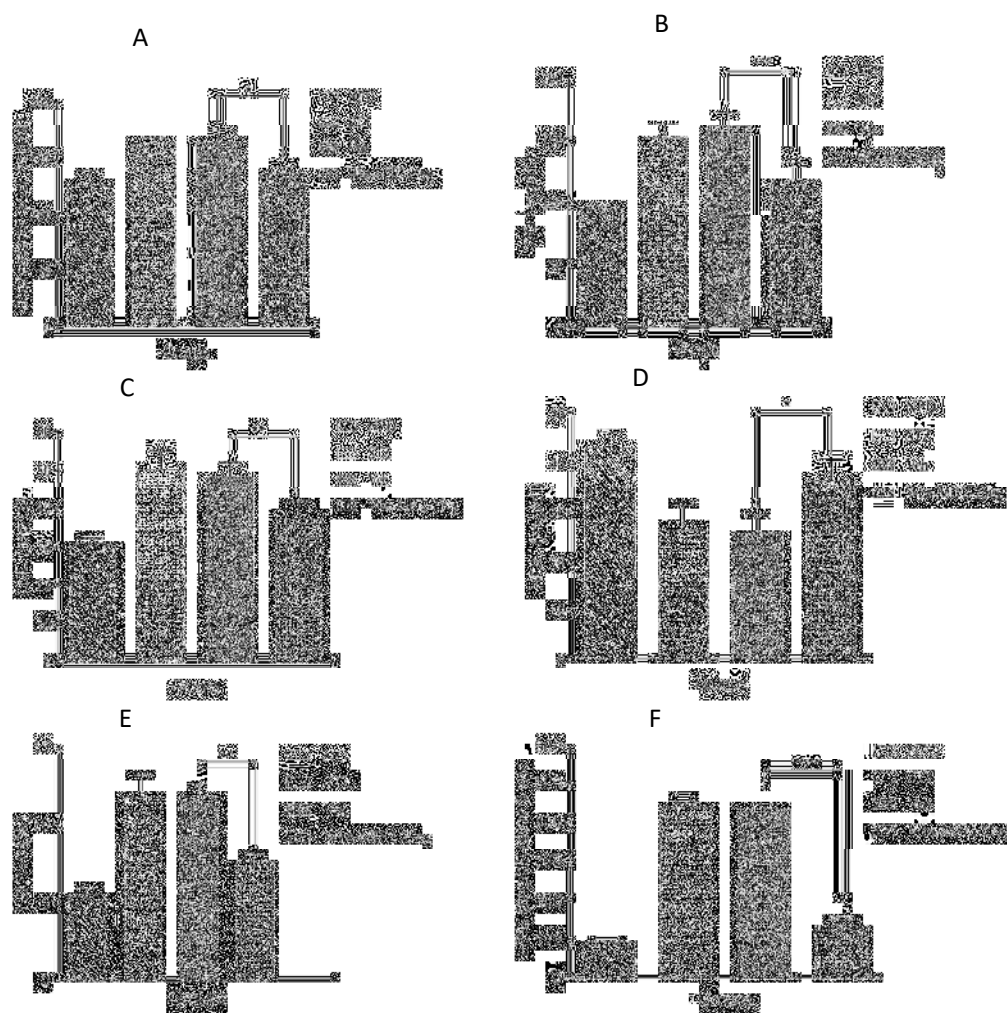


Figure 14: Blood biochemical parameters of T2DM animal (a) Cholesterol (b) Triglycerides (c) Low density lipid (d) High density lipid (e) Hb1Ac (f) fasting blood glucose (post 5th week treatment). Data were presented as mean $\pm$ SD and analyzed using One-Way ANOVA with Tukey post-hoc test. $p < 0.05$ was considered significant. (* $p < 0.5$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.0001$). (All the data points were obtained from the single study).

3.3.3 Recombinant Wba-MIF-2 treatment decreases the pro-inflammatory and increases the anti-inflammatory cytokines in T1DM animals

Serum cytokine levels of the various animal groups are depicted in Figures 15 and 16. Intraperitoneal administration of 25 µg Wba-MIF2 recombinant protein significantly influenced both pro-inflammatory and anti-inflammatory cytokine levels in streptozotocin-induced type 1 diabetes mellitus (T1DM) animal models ($p < 0.05$). After 5 weeks of the Wba-MIF2 recombinant protein treatment, a significant down-regulation ($p < 0.05$) of pro-inflammatory cytokines viz., IL-1 β (35.64 ± 0.5051 vs 44.64 ± 4.546), IL-2 (22.39 ± 0.4714 vs 29.28 ± 2.593), IL-6 (86.69 ± 3.447 vs 120.6 ± 1.326) TNF- α , (309.5 ± 2.121 vs 502.0 ± 12.73) and IFN- γ (30.80 ± 1.697 vs 40.80 ± 1.131) was observed in protein treated diabetic animals, as compared to vehicle-treated group (Figure 15). On the other hand, a significant up-regulation of anti-inflammatory cytokines viz., IL-4 (3.870 ± 0.3074 vs 2.957 ± 0.2460), and IL-10 (177.5 ± 3.536 vs 116.5 ± 14.85) was observed in protein treated diabetic animals, compared to the vehicle-treated animal groups ($p < 0.05$) (Figure 16).

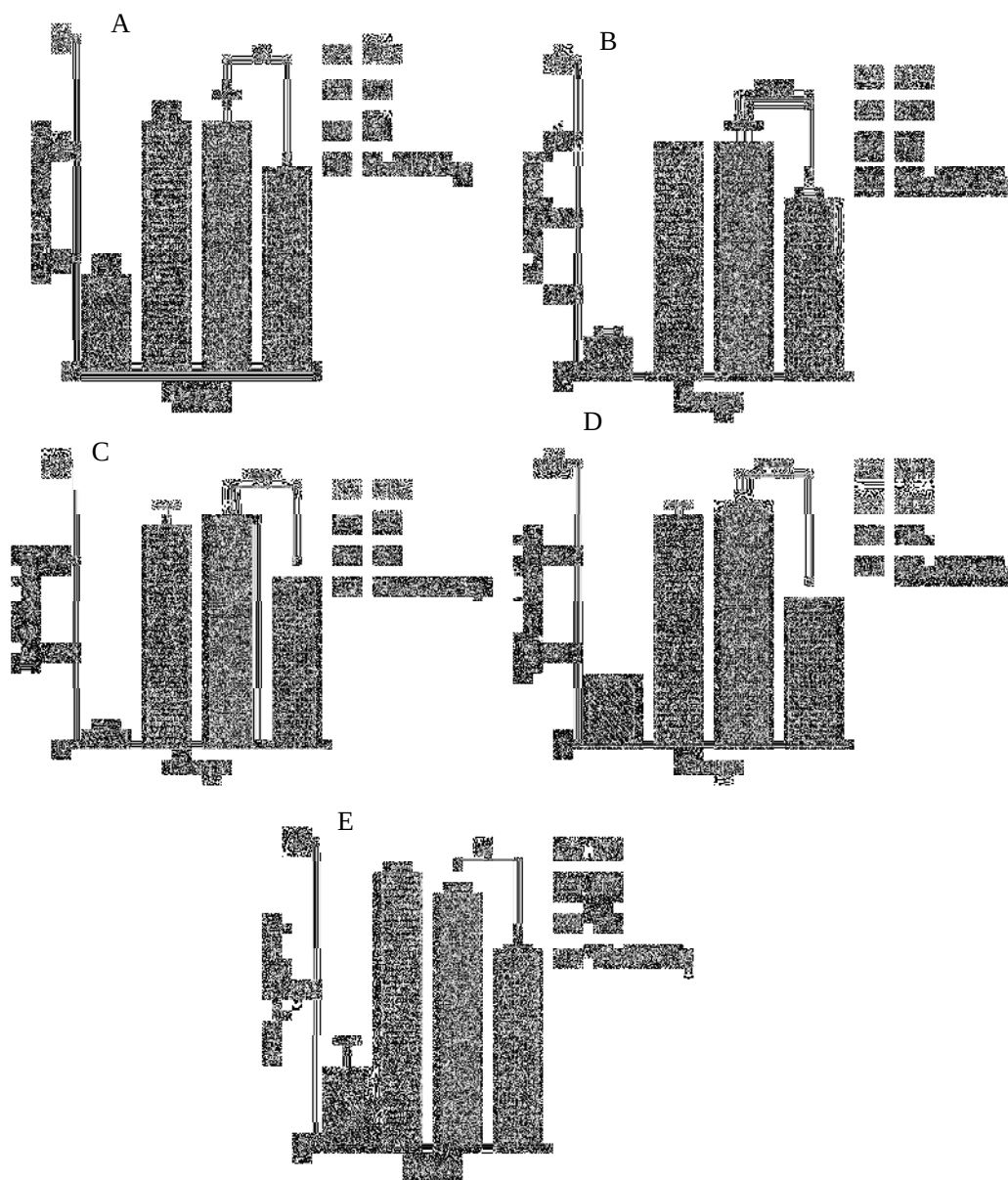


Figure 15: Pro-inflammatory cytokines level in sera samples (n=3) of T1DM animals. Panel A to E represent IL-1 β , IL-2, IL-6, TNF- α , IFN- γ levels. Data were presented as mean $\pm$ SD and analyzed using One-Way ANOVA with Tukey post-hoc test. $p < 0.05$ was considered significant. (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.0001$). (All the datapoints were obtained from the single study).

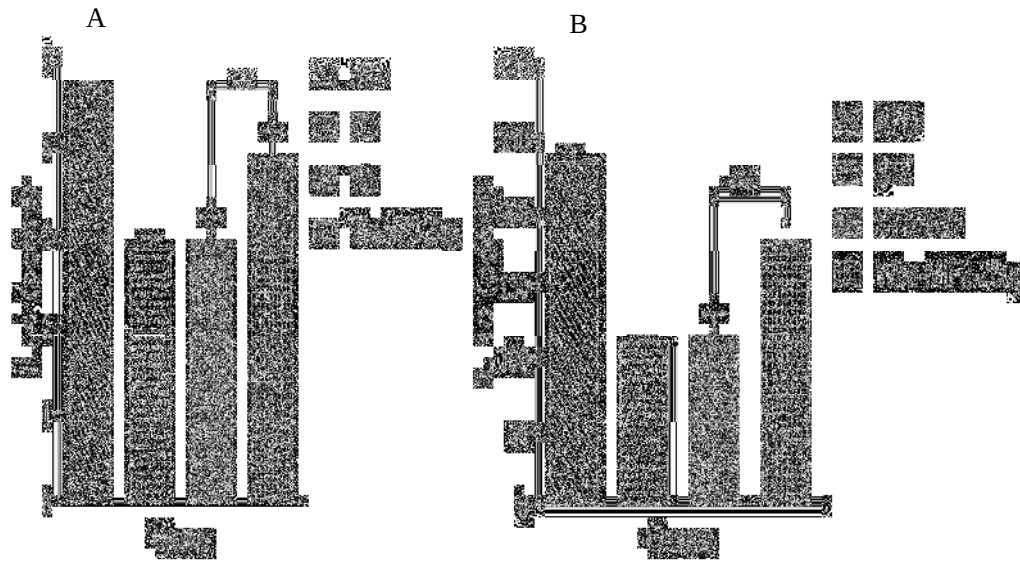


Figure 16:Ant-inflammatory cytokines level in sera samples (n=3) of T1DM animals (A) IL-4 levels and (B) IL-10 levels. Data were presented as mean $\pm$ SD and analyzed using One-Way ANOVA with Tukey post-hoc test. $p < 0.05$ was considered as significant. (** $p < 0.001$, and *** $p < 0.0001$). (All the datapoints were obtained from the single study).

3.3.4 Rrecombinant Wba-MIF-2 decreases the expression of pro-inflammatory and increase the anit-inflmmatory cytokine genes in T1DM animals

Treatment with recombinant Wba-MIF2 significantly altered the expression of genes associated with inflammation. A marked decrease in the expression of pro-inflammatory cytokine genes was observed in the spleen tissue of animals treated with recombinant Wba-MIF2 five weeks post-intervention as (IL-1 - 1.128 ± 0.4840 vs 5.319 ± 0.4825 ; IL-2 - 1.907 ± 0.2106 vs 9.201 ± 1.088 ; IL-6 - 3.075 ± 1.111 vs 12.20 ± 2.772 ; TNF- α - 1.278 ± 0.2830 vs 9.242 ± 0.4593 and IFN- γ - 1.382 ± 0.9577 vs 6.284 ± 2.077), in comparison to vehicle groups (Figure 17). In contrast, animals treated with recombinant Wba-MIF2 exhibited a significant increase ($p < 0.05$) in the expression of anti-inflammatory cytokine genes IL-4 (0.2469 ± 0.04396 vs $1.539 \pm$

0.3655) and IL-10 (1.066 ± 0.1368 vs 0.4464 ± 0.02170) within spleen tissue compared to both diabetic and control groups (Figure 18).

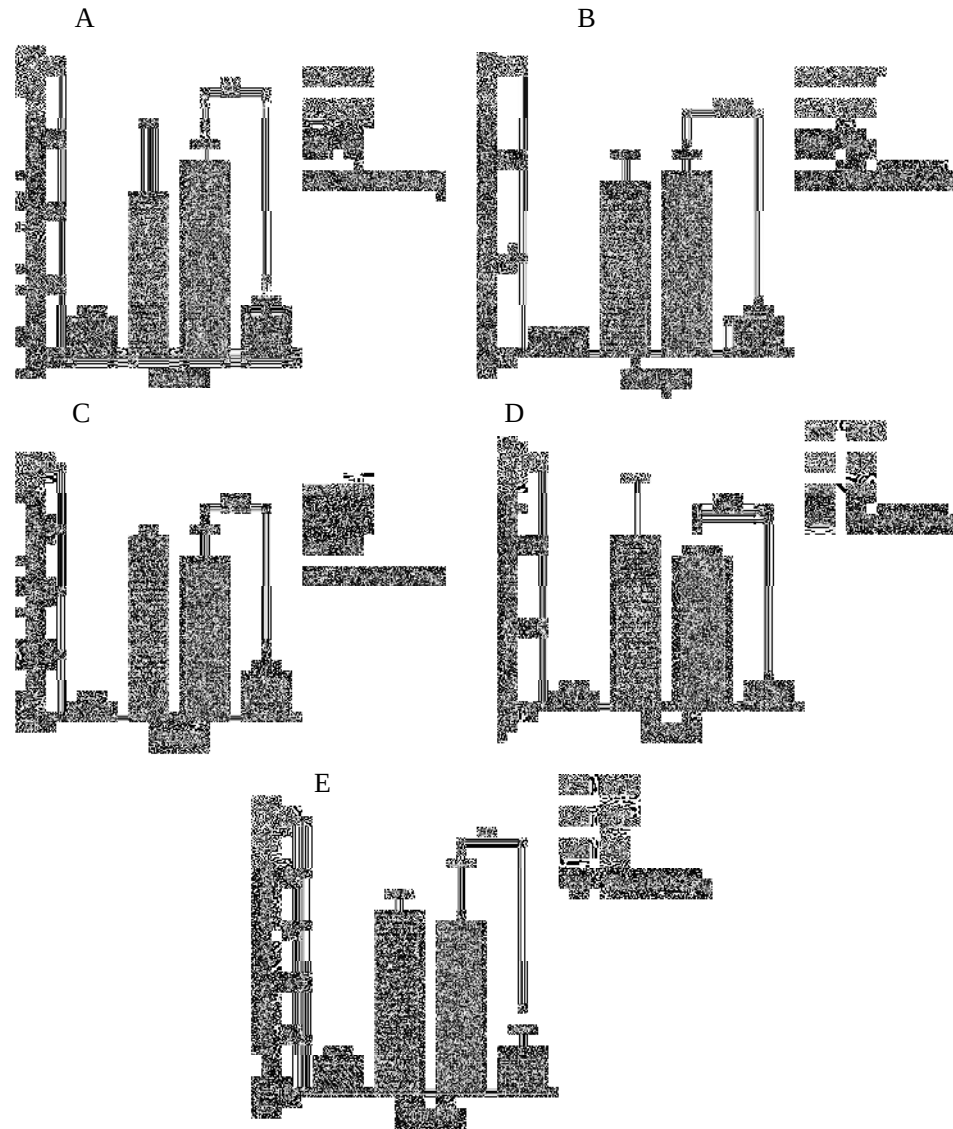


Figure 17: Expression (mRNA) level of pro-inflammatory cytokines genes in spleen tissue (n=3) of T1DM animals. Wba-MIF2 recombinant protein affected a significant reduction in proinflammatory gene expression compared to diabetic and vehicle-treated group. The data were presented as mean $\pm$ SD and $p < 0.05$ was considered significant. (** $p < 0.001$, *** $p < 0.0001$). (All the datapoints were obtained from the single study).

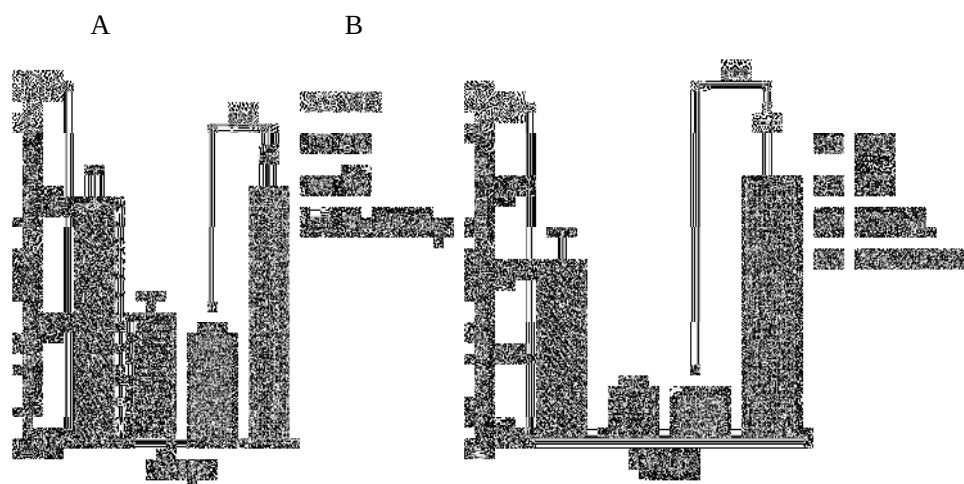


Figure 18: Expression (mRNA) of anti-inflammatory cytokines genes in spleen tissue (n=3) of T1DM animals. Wba-MIF2 recombinant protein caused significant up-regulation of mRNA level of anti-inflammatory genes compared to the vehicle-treated group. The data were presented as mean $\pm$ SD. One-way ANOVA with Tukey's test was performed and $p < 0.05$ was considered significant. (***) $p < 0.0001$; (All the datapoints were obtained from the single study).

3.3.5 Histopathological studies of T1DM animals

Histopathology reports showed that, the infiltration of macrophages and damage of beta cells due to inflammation was observed in the pancreatic tissue of diabetic animals. Vehicle (PBS) treated animals group showed hypertrophied with cytoplasmic vacuolation beta cells, less number of beta cells, more infiltrated macrophage cells and more beta cells islets damage (Figure 19.c). Wba-MIF2 treated animals showed less number of hypertrophied with cytoplasmic vacuolation beta cells, more number of intact beta cells, less infiltrated macrophage cells with less beta cells islets damage and most of the beta cells have active nuclei as compared to the vehicle treated animals group (Figure 19.d). The pancreatic tissues of a diabetic group of animals showed more damage of islet of beta cell, more number of macrophage

cell infiltration, and a low number of islets reported. On the contrary, significantly less beta cell damage, low macrophage cell infiltration, and more intact islets were observed in the recombinant Wba-MIF2-treated diabetic animal results were shown in Figure 19.

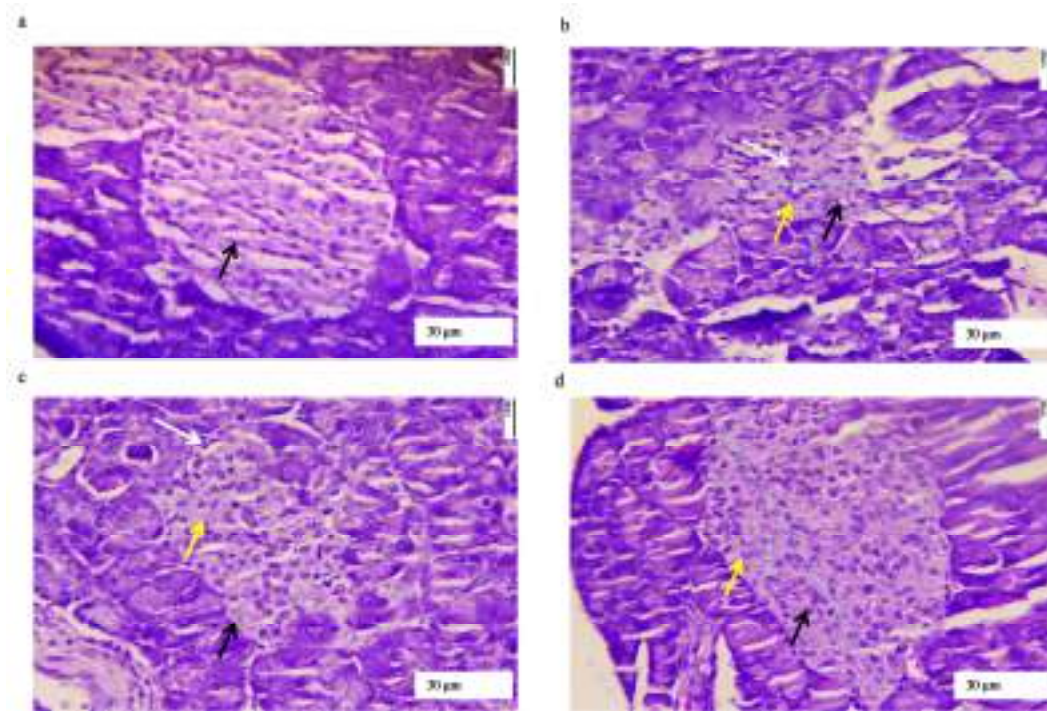


Figure 19: Histopathology of pancreatic tissue of T1DM animals. (a) Control animals exhibited intact islets of beta cells (indicated by black arrow), (b& c) Animals with diabetes treated with a vehicle control (PBS) exhibited enlarged pancreatic islet cells with cytoplasmic vacuoles (indicated by white arrows). These islets contained a reduced number of beta cells (black arrows) and increased infiltration of lymphocytes and macrophages (yellow arrows), resulting in heightened damage to the beta cell islets. (d) Animals treated with Wba-MIF2 exhibited a decreased incidence of beta cell hypertrophy with cytoplasmic vacuoles. Additionally, these animals displayed an increased number of intact beta cell islets, as indicated by black arrows. The infiltration of lymphocytes and macrophages into the beta cell cytoplasm, marked by yellow arrows, was reduced, resulting in less islet damage compared to the vehicle-treated control group. Notably, a higher proportion of beta cells in the Wba-MIF2 group demonstrated active nuclei. (H & EX400)

3.4 Type II diabetes animal model

Type II diabetes animal model was developed successfully using single low dose (70mg/kg body wt.) of STZ injection i.p with HFD diet regimen. Animals had blood glucose level more than 250mg/dl for two consecutive readings were considered as a diabetic. T2DM model development stages are shown in Figure 20 [234].

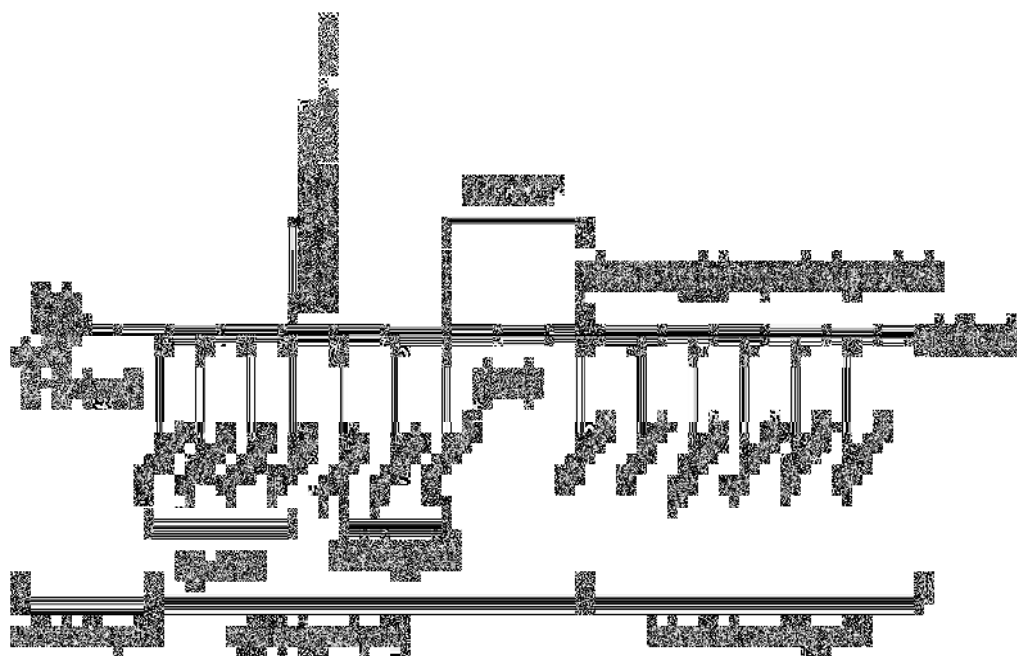


Figure 20: T2DM animal model development using combination of HFD with single low dose STZ.Wba-MIF2-15 μ g recombinant protein was tested for its immunomodulation activity.

3.4.1 Oral glucose and insulin tolerance test analysis

To confirm the establishment and progression of T2DM, animals were tested for its glucose tolerance. Glucose clearance rate was measured in NPD (group I) vs HFD (group IIb) vs HFD +STZ induced diabetic animals Rate of glucose clearance was less in the HFD+STZ induced diabetic animals as compared to non- diabetic

normal animals. Additionally, insulin tolerance test also showed that glucose clearance rate is slow in HFD+STZ animals compared to HFD and NPD group animals (Figure 21).

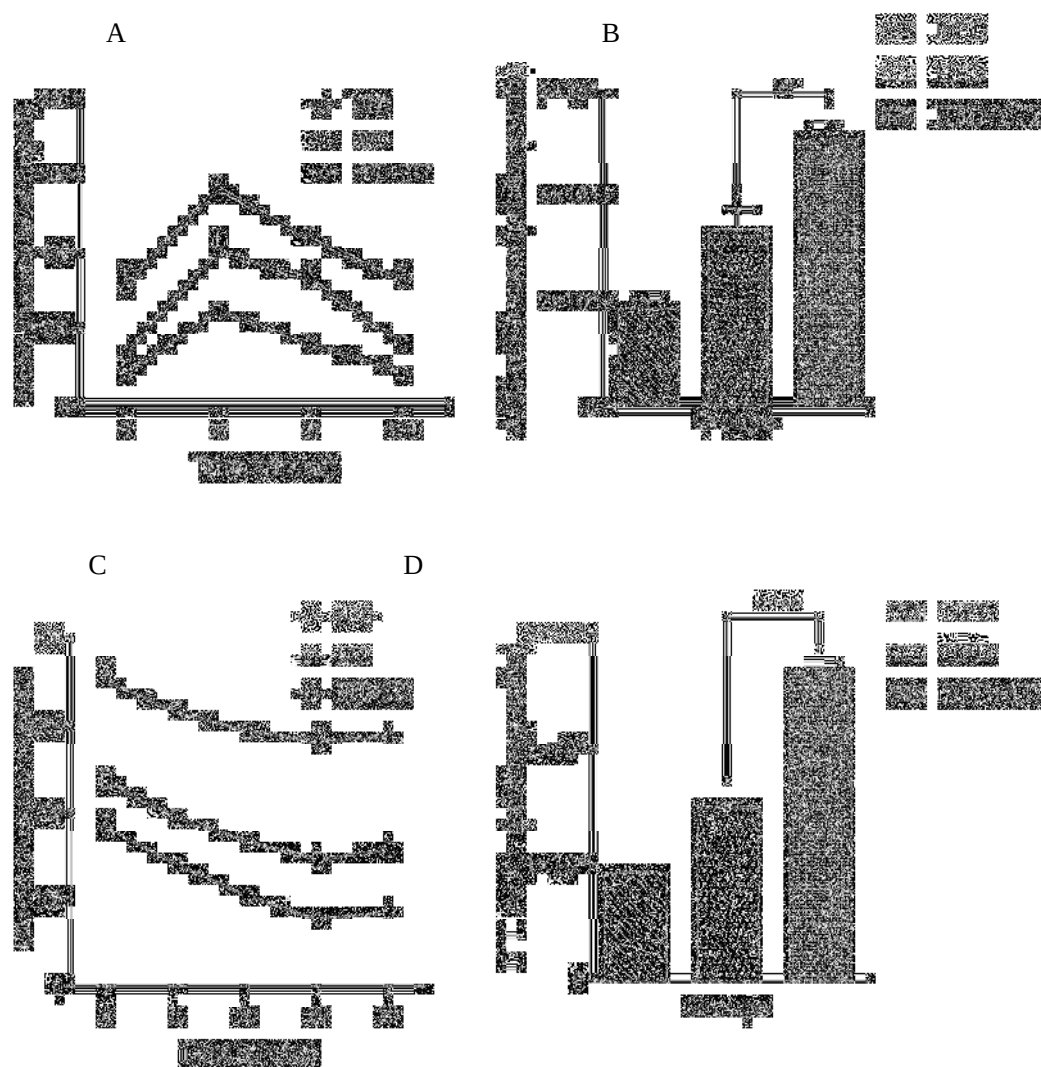


Figure 21:T2DM animal model development OGTT, ITT and respective AUC. A) Glucose clearance rate, (B) area under the curve was analyzed. Data points were represented in mean $\pm$ SD (n=6 animals) and **p<0.05 considered as significant. (C) glucose clearance rate in insulin tolerance test, (D) area under the curve for insulin tolerance test. Data points were represented in mean $\pm$ SD (n=6 animals) and **p<0.05 and ****p<0.001 considered as significant. *All the datapoints were obtained from the single study.*

3.4.2 WbaMIF-2 treatment improved the blood biochemistry in T2DM animals

Overall physiological changes were improved in the HFD+STZ+Wba-MIF-2 group compared to HFD+STZ group and HFD group. The cholesterol level in HFD+STZ+Wba-MIF-2 group was significantly reduced (182.3 ± 8.103 , $p < 0.01$) compared to HFD+STZ+vehicle group (216.5 ± 6.560 , $p < 0.01$). Similarly, the level of TG and LDL were also significantly reduced in Wba-MIF-2 treated group (227.0 ± 10.59 , $p = 0.001$), (43.47 ± 3.386 , $p < 0.01$) compared to vehicle (266.4 ± 15.68 , $p < 0.001$), (58.02 ± 5.758 , $p < 0.01$) respectively. In contrast, HDL was increased significantly in Wba-MIF-2 treated group animals (37.97 ± 4.032 , $p < 0.05$) compared to vehicle group (25.36 ± 2.731 , $p < 0.05$). Additionally, Hb1AC and FBG were increased significantly in vehicle group animals (8.833 ± 0.6801 , $p < 0.05$) (379.0 ± 24.02 , $p < 0.0001$) compared to Wba-MIF-2 treated group animals (7.155 ± 0.4548 , $p < 0.05$) (174.8 ± 10.34 , $p < 0.0001$) respectively (Figure 22).

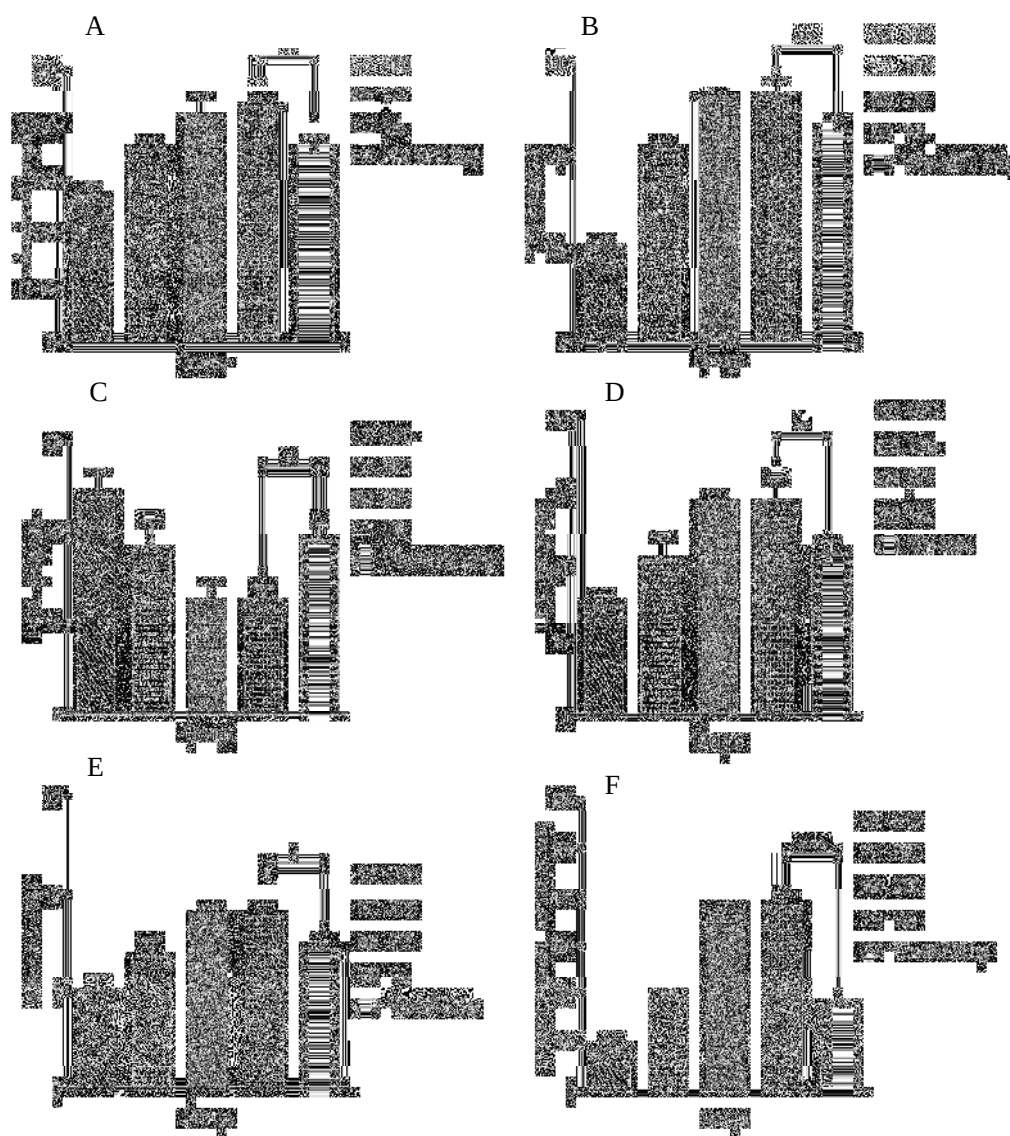


Figure 22: Blood biochemical parameters of T2DM animal. (a) Cholesterol (b) Triglycerides (c) High density lipid (d) Low density lipid (e) Hb1Ac (f) Fasting blood glucose. Data were presented as mean $\pm$ SD and analyzed using One-Way ANOVA with Tukey post-hoc test. $p < 0.05$ was considered significant. (* $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, **** $p < 0.0001$). (All the datapoints were obtained from the single study).

3.4.3 Wba-MIF-2 treatment decreases the pro-inflammatory and increases the anti-inflammatory cytokines mRNA expression level

The genes related to pro and anti-inflammatory cytokines was significantly changed due to Wba-MIF2 recombinant protein injected (i.p.) in multiple low dose (70mg/kg body wt.) of STZ-induced type I diabetic mouse model. Five-week post-injection of 25µg of Wba-MIF2 recombinant protein, the results shown a significant fold change of mRNA level in comparison of vehicle vs treated group of the pro-inflammatory cytokines; IL-1 (3.806 ± 0.5747 vs 1.592 ± 0.4747), IL-2 (2.433 ± 0.1467 vs 1.784 ± 0.2027), IL-6, (4.068 ± 0.3999 vs 2.371 ± 0.6333), TNF- α , (2.990 ± 0.1993 vs 1.669 ± 0.1315), IFN- γ (2.205 ± 0.2871 vs 1.204 ± 0.3369). On the contrary, anti-inflammatory cytokines IL-4 (0.2574 ± 0.0143 vs 0.6339 ± 0.0927), IL-10 (0.2955 ± 0.01456 vs 0.9877 ± 0.1771) genes was significantly ($p < 0.05$) upregulated in the adipose tissue (Figure 23 and 24).

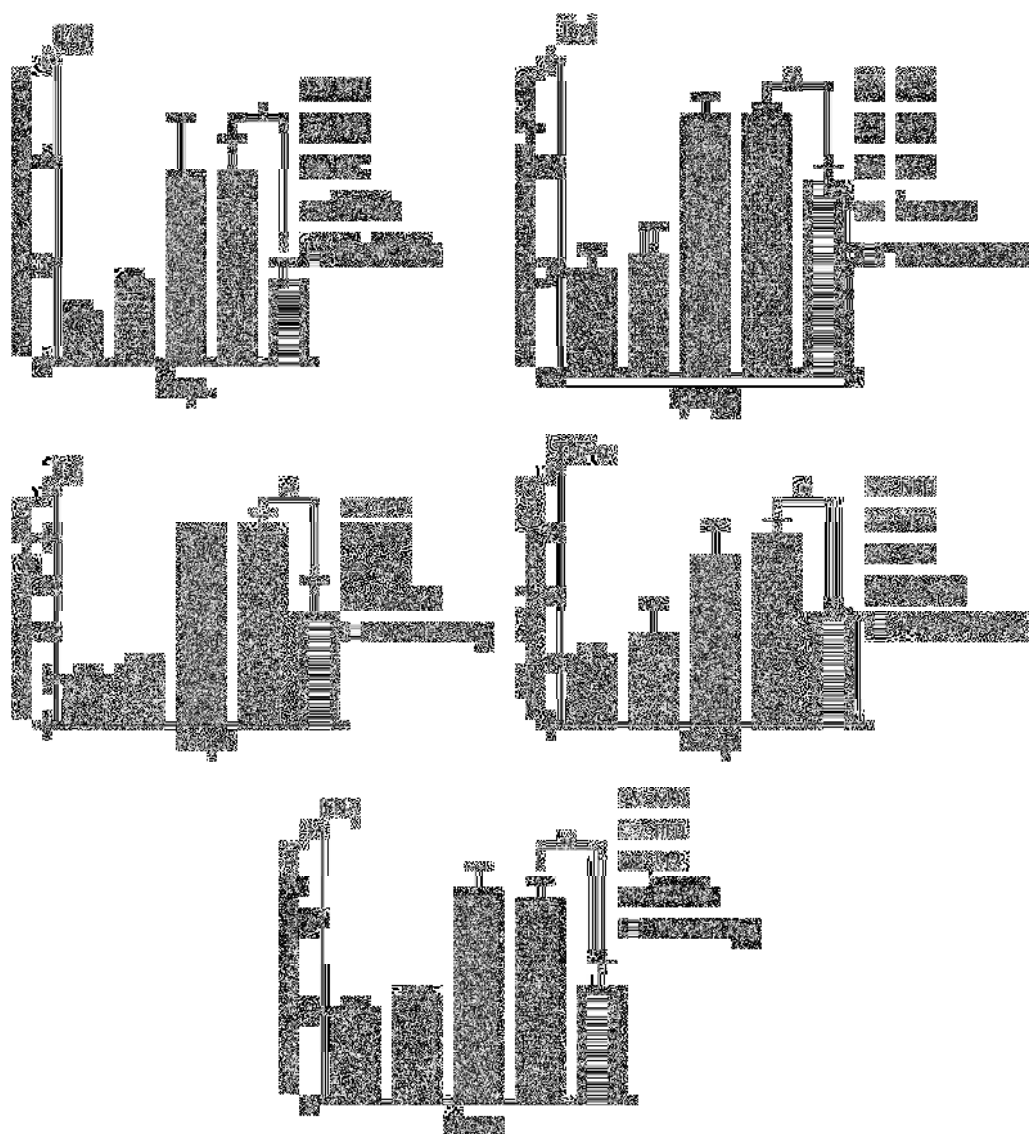


Figure 23: Expression (mRNA) level of pro-inflammatory cytokines genes in spleen tissue (n=3) of T2DM animal. Parasitic Wba-MIF2 protein had shown a significant reduction of proinflammatory gene expression compared to vehicle vs Wba-MIF2 treated groups. Gene belongs to proinflammatory cytokines presented in figure from A to E for IL-1 β , IL-2, IL-6, TNF- α , IFN- γ was significantly reduced in the treated group as compared to vehicle group respectively. All data were presented in mean $\pm$ SD and $p < 0.05$ was considered significant. (* $p < 0.05$, ** $p < 0.001$). (All the datapoints were obtained from the single study).

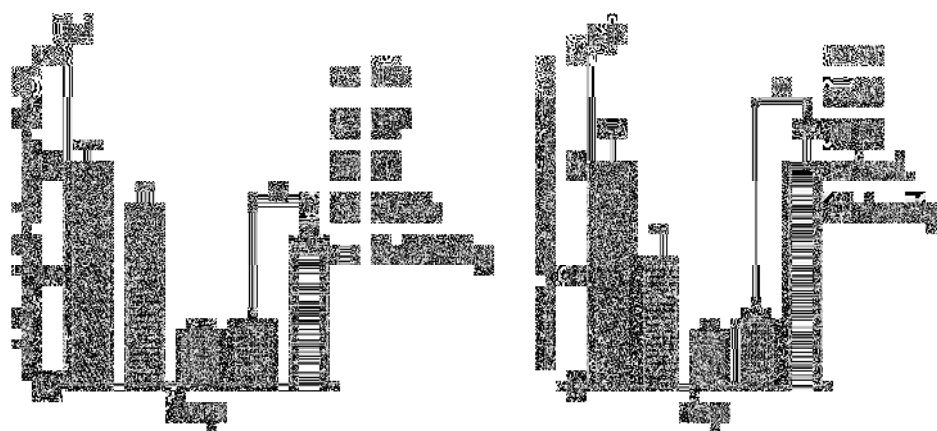


Figure 24: Expression (mRNA) level of anti-inflammatory cytokines genes in spleen tissue (n=3) of T2DM animals. Parasitic Wba-MIF2 recombinant protein had shown a significant up-regulation of mRNA level of anti-inflammatory gene compared to vehicle vs Wba-MIF2 treated groups. Gene belongs to anti-inflammatory cytokines presented in figure A and B for IL-4, IL-10, was significantly up-regulated in the treated group compared to vehicle group respectively. All data were presented in mean $\pm$ SD. One-way ANOVA with Tukey's test was performed and $p < 0.05$ was considered significant. (** $p < 0.001$, *** $p < 0.0001$). (All the datapoints were obtained from the single study).

3.4.4 Wba-MIF2 treatment decreases the pro-inflammatory and increases the anti-inflammatory cytokines in T2DM animals

Cytokine analysis in sera sample is essential to find out the Th1 and Th2 immune response by parasitic homolog vaccine antigens. Intraperitoneal injected 25 μ g Wba-MIF2 recombinant protein had shown a significant effect on the proinflammatory and anti-inflammatory cytokines in STZ induced type I diabetes. After 5 weeks of the injection cytokines level in diabetic condition was analyzed and compared the results between vehicle vs treatment group. A significant ($p < 0.05$) down regulation of pro-inflammatory cytokines levels of IL-1 β , (19.13 ± 4.808 vs 21.23 ± 9.596), IL-2, (17.89 ± 0.1571 vs 16.83 ± 0.471), IL-6, (83.06 ± 1.326 vs 58.56 ± 9.811), TNF- α , (292.2 ± 0.2121 vs 241.6 ± 3.606), IFN- γ (60.40 ± 1.131 vs

48.80 ± 1.143) and significantly ($p < 0.05$) upregulation of anti-inflammatory cytokines IL-4 (2.522 ± 0.3689 vs 3.739 ± 0.2460), IL-10 (99.10 ± 2.828 vs 139.5 ± 14.85) in the sera sample of animals (Figure 25 and 26).



Figure 25: Pro-inflammatory cytokines level in sera samples (n=3) of T2DM animals: Graph (A to E) represented IL-1 β , IL-1 β , IL-2, IL-6, TNF- α , IFN- γ level was found significantly reduced in Wba-MIF2 treated animals. All data were presented in mean $\pm$ SD and analyzed using One-Way ANOVA with Tukey post-hoc test and $p < 0.05$ was considered as significant. (* $p < 0.05$, ** $p < 0.001$). (All the datapoints were obtained from the single study).

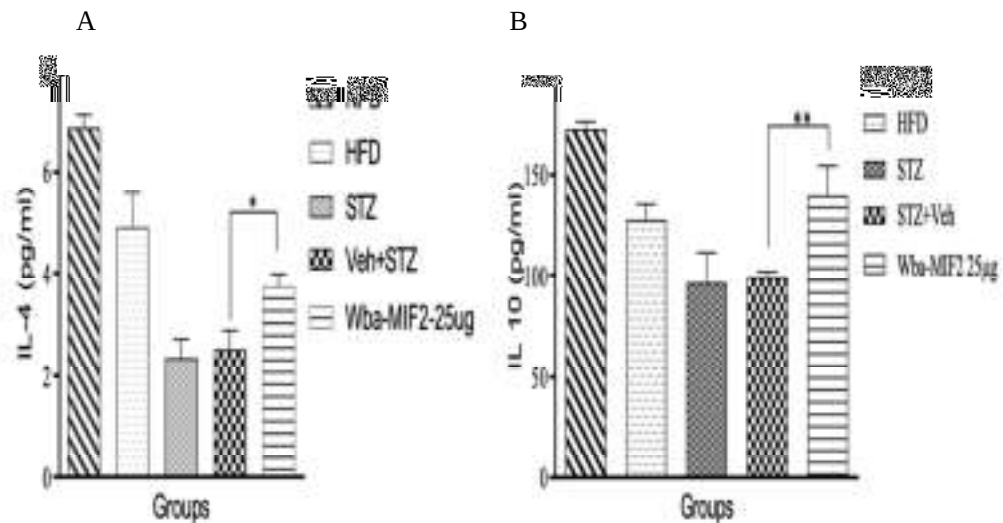


Figure 26: Anti-inflammatory cytokines level in sera samples (n=3) of T2DM animals. (A) IL-4 level and (B) IL-10 level was significantly up-regulated in Wba-MIF2 treated animals compared to vehicles treated group. All data were presented in mean $\pm$ SD and analyzed using One-Way ANOVA with Tukey post-hoc test and $p < 0.05$ was considered as significant. (* $p < 0.5$, ** $p < 0.001$). (All the datapoints were obtained from the single study).

3.4.5 Histopathology of T2DM animals

Histological analysis of the pancreatic tissue revealed inflammation-induced infiltration of macrophages and damage to beta cells in diabetic animals. Normal control animal's pancreatic islets were looking health and without inflammation (Figure 27. a). HFD treated animal's pancreatic tissue also shown inflamed and but less intact as compared to the Normal control group (figure 27. b). The STZ and PBS treated group showed several abnormalities; beta cells were enlarged (hypertrophied) with clear spaces inside the cytoplasm (cytoplasmic vacuolation). Fewer healthy beta cells were present, increased numbers of macrophages were observed, suggesting inflammatory damage to the clusters of beta cells (islets) were evident (Fig. 27.c and d). In contrast, the Wba-MIF2 treated group displayed; fewer enlarged and

vacuolated beta cells, more healthy beta cells with intact cytoplasm, significantly less macrophage infiltration, indicating less inflammation and less damage to the beta cell islets and most beta cells had visible nuclei, suggesting increased activity compared to the vehicle (PBS) treated group (Figure 27.e). Overall, the findings demonstrate that Wba-MIF2 treatment protected beta cells in diabetic animals by reducing inflammation and promoting their health.

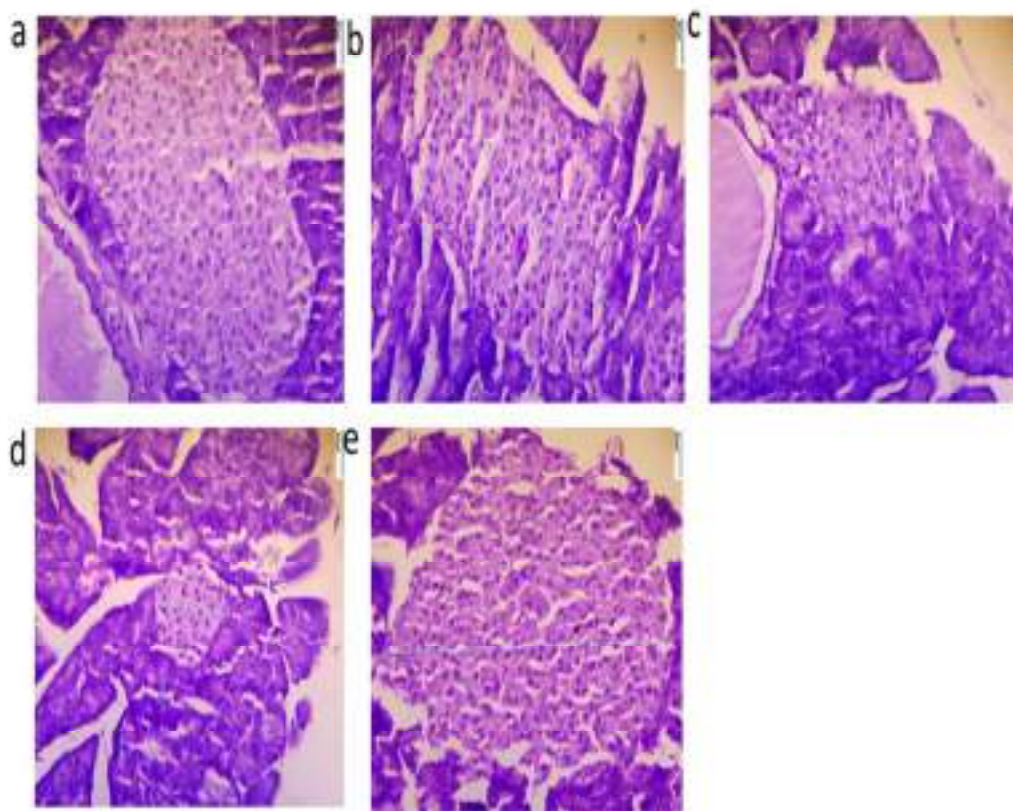


Figure 27: Histopathology of pancreatic tissue of T2DM animals. (a) normal animals show intact islets and healthy beta cells, (b) HFD fed animals, (c) STZ+HFD treated animals, (d) STZ+HFD + Veh (PBS) treated animals and (e) STZ+HFD+Wba-MIF2-25µg recombinant protein treated animals.

3.5 Phosphoproteins signaling responses mediated by Wba-MIF2 in RAW 264.7 macrophage cells

In this current investigation, we utilized an LC-MS/MS-based phosphoproteomics approach to identify the mechanism involved in Wba-MIF2-mediated signaling of RAW 264.7 macrophage cells. We also compared signaling between h-MIF and Wba-MIF2. The comprehensive workflow utilized in this study is depicted in Figure 28.A. The Signaling response mediated by Wba-MIF2 was measured in macrophages. To unveil the time-dependent impact of Wba-MIF2 on macrophages, we investigated the changing phosphorylation patterns of established signaling molecules, specifically p-44/42 MAPK (pERK) and pAKT, at five different time intervals followed by Wba-MIF2 stimulation. Increased phosphorylation of 44/42 MAPK and AKT was observed at 5 min, which decreased at 10 min. However, an increased phosphorylation was observed at 15 and 30 min. The peak phosphorylation of 44/42 MAPK was observed at 5 min in both Wba-MIF2, and h-MIF stimulated cells. However, the peak phosphorylation of AKT was observed at 5 min in response to h-MIF and at 30 min in Wba-MIF2. Based on these results, we chose 5 for h-MIF and 15 min for Wba-MIF2 for our subsequent experiments (Figure 28.B).

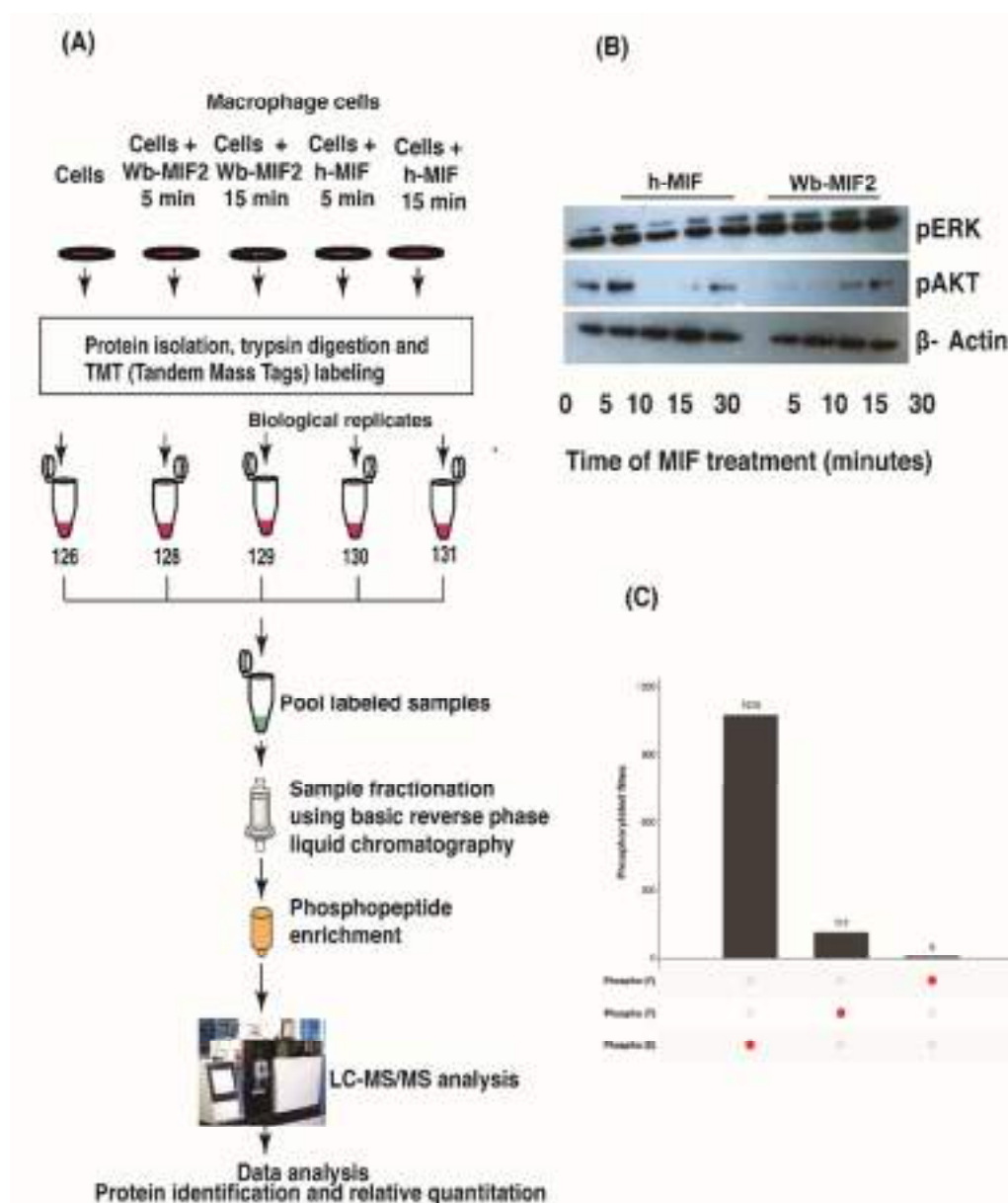


Figure 28: Phosphoproteomics work flow and Wba-MIF2 mediated signalings.

(A) Experimental workflow used for the study, (B) Western blotting results show MIF induces phosphorylation of MAPK 44/42 (ERK) and AKT. h-MIF: human MIF; Wba-MIF2: MIF2 from *Wuchereria bancrofti*. pERK phosphorylation at Thr202/Tyr204. pAKT phosphorylation at Ser473. β -Actin was used as internal control. (C) Total phosphocites identified in the present study. Y-axis denotes number of phosphocites and X-axis denotes phosphorylation at serine, threonine and tyrosine residues.

3.5.1 Quantitative temporal changes of protein phosphorylation in response to Wba-MIF2 stimulation.

We conducted a multiplexed quantitative phosphoproteome analysis to gain insights into protein dynamics and examine the signaling responses influenced by Wba-MIF. Additionally, we also compare the signaling response by Wba-MIF2 and h-MIF. RAW 264.7 macrophages were treated with Wba-MIF2 and h-MIF at different time points (0, 5, and 15 min). Upon treatment, cell samples were lysed in a lysis buffer having SDS, protease inhibitors, and phosphatase inhibitors. Subsequently, the samples underwent trypsin digestion and a C18 purification step. Equal quantities of peptide digests from each timepoint were labeled using a six-plex TMT labeling technique. Following this, phosphopeptides were enriched through TiO₂-affinity purification, and the resulting enriched phosphopeptides were subjected to analysis using an Orbitrap Fusion mass spectrometer. The database searches of the raw files obtained from LC-MS/MS analysis of the RAW 264.7 macrophages treated with Wba-MIF2 and h-MIF protein led to 0.74 million (747,866) MS/MS scans from the total proteomic experiment resulted in the identification of 0.25 million (252,151) peptide-spectrum matches (PSMs), and 19,505 peptides corresponding to 3545 proteins. Similarly, the 0.67 million (676,918) MS/MS scans from the phospho proteomics experiment resulted in the discovery of 38604 PSMs and 3488 peptides correlating to 1184 proteins. Based on the ptmRSbestsite localization probability cutoff of $\geq 75\%$, we observed 1201 phosphorylated sites on 467 proteins. Out of 1201 phosphorylated sites, 1075, 117, and 9 were found on serine (S), threonine (T), and tyrosine (Y) residues, respectively (Fig. 4C). Further, relative quantification was performed on 3253 proteins from the total proteomic experiment and 1150 phosphopeptides. Our mass spectrometry phosphoproteomics data revealed that all the

phosphopeptides identified across all the conditions mapped to 5 clusters and are differentially expressed (Figure 29.A). We have also identified differentially expressed phosphoproteins upon Wba-MIF2 treatment. Among the 3488 phosphopeptides identified, 54 peptides were significantly hyperphosphorylated (≥ 1.25 -fold), and 15 peptides were significantly hypo-phosphorylated (≤ 0.86 -fold) corresponding to 54 and 15 proteins, respectively, upon Wba-MIF2 treatment of RAW 264.7 cells. Of these significantly altered proteins, 17 were hyperphosphorylated, and three were hypo-phosphorylated at 5 min. Similarly, 37 proteins were hyperphosphorylated, and 12 were hypo-phosphorylated at 15 min Wba-MIF2 induction. The heatmap illustrates the noteworthy changes in phosphoproteins following exposure to Wba-MIF2 treatment (Figure 29.B).

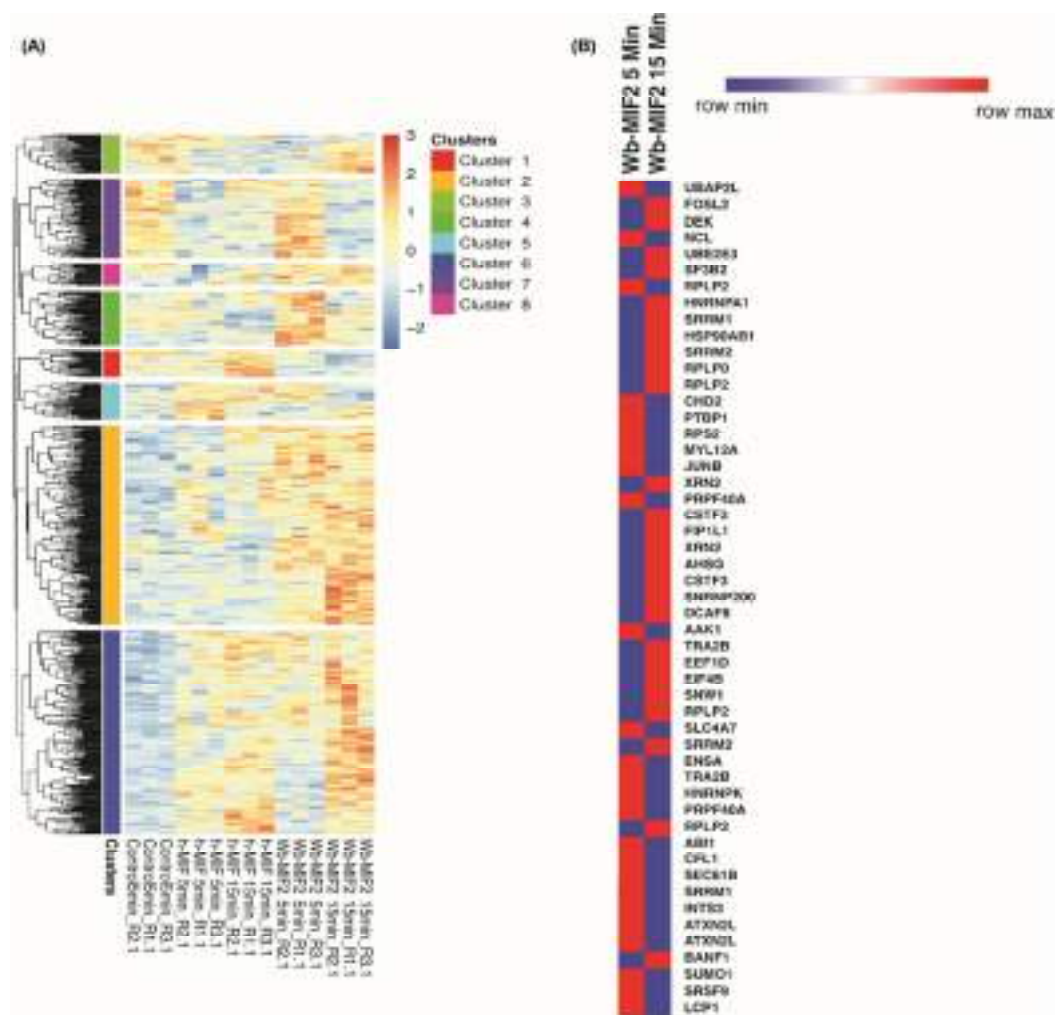


Figure 29: MIF induced phosphoproteins at 5 and 15 minutes. (A) Differentially expressed phosphopeptides across different time points induced by h-MIF and Wba-MIF2. (B) Differentially expressed phosphoproteins upon Wba-MIF2 treatment to RAW264.7 cells.

3.5.2 Functional and subcellular classification of phosphoproteins identified as targets of Wba-MIF2 stimulation

Phosphoproteins have a role in the structural and functional characteristics of cells and tissues and the coordination of several metabolic and signaling pathways. We performed functional annotation of identified phosphoproteins using the

PANTHER tool to categorize phosphoproteins based on their biological processes, molecular functions, and subcellular localization to understand better the functionality of the identified dysregulated phosphoproteins in response to Wba-MIF2 in macrophages. The subcellular localization analysis illustrated in figure 30 revealed that a majority of the phosphoproteins belonged to the cytosol (GO:0005829) (60%), extracellular exosome (GO:0070062) (34%), nucleoplasm (GO:0005654) (39%), cytoplasm (GO:0005737) (49%), membrane (GO:0016020) (34%), nucleus (GO:0005634) (46%), endoplasmic reticulum (GO:0005783) (10%) and ribosome (GO:0005840) (5%).

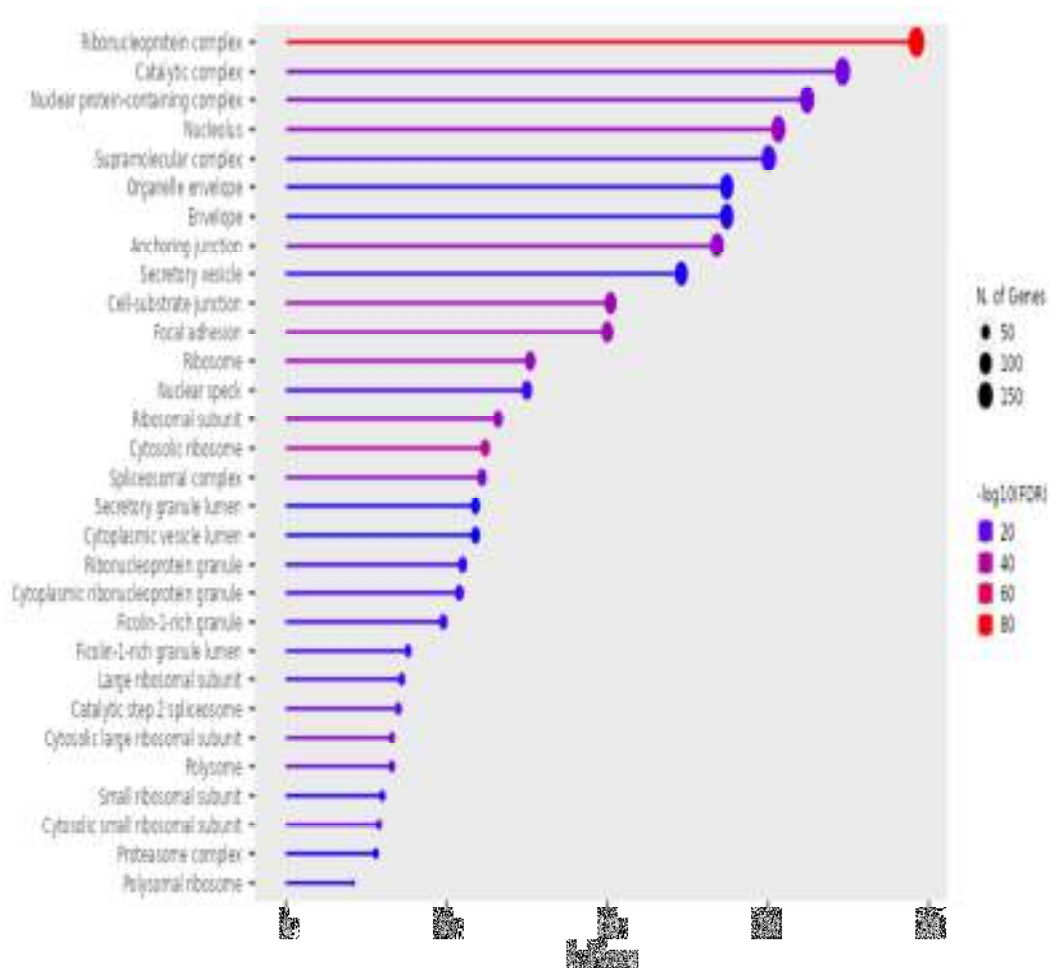


Figure 30: Wba-MIF2 mediated subcellular localization of phosphoproteins

The analysis of the biological process in figure 31 revealed most of the phosphoproteins to be involved in the cytoplasmic translation (GO:0002181) (6%), translation (GO:0006412) (8%), mRNA splicing (GO:0000398) (10%), and RNA processing (GO:0006364) (7%). Apart from these, they are also involved in the tricarboxylic acid (TCA) cycle (GO:0006099), intracellular protein transport (GO:0006886), protein transport (GO:0015031), cytoskeleton organization (GO:0007010).

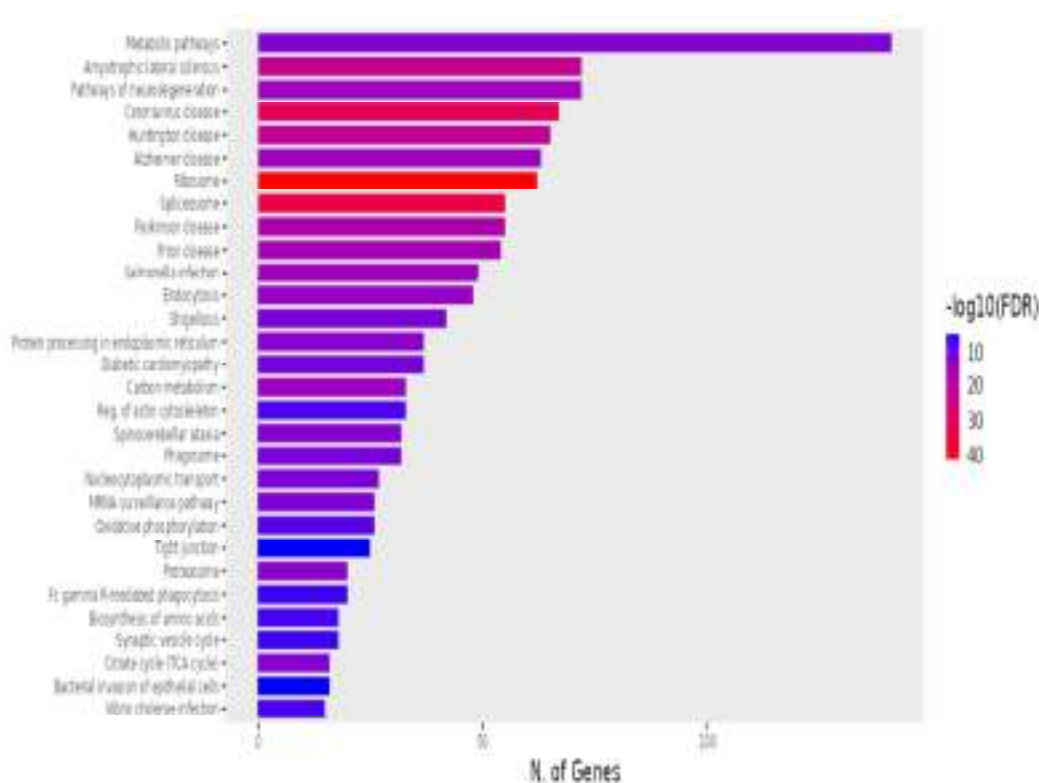


Figure 31: Wba-MIF2 mediated GO enrichment for biological process of phosphoproteins

The molecular functional analysis in figure 32 pointed out that the majority of phosphoproteins belonged to protein binding (GO:0005515) (89%), RNA binding (GO:0003723) (34%), ATP-binding (GO:0005524) (14%), protein binding (GO:0042802) (14%), DNA binding (GO:0003677) (9%), cadherin binding

(GO:0045296) (8%), ATPase activity (GO:0016887) (6%), protein homodimerization activity (GO:0042803) (6%) among others.

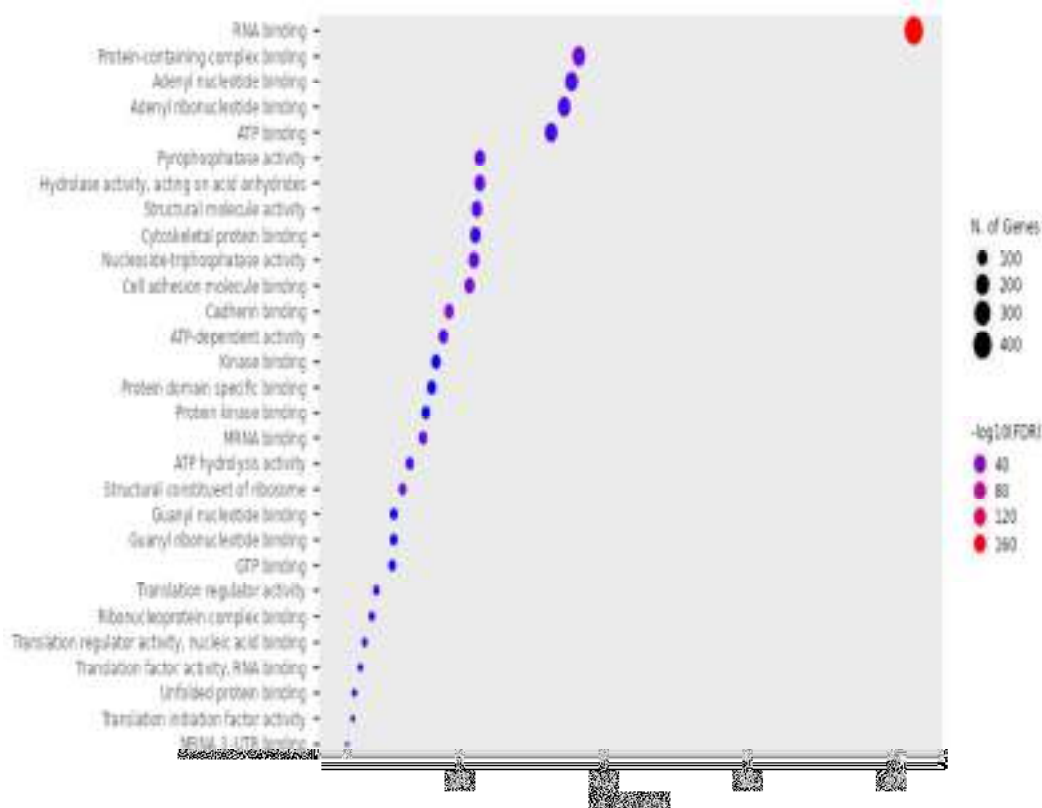


Figure 32: Wba-MIF2 mediated GO enrichment for molecular function of phosphoproteins.

3.5.3 Wba-MIF2 stimulated pathways enrichment analysis

Protein dynamics is a key factor in regulating several cellular pathways. We conducted pathway enrichment analysis using both David and Panther tools. We identified several significant pathways ($p < 0.05$) (Figure 33). We have shortlisted essential pathways such as Spliceosome, mRNA surveillance pathway, IL-17 signaling pathway, VEGF signaling pathway, Sphingolipid signaling pathway, Insulin signaling pathway, PI3K-Akt signaling pathway, and mTOR signaling pathway. The proteins involved in these pathways are given in Supplementary Table 7.

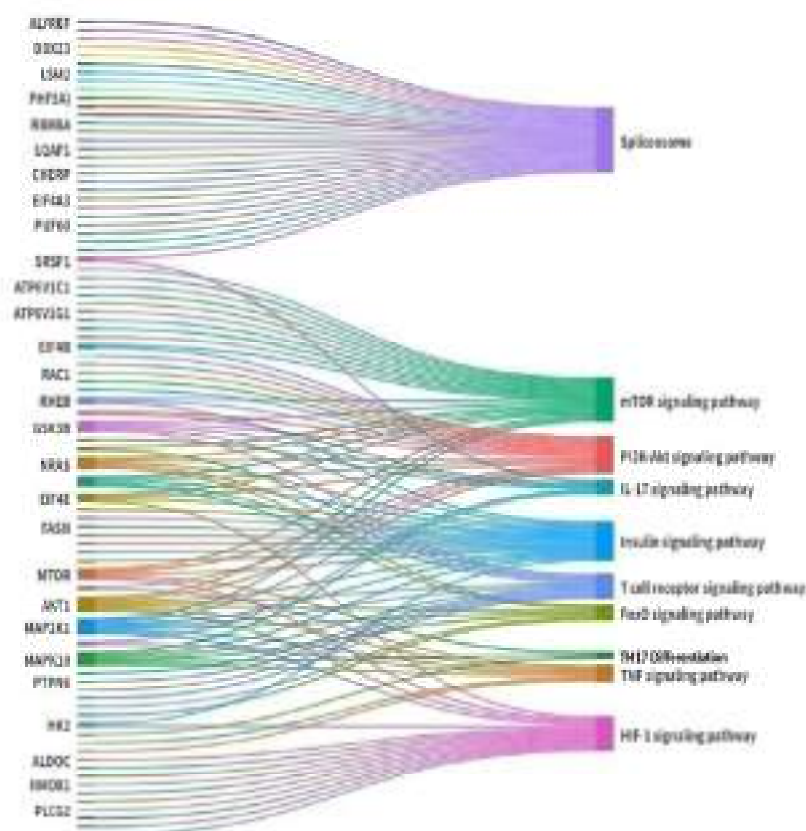


Figure 33: Pathways altered by Wba-MIF2. A schematic diagram depicting the categorization of proteins that exhibited differential regulation after Wba-MIF2 treatment.

3.5.4 Wba-MIF2 stimulated transcription factor enrichment analysis

Several proteins called transcription factors are involved in the process of transcription of DNA. Numerous phosphoproteins serve as transcription factors. We enriched transcription factors by using the online eXpression2Kinases (X2K; <http://amp.pharm.mssm.edu/X2K/>) tool to gain more insight into Wba-MIF2 mediated signaling. Our analysis leads to the enrichment of several transcription factors, a few of them are TAF1, ATF2, MYC, BRCA1, CREB1, CHD1, MYC, MAX, YY1, TAF7, TCF3, E2F4, E2F1, PML, NRF1, NELFE (Figure 34). A detailed list of transcription factors enriched is provided in Supplementary Table 8.

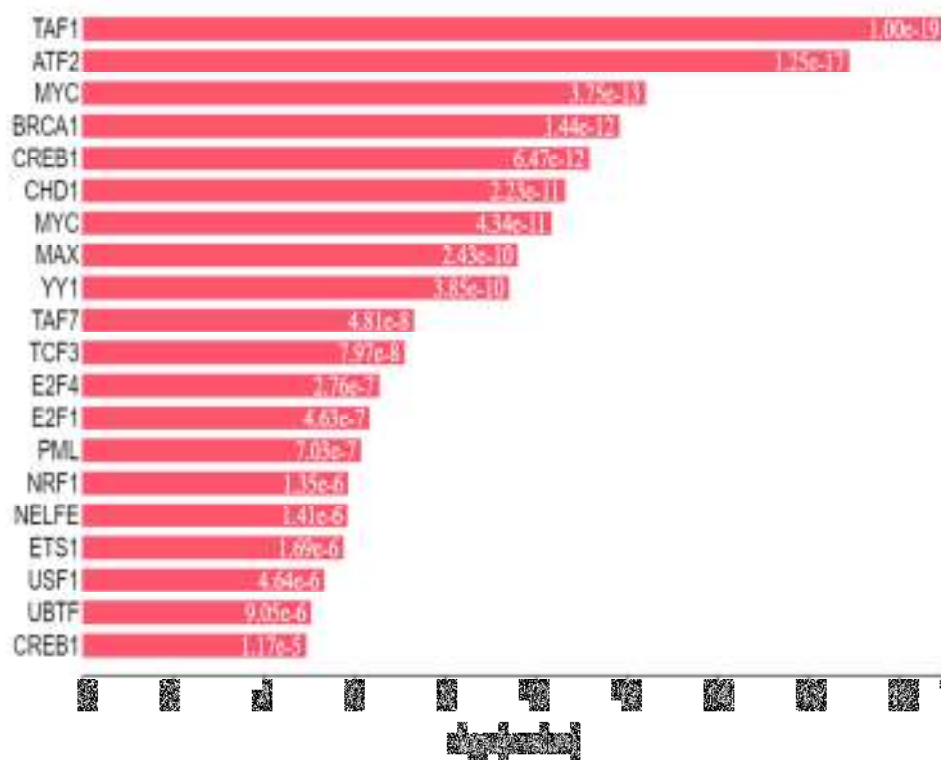


Figure 34: Transcription factor enrichment from dysregulated phosphoproteins identified upon Wba-MIF2 treatment.

3.5.5 Wba-MIF2 stimulated kinase enrichment analysis

In addition to the enrichment of transcription factors, we conducted kinase enrichment analysis using X2Kweb, an online tool for identifying dysregulated phosphorylated proteins following Wba-MIF treatment. X2Kweb employs computational methods to predict the involvement of upstream cell signaling pathways. Our analysis revealed an enrichment of CDK5, CDC2, DNAPK, and CDK2 as downstream kinases in the Wba-MIF-induced signaling cascade (Figure 35.A). These findings suggest that the signaling cascade mediated by Wba-MIF2 in macrophages may involve CDK5, CDC2, DNAPK, and CDK2, subsequently engaging MYC, TCF3, MAX, ATF2, TAF7, YY1, CREB1, BRCA1, and TAF1 transcription factors (Figure 35.B)

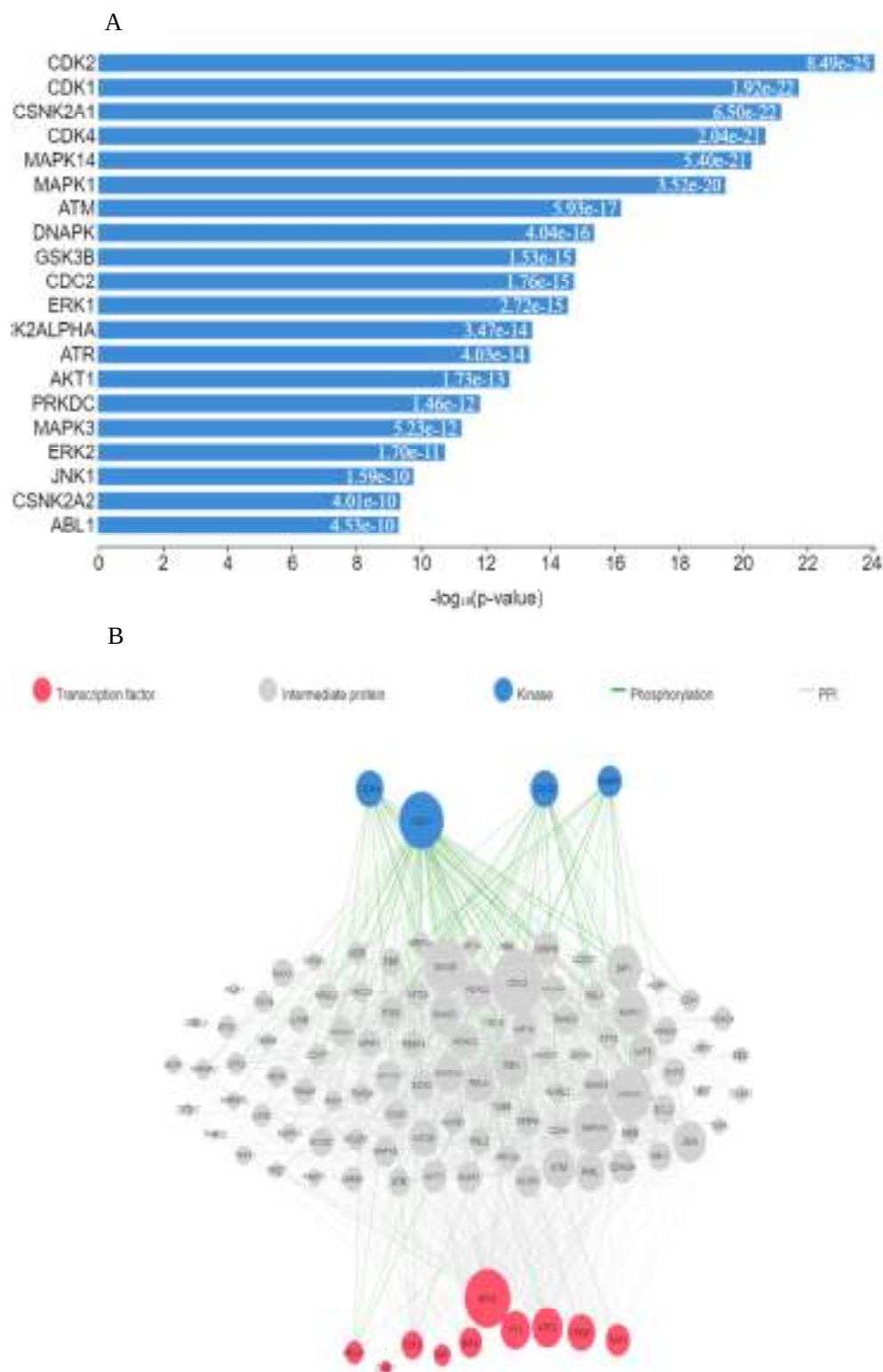


Figure 35: Wba-MIF2 mediated alterations in the phosphoproteome of macrophage cells.

Kinases are enriched among the identified phosphoproteins in macrophages exposed to Wba-MIF2. B) the expression kinases analysis of phosphorylated proteins presents a network illustrating the enriched kinases, protein-protein interactions, and transcription factors within the phosphorylated dataset of macrophages treated with Wba-MIF2.

3.5.6 Comparison between h-MIF and Wba-MIF2 treatment

As a pleiotropic protein, macrophage migration inhibitory factor (MIF) contributes to inflammatory and immunological reactions. MIF was initially identified as a lymphokine involved in tumoricidal action, delayed hypersensitivity, and several macrophage related activities, such as phagocytosis and dissemination. In the present study, we compared signaling mediated by Wba-MIF2 and h-MIF to see the efficiency of modulating immunological response compared to h-MIF. We have compared the phosphorylation status of the proteins dysregulated by h-MIF and Wba-MIF2. We also compared differentially expressed proteins from treatment with Wba-MIF2 and h-MIF. Our results show that h-MIF and Wba-MIF2 regulated the exact signaling mechanism; however, Wba-MIF showed some advantages over the h-MIF. The table 9 shows the number of differentially expressed proteins in each comparison. Figure 36 represents the heat map corresponding to the expression of proteins and phosphoproteins in various conditions.

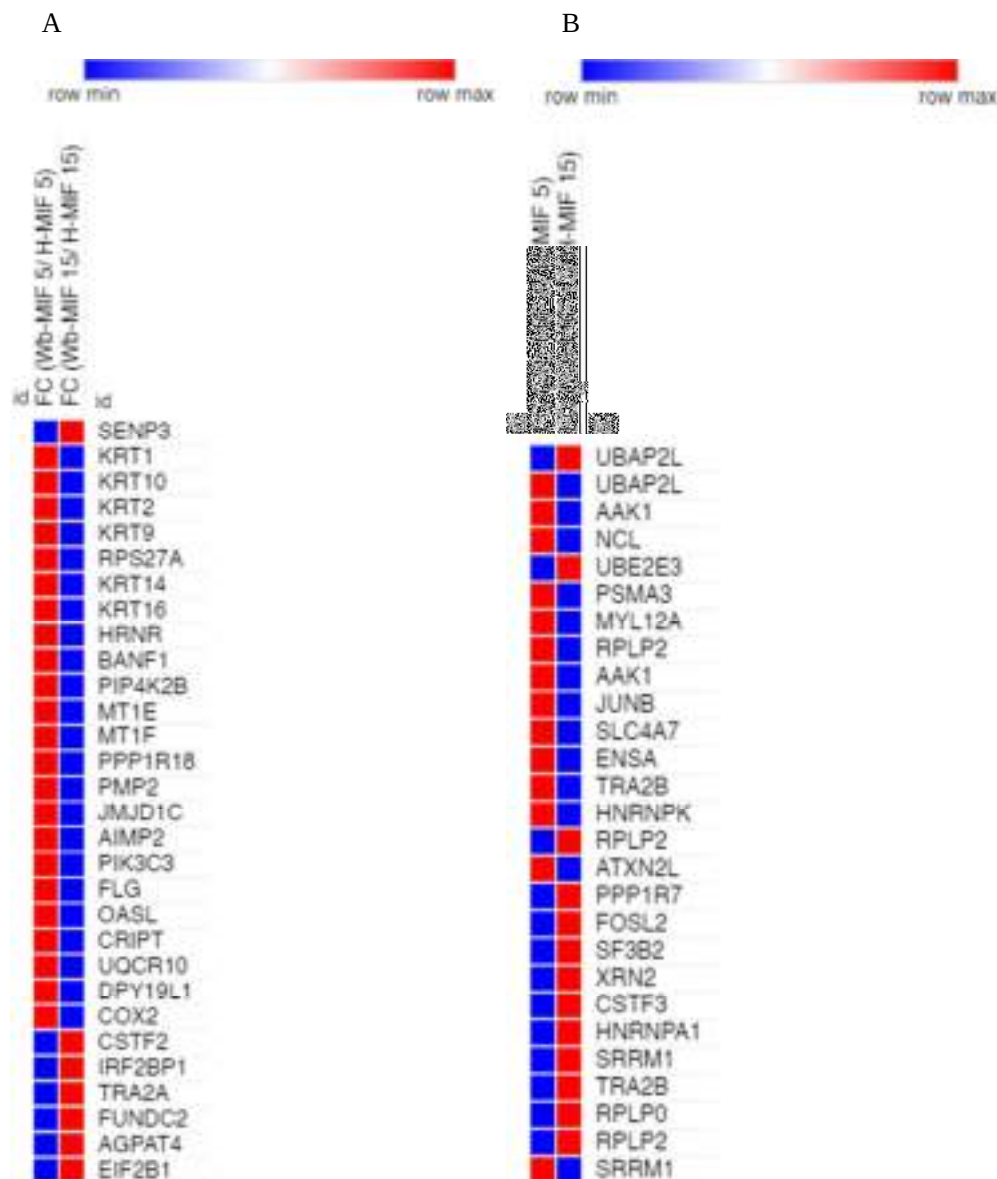


Figure 36: Wba-MIF2 and h-MIF mediated differential proteins and phosphoproteins expression. A comparison between Wba-MIF2 and h-MIF is carried out. The heat map shows a comparison between Wba-MIF2 and h-MIF. A: at the protein level and B: at the phosphoprotein level.

Table 7.Summary of differentially expressed proteins and Phosphoproteins

Comparison	Total-Proteome		Phospho-Proteome	
	Up-regulated (FC ≥ 1.25 and p-value < 0.05)	Down- regulated (FC ≤ 0.80 and p-value < 0.05)	Hyper phosphorylated (FC ≥ 1.25 and p-value < 0.05)	Hypo phosphorylated (FC ≤ 0.80 and p-value < 0.05)
Human MIF 5min vs Control 5min	10	4	18	9
Human MIF 15min vs Control 5min	20	0	38	8
Wba-MIF2 5min vs Control 5min	9	0	21	2
Wba-MIF2 15min vs Control 5min	13	9	70	13
Wba-MIF2 5min vs Human MIF 5min	3	5	22	9
Wba-MIF2 15min vs Human MIF 15min	2	16	37	16

Table 8. Partial list of the differentially regulated phosphoproteins upon Wba-MIF2 stimulation.

Gene Symbol	Phosphosite Modification	Fold Change (Wba-MIF Treated/Untreated)	
		5 Min	15 Min
UBAP2L	hYEDGYPGGSDNYGSLsR	1.411489	1.52387
UBAP2L	tGDLGIPPNPEDRsPsPEPIYNSEGk	1.46933	1.272354
AAK1	sATTTPsGsPR	1.446567	1.404682
NCL	MAPPPKEVEEDsEDEEMsEDEEDDsGEEV VIPQK	1.697519	1.376107
UBE2E3	QRsDDESPSTSSGSSDADQR	1.291608	1.502007
PSMA3	ESLKEEDsDDDNM	1.57672	1.368004
MYL12A	AtsNVFAMFDQSQIQEFK	1.895523	1.123517
RPLP2	KEEsEEsDDDMGFGLFD	2.674506	1.907799
SUMO1	sDQEAKPSTEDLGDKK	1.256548	1.117476
AAK1	SATTTPSGsPR	1.462033	1.352155
SRSF9	GsPHYFSPFRPY	1.260935	1.227728
JUNB	SRDAtPPVsPINMEDQER	1.332762	1.056738
SLC4A7	KHsDPHLLER	1.25897	1.254215
LCP1	GsVSDEEMMELR	1.299723	1.163718
ENSA	YFDsGDYNMAK	1.424717	1.355641

TRA2B	HsHsHsPMSTR	1.33301	0.900644
HNRNPK	DYDDMsPR	1.452556	1.3857
RPLP2	KEEsEEsDDDMGFGLFD	0.710428	0.82614
ATXN2L	STSTPTsPGPR	0.84023	0.729607
BANF1	ttsQKHR	0.856183	0.935541
PPP1R7	RVEsEEsGDEEGK	1.115837	1.64886
FOSL2	GsSGsPAHAESYSSGGGGQK	1.188398	1.784538
FIP1L1	HEsEEGDSHR	1.065067	1.269234
SF3B1	GSEtPGAAtPGSK	1.238508	1.265721
DEK	KEsEsEDssDDEPLIK	1.077535	1.303458
HSP90AB1	IEDVGsDEEDDSGKDK	1.124545	1.259734
SF3B2	GFEEHKDsDDDssDDEQEK	1.102707	2.521526
NCL	EVEEDsEDEEMsEDEEDDssGEEVVIPQK	1.208602	1.31725
XRN2	AEDsDsEPEPEDNVR	1.101169	1.369278
AHSG	CDSSPDsAEDVR	1.227368	1.279914
CSTF3	RPNEDsDEDEEK	1.059664	1.380998
SNRNP200	EAEtDsDsD	1.037118	1.254622
DCAF8	DQDssDDER	1.014164	1.292311
TRA2B	SGsAHGSGK	1.139667	1.308488
HNRNPA1	SEsPKEPEQLR	1.055408	1.371693
EEF1D	ATAPQTQHVSPMR	1.241908	1.292038

EIF4B	HPsWRsEETQER	1.158034	1.284627
SNW1	GPPsPPAPVMHsPSR	1.247163	1.291559
SRRM1	sVsGsPEPAAK	1.131133	1.52958
HSP90AB1	IEDVGsDEEDDsGKDK	1.164239	1.333718
RPLP2	KEEsEEsDDDMGFGLFD	0.877865	1.269058
SRRM2	sLtRsPPAIR	1.03753	1.276944
TRA2B	SGsAHGsGK	1.145602	1.325036
SRRM2	GRsGssSER	1.007992	1.287511
RPLP0	EEsEEsDEDMGFGLFD	0.953114	1.829203
RPLP2	EEsEEsDDDMGFGLFD	1.073276	1.473818
SRRM1	TRHsPtPQQSNR	0.862256	0.779392
INTS3	KGsSAVGsDsD	0.930316	0.831951
ATXN2L	STsTPTSPGPR	0.911049	0.813614
PTBP1	TDSsPNQAR	1.008052	0.815196
RPS2	THTRVsVQR	1.09841	0.835654

Table 9. A partial list of the comparison of the dysregulated phosphoproteins upon Wba-MIF2 and h-MIF stimulation.

Gene Symbol	Phosphosite Modification	Fold Change (Wba-MIF2/h-MIF treated)	
		5 Min	15 Min
PPP1R7	RVEsEEsGDEEGK	1.045444	1.472339
MYL12A	AtsNVFAMFDQSQIQEFK	0.993107	1.411578
SF3B2	GFEEEHKDsDDDssDDEQEK	1.125618	2.356023
NCL	EVEEDsEDEEMsEDEEDDssGEEVVIPQK	1.09853	1.573233
RPLP2	KEEsEEsDDDMGFGLFD	1.773414	1.358587
DCAF8	DQDssDDER	1.005756	1.411046
SRRM1	sVsGsPEPAAK	1.071024	1.36456
RPLP0	EEsEEsDEDMGFGLFD	1.061882	1.608899
RPS6	LSsLRAsTSK	1.293021	1.28208
RPLP2	EEsEEsDDDMGFGLFD	1.262551	1.432403
ABI1	THsGSSGGSGSR	1.051394	0.828613
HDAC2	MLPHAPGVQMQAIPEDAVHEDsGDEDGEDPD KR	1.077086	0.795191
NCL	MAPPPKEVEEDsEDEEMsEDEEDDssGEEVVIP QK	1.356751	1.061793
MYL12A	AtsNVFAMFDQSQIQEFK	1.649576	1.030301
TRA2A	sRsHsPMSNR	1.335105	0.956655

RPLP2	EEsEEsDDDMGFGLFD	1.262551	1.432403
TRA2B	HsHsHsPMSTR	1.657835	0.895281
ZC3H13	NEGsPsPRQsPK	1.351157	1.042408
RPLP2	KEEsEEsDDDMGFGLFD	0.818895	1.072395
PI4KB	sVENLPECGITHEQR	0.810001	1.180612
C2CD5	SQsEssDEVTELDLSHGK	0.855485	1.108013
HNRNPD	IDASKNEEDEGHNSsPR	0.831065	0.822662
AKAP13	FLSHsTDsLNK	0.858982	0.879473
RPLP2	KEEsEEsDDDMGFGLFD	0.805454	1.003064

3.6 Results of the bioinformatics studies

3.6.1 Modelling of MIF2 trimeric form

Among the 100 modelled structures, a DOPE score of -44318.52 was obtained as lowest for a structure and was preceded for stereochemical check. Ramachandran plot for the chosen model showed 93.7% of residues to be in the favoured region with no residues in the disallowed or additionally allowed region (Figure 37). The model also scored ProSA Z-score of -6.25, LG score of 6.85 and MaxSub score of 0.631 indicating the good quality of the modelled structure.

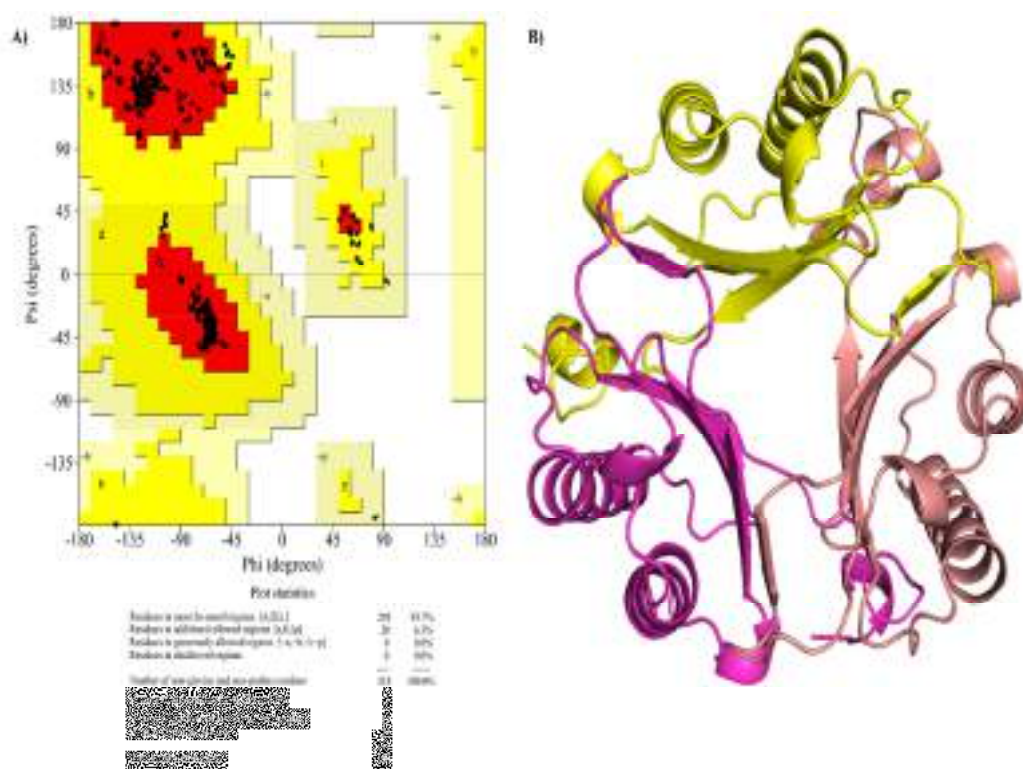


Figure 37: Ramachandran plot and Cartoon Representation of MIF2 protein.

(A) Ramachandran plot of the modelled MIF2 protein (B) Cartoon Representation of the modelled MIF2 protein coloured as: Chain-C (pink), Chain-D (yellow) and Chain-E (Salmon).

3.6.2 MIF2-Insulin docking:

The HDOCK result of the MIF2-Insulin docking with the top best model showed a docking score of -196.98 (Figure 38.A). Further, this complex was subjected to binding energy scoring using PRODIGY server, which inferred a significant ΔG value to be -10.2 kcal/mol. Subsequently, the protein-protein intermolecular interactions were visualized using DIMPLOT. This inferred 6 H-bonded interactions and extensive hydrophobic interactions among MIF2-Insulin docked complex (Figure 38.B).

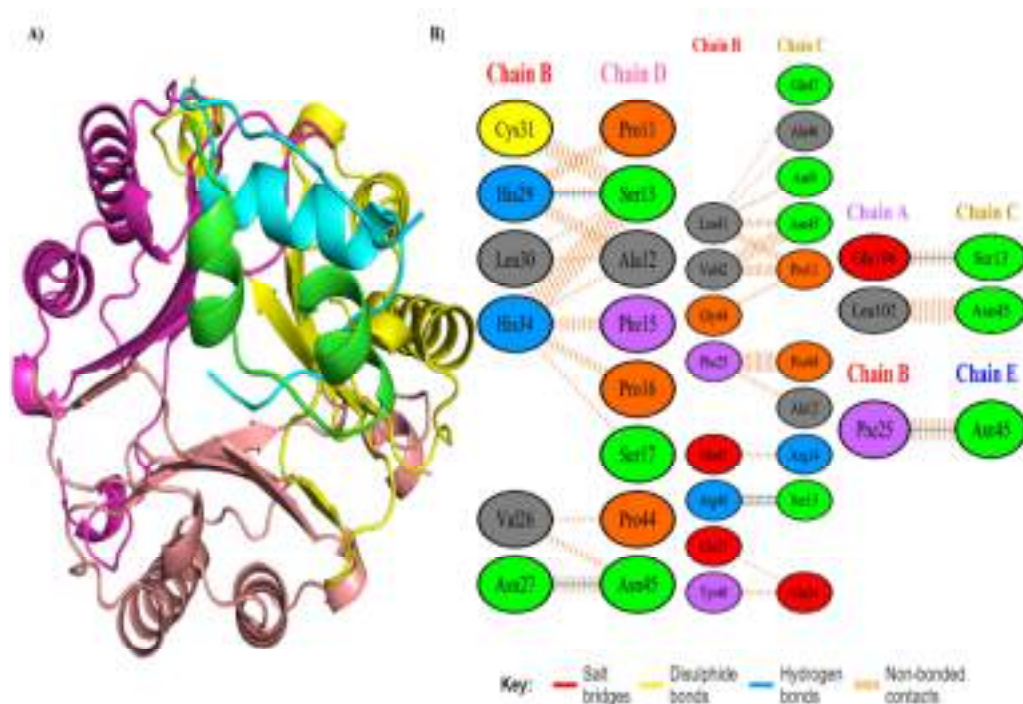


Figure 38: HDock and Dimplot based docking complex of MIF2 and Insulin.

(A) HDock based docked complex of MIF2 and Insulin shown as Cartoon representation coloured as: Insulin Chain-A (Green), Chain-B (Cyan) and MIF2 protein Chain-C (Pink), Chain-D (Yellow), Chain-E (Salmon). (B) Dimplot showing the protein-protein interactions between MIF2 (Chain-C, D, E) and Insulin (Chain-A, B).

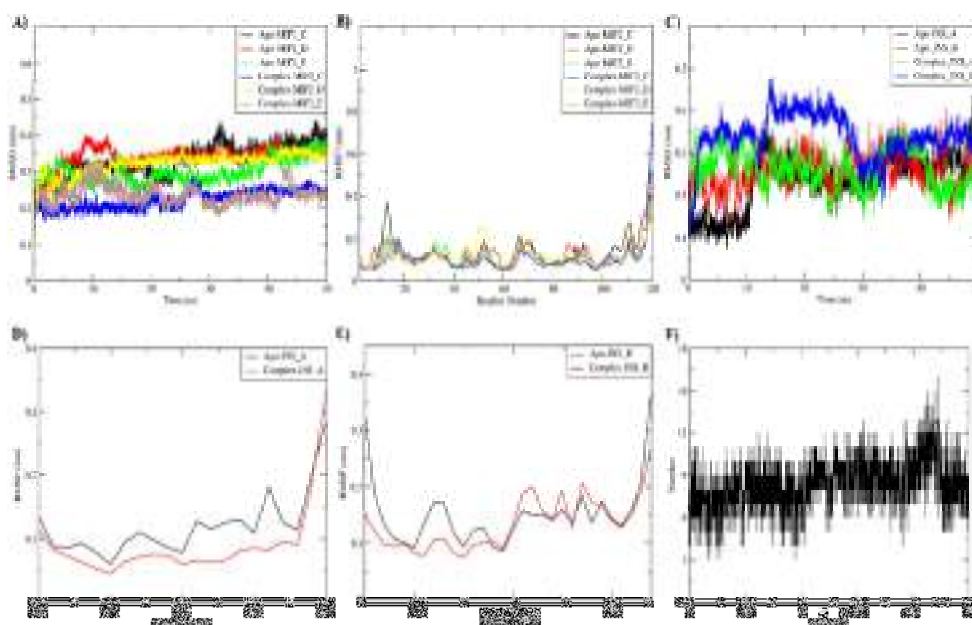


Figure 39: RMSD and RMSF plot of MIF2-Insulin complex. (A) RMSD and (B) RMSF plot of MIF2 *Apo* and Complex (Insulin) with respect to Chain-C,D,E, (C) RMSD plot of Insulin *Apo* and Complex with different Chain-A,B (D) RMSF plot of Insulin Chain-A *Apo* and Complex (E) RMSF plot of Insulin Chain-B *Apo* and Complex (F) H-bond interactions plot between MIF2-Insulin during the simulation period.

From the RMSD graphs of MIF2 (*Apo*) and MIF2-Insulin complex (Figure 39.A), it can be observed that RMSD of Chain-C which harbours more interacting residues with the insulin to feature lesser RMSD deviation in complex when compared to its *Apo* form. Even in case of MIF2 (Chain-E), the complex showed lesser deviations than the *Apo* during the simulation period. From the RMSF graphs, it can be observed that MIF2 residues interacting with Insulin were found to be less fluctuating when compared to *Apo* form (Figure 39.B). Similarly, in case of insulin, the residues (spanning Chain-B) that forms major interactions with MIF2 were found to fluctuate lesser when compared to its *Apo* form (Figure 39.E). Even the Chain-A residues of Insulin were found to fluctuate less in the MIF2-Insulin complex than the

Apo form (Figure 39.D). The RMSD graph of Insulin *Apo* and in complex with MIF2 inferred that the Chain-B of Insulin to confer comparatively fewer deviations during the simulation period (Figure 39. C). From the MD simulation-based H-bond interaction analysis between MIF2 and Insulin complex, it could be observed that an average of 8 H-bonded interactions to form between this complex during the simulation period (Figure 39.F). Based on all these observations, it can be concluded that MIF2-Insulin complex to have maintained stable interactions during the simulation period.

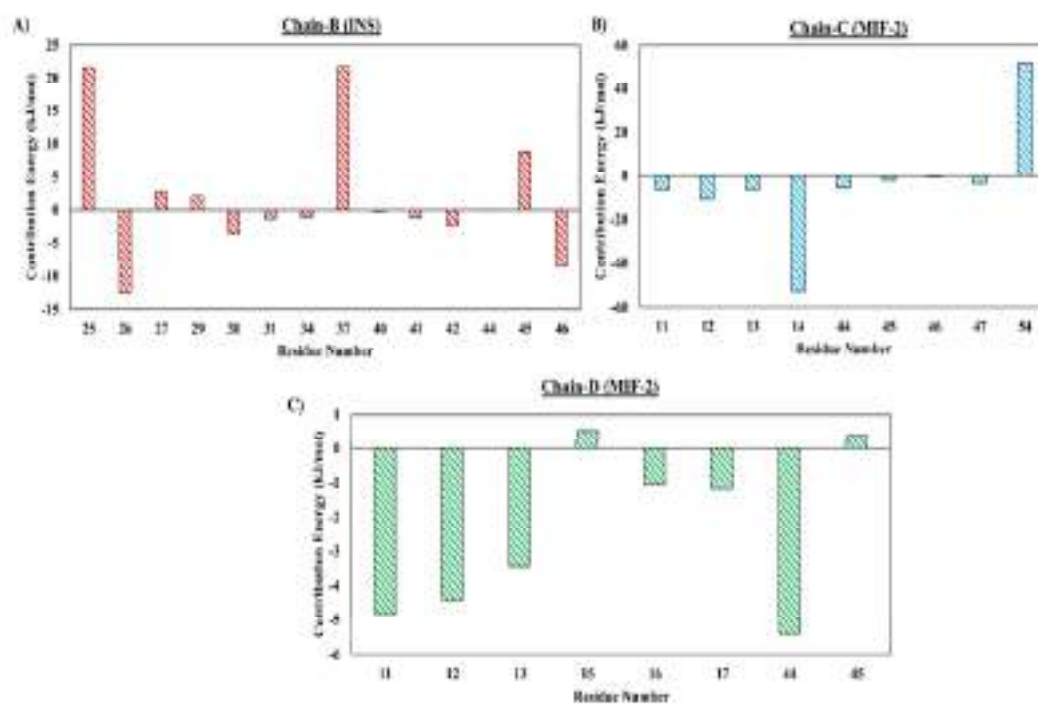


Figure 40: Per-residue Energy Contribution during simulation period of the MIF2-Insulin complex. Insulin Chain-B (A), MIF2-Chain-C (B), MIF2-Chain-D (C).

From the mmpbsa calculation of MIF2-Insulin complex interactions for the simulation period of 50ns, following energetics were inferred: van der Waals energy of -230.211 $\pm$ 0.393 kJ/mol, Electrostatic energy of -350.718 $\pm$ 0.980 kJ/mol, Polar

solvation energy of 601.656 $\pm$ 1.374 kJ/mol, SASA energy of -30.739 $\pm$ 0.071 kJ/mol and Binding energy of -9.982 $\pm$ 1.061 kJ/mol. The per residue contribution energy based on major interacting residues featured in DIMPLOT (Figure 39.C) alone are shown in Figure 40. From these graphs, the residues involved and its contribution energy are inferred as follows: Insulin Chain-A L102(-5.44kJ/mol), Chain-B V26(-12.41 kJ/mol), L30(-3.58 kJ/mol), V42(-2.36 kJ/mol), R46(-8.4781 kJ/mol) and MIF2 Chain-C P11(-6.49 kJ/mol), A12(-10.71 kJ/mol), S13(-6.34 kJ/mol), R14(-52.65 kJ/mol), P44(-5.65 kJ/mol), Chain-D P11(-4.84 kJ/mol), A12(-4.4 kJ/mol), S13(-3.45 kJ/mol), P16(-1.07 kJ/mol), S17(-1.18 kJ/mol), P44(-5.39 kJ/mol), Chain-E N45(-1.0 kJ/mol). Based on all these analyses it can be concluded that the Wba-MIF2 and Insulin to have formed stable intermolecular interactions, and the residues contributing to these interactions also to feature ideal energy contributions towards maintaining a stable complex during the production run.

3.6.3 Peptide identification and interaction analysis

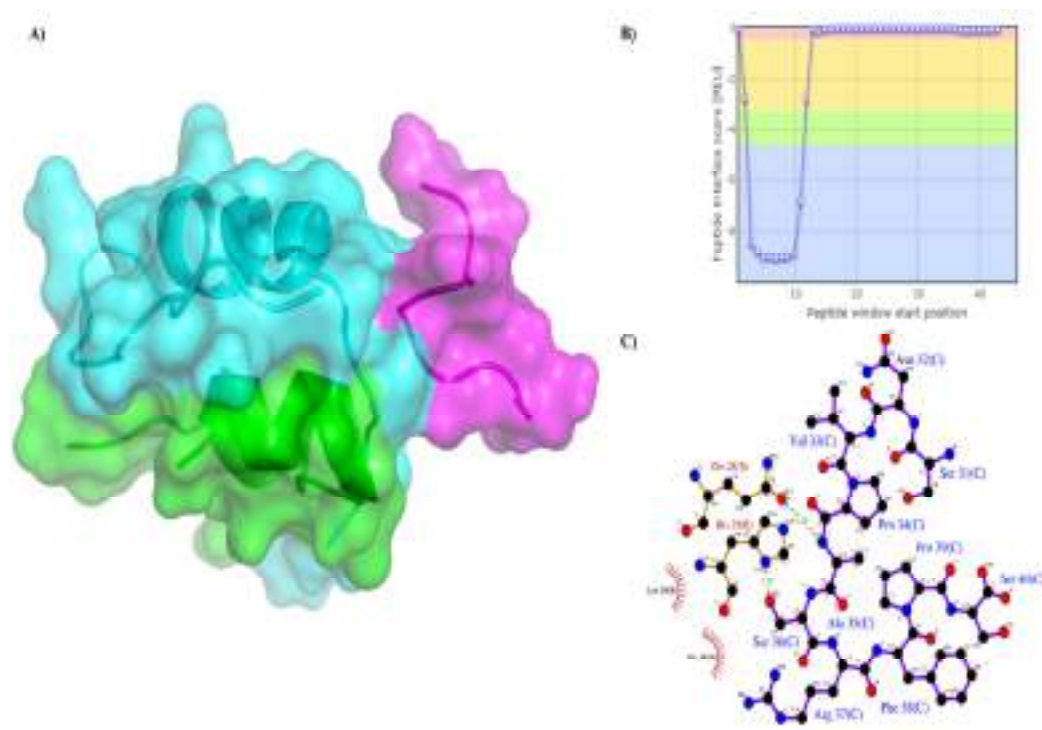


Figure 41: Complex of Insulin- Wba-MIF2 derived peptide and its peptiderive Cartoon representation. (A) Complex of Insulin and Peptide derived from MIF2-Insulin complex, peptiderive shown in Cartoon representation along with surface transparency with colouring as Insulin Chain-A(Green), B(Cyan) and Peptide Chain-C(Pink). (B) Peptide interface score with respect to each peptide position obtained from Peptiderive of MIF2-Insulin complex. The fraction of peptide region contributing the interface score with respect to the total interface score is given in background colour as red:<5%, yellow:5-35%, green:35-50%, blue:>50%. (C) Ligplot showing interactions of the complex between the Insulin (Chain-A, B) and Peptide (Chain-C) derived from peptiderive.

The final complex obtained from the MD simulation of 50ns was subjected to peptide identification through Peptiderive protocol in Rosie server (delimiting the length to 10-mer). The peptide region of MIF2 (Chain-C) which contributes to the highest interaction in the MIF-2-insulin complex, as observed during the simulation period was like the results derived from peptiderive (Figure 41.A). Hence, based on

this consensus agreement, MIF2 Chain-C (31-40 residues) region was chosen as the best scoring peptide with the sequence, **SNVPASRFPS**, as it featured a significant interface score of -9.082 REU with a relative interface score of 97.88% (Figure 41.B). This peptide was also found to have a good solubility profile as per innovogen prediction. Moreover, other sequence-based predictions also inferred that this peptide to be non-allergenic, non-haemolytic and less cell penetrative, thereby favoring binding with insulin in the extracellular milieu. The protein-peptide complex also showed that the MIF2 derived peptide to have interactions with the Insulin that were also observed in the MIF-2-insulin complex. The derived peptide, in complex with Insulin was subjected to MD simulation for 50ns and the stability of complex formation was calculated.

On comparative analysis of RMSD plots (Figure 42.A) of Insulin in *Apo* form and in complex with derived peptide, it can be observed that Insulin fluctuations were marginally higher in the case of peptide bound form in comparison with its *Apo*. From the RMSF graph (Fig. 10B & C), it can be observed that the insulin residues forming interactions with the peptide were shown to have marginally higher fluctuations in comparison with the *Apo* form. The peptide residues also showed lesser fluctuation of $<3\text{\AA}$ during the simulation period. Moreover, the peptide residues interacting with the Insulin also showed lesser fluctuations. An average of 4 H-bond formation was observed in Insulin-peptide complex during the simulation period (Figure 42.D). On cumulative analysis of all these results, it can be inferred that MIF-2 derived peptide can potentially interact with insulin and form a stable complex with optimal intermolecular interactions.

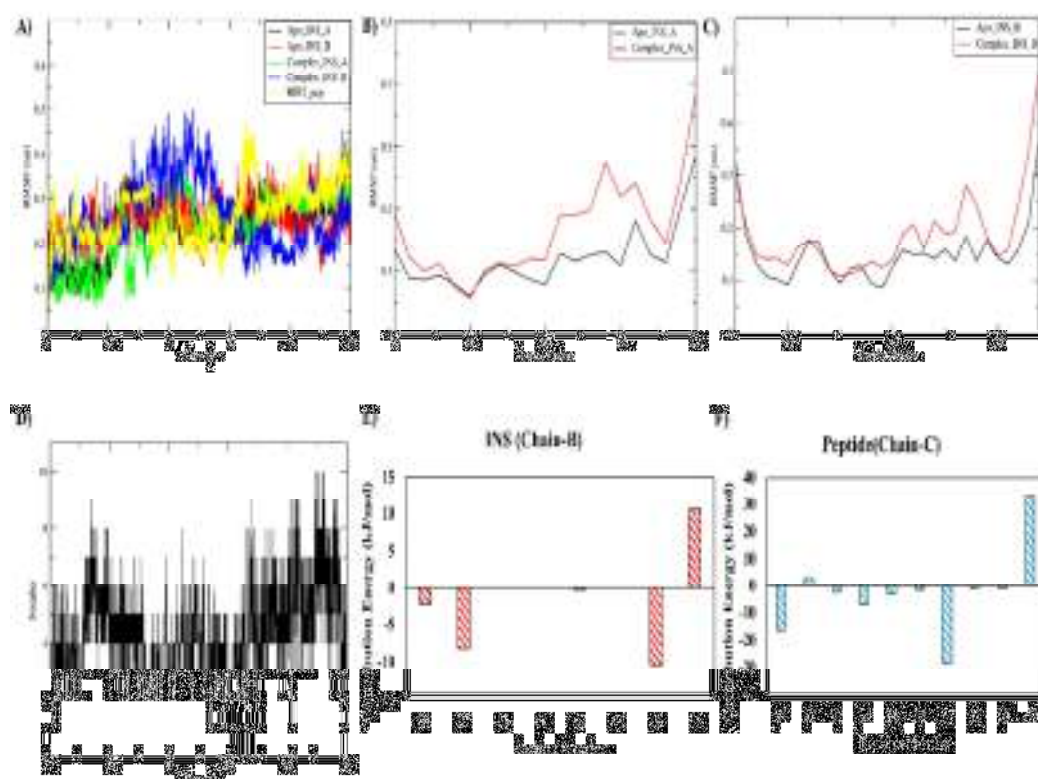


Figure 42: RMSD and RMSF plot of Insulin-Wba-MIF2 derived peptide. (A) RMSD and (B, C) RMSF plot of Insulin Apo and Complex (Peptide) with respect to Insulin (Chain-A, B), (D) H-bond interactions plot between Insulin-Peptide during the simulation period, Per residue Energy Contribution during simulation period: Insulin (Chain-B) (E), Peptide (Chain-C) (F).

3.6.4 *In-vitro* oxidoreductase activity of the recombinant Wb-MIF2 and synthetic peptide

Synthetic peptide of Wba-MIF2 was tested for its oxidoreductase activity and found active. Wild type Wba-MIF2 was used as reference for the activity and synthetic peptide was tested for its oxidoreductase activity. Insulin was reduced significantly as compared to control and full length Wba-MIF2 protein, as results were analysed using one way ANOVA and Tukey's multiple comparison test (Figure 43.A and B).

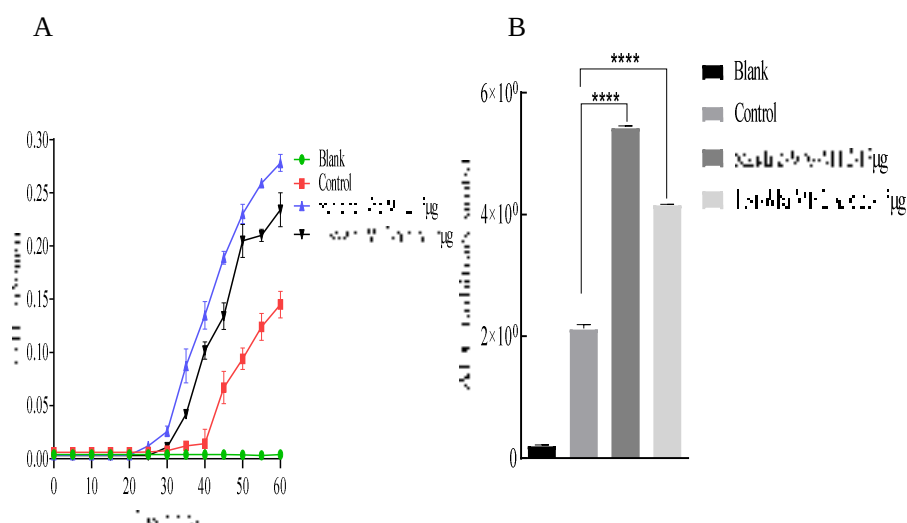


Figure 43: Oxidoreductase activity of the synthetic peptide of Wba-MIF-2. (a) Oxidoreductase activity of the synthetic peptide: blank (insulin), control: (insulin + DTT), internal standard: (insulin + Wba-MIF-2 recombinant protein + DTT), enzymatic reaction: (insulin + synthetic peptide of Wba-MIF-2 + DTT). (b) Area under the curve was calculated and analysis was performed using One Way ANOVA and Tukey multiple comparison test shows that peptide is significantly ($p < 0.0001$) reducing the insulin as compared to control and standard recombinant Wba-MIF2 protein.

4. DISCUSSION

This study investigated the ability of recombinant Wba-MIF2 to modulate the immune response in mouse models of T1DM induced by STZ and type 2 diabetes T2DM induced by a high-fat diet and STZ. Administration of Wba-MIF2 notably increased anti-inflammatory cytokine levels (IL-4 and IL-10) while decreasing pro-inflammatory cytokine (IL-1 β , IL-2, IL-6, IFN- γ , and TNF- α) levels in animal models of type I and type II diabetes. Histopathological examination of diabetic pancreata revealed an anti-inflammatory effect of Wba-MIF2. This suggests that Wba-MIF2 treatment may mitigate the progression of both type 1 and type 2 diabetes by inhibiting the infiltration of activated macrophages. Pancreatic tissue exposed to Wba-MIF2 exhibited significantly reduced infiltration of lymphocytes and macrophages. Emerging research highlights the potential of parasite-derived molecules to modulate the host immune response. Several studies have demonstrated that the waste products and secretions produced by various parasites can suppress the host's immune system, enabling these organisms to establish a persistent infection [258]. Helminth parasites can survive within their hosts for extended periods by inducing the production of immune-regulatory molecules. These regulatory factors manipulate the host's intricate immune system, contributing to the parasite's long-term persistence [259]. Onchocystatin, a protein produced by the parasitic worm *Onchocerca volvulus*, has been shown to influence the human immune system. It does this by shifting the immune response away from a pro-inflammatory state (Th1) towards a more anti-inflammatory response (Th2/Th3)[260]. Recent findings indicated that administering a recombinant form of the *W.bancrofti* MIF-2 protein mitigated colitis symptoms in mice subjected to a dextran sulfate sodium model[232]. Our findings indicated that Wba-MIF2 mitigated disease progression in both T1DM and T2DM mouse models,

aligning with previous research on the immunomodulatory properties of parasites[192]. A research study found that infection with the parasite *L. sigmodontis* and treatment with an extract from adult worms' decreased inflammation in adipose tissue caused by a HFD and enhanced the body's ability to regulate blood sugar levels[192]. These results point to a potential mechanism of action for Wba-MIF2, involving a shift in the immune response from a Th1 to a Th2 phenotype [261]. Our prior research investigated the ability of Wba-MIF2 to regulate the immune system. We analyzed serum samples obtained from people living in regions affected by lymphatic filariasis[28, 29]. To investigate the potential impact of Wba-MIF2 on Th1-mediated inflammatory processes, we compared its effects in animal models of type 1 and type 2 diabetes. These models were established using multiple low-dose STZ and HFD combined with streptozotocin, respectively. Our findings indicated that recombinant Wba-MIF2 influenced the gene expression of anti- and pro-inflammatory cytokines in the splenocytes cells of T1DM and T2DM animals [261]. Notably, we observed a significant reduction in Th1 cytokines (IL-1 β , IL-2, IL-6, IFN- γ , and TNF- α) following Wba-MIF2 administration. These Th1 cytokines are well-established as key inflammatory factors in both T1DM and T2DM. Additionally, Wba-MIF2 treatment led to increased IL-4 and IL-10 cytokine levels in spleen cells Figure 17 and 23). IL-10, also known as cytokine synthesis inhibitory factor, is an anti-inflammatory molecule that suppresses the production of pro-inflammatory cytokines[262]. Primarily produced by macrophages, monocytes, and regulatory T cells, this substance has been shown to stimulate and promote further development [261, 263]. Wba-MIF2 elevated the IL-10, that may mediated its anti-inflammatory effect via Treg-cells [10], we hypothesize that Treg cells may contribute to the anti-inflammatory effects of Wba-MIF2[232]. Macrophage and lymphocyte infiltration within the pancreatic tissue was evaluated. Our findings revealed a significant reduction in these

immune cell populations in Wba-MIF2-treated mice compared to the diabetic control group (Figure 19.d and 27.c). IL-10 and TGF- β are both known to suppress inflammation. They are produced in abundance during persistent infections and play a crucial role in supporting parasite survival and the function of regulatory T cells [264]. There is currently limited knowledge about helminth-derived molecules that mimic host immune molecules and their potential role in recruiting host immune cells such as macrophages, monocytes, neutrophils, and regulatory T cells. It remains to be determined whether parasite-produced substances can influence macrophage differentiation and function both in the organism and in controlled laboratory settings, potentially in response to antigenic stimuli [265, 266]. Our findings revealed a correlation between the gene expression of pro- and anti-inflammatory cytokines in splenocytes and the corresponding cytokine levels in the blood of the animals. Wba-MIF2 treatment led to a significant upregulation of IL-4 and IL-10 gene expression in spleen tissue. Conversely, there was a marked downregulation in the expression of IL-1 β , IL-2, IL-6, IFN- γ , and TNF- α gene within the same tissue. Helminths can manipulate dendritic cell function through direct contact and by influencing host-produced molecules [267]. Our findings indicated that Wba-MIF2 stimulated the production of IL-4 and IL-10, suggested its potential role as a parasite-derived immunoregulatory factor in diabetic patients. IL-4 is well-established as a key cytokine in Th2-mediated immune responses to parasitic infections[268]. Additional research is needed to understand how IL-4 and Wba-MIF2 interact in diabetes and immune function. hMIF influenced the immune system and participated in all inflammatory processes and can intensify inflammation triggered by superoxide [269], inflammatory cytokines [141], and nitric oxide [269]. Studies have shown that MIF promotes the proliferation of lymphocyte and macrophage cells[179]. It has been previously reported that hMIF is involved in drawing neutrophils to inflammatory

foci, thus intensifying the severity of acute inflammation [270]. However, the functions of MIF homologs from various helminth parasites differ, particularly those classified as Type 2 MIF [271]. Parasitic MIF (rAceMIF or type 2 MIF) exhibits a structural similarity to human MIF (hMIF) and type 1 MIF. All these MIFs interact with the same receptor complex, CD74-CD44. Nevertheless, the binding affinity of rAceMIF to this receptor is significantly weaker, being four to five times lower compared to hMIF [272, 8]. In contrast, the reported inhibitors of mammalian MIF, such as ISO-1, were ineffective against the parasitic MIF2 enzyme[159].

We hypothesize that the parasite's MIF protein interacts directly with the human MIF receptor CD74, interfering with the host's MIF function. Our previous work has categorized Wba-MIF as a type 2 MIF protein [29]. Studies suggest that recombinant mouse macrophage migration inhibitory factor (rMouMIF) can exacerbate clinical symptoms in ovalbumin-induced airway inflammation. However, the administration of recombinant rAs-MIF has been shown to markedly reduce these effects[273]. Notably, hMIFs and type 1 nematode MIFs share conserved amino acid sequences, while type 2 nematode MIFs, such as Wba-MIF2, exhibit relatively diverse amino acid compositions [273, 29]. This conserved region has been identified as forming of intramolecular disulfide bond and is essential for the oxidoreductase activity, which relies on the presence of cysteine [42, 147]. The data indicated that the oxidoreductase capability of Wba-MIF2 is critical for immune processes. To delve deeper into this, we have engineered a Wba-MIF2 mutant devoid of oxidoreductase function and are examining its influence on immunomodulation.

Our study suggested that the Wba-MIF2 protein may be effective in mitigating Type I and II diabetes. This aligns with the Chennai Urban Rural Epidemiology Study (CURES), which found lower levels of inflammatory markers (IL-6 and TNF- α) in

individuals with lymphatic filariasis compared to those without infection. Additionally, CURES revealed an inverse correlation between lymphatic filariasis and both Type I and Type II diabetes [249, 274].

To comprehensively understand how Wba-MIF2 influenced the host's immune response, future research should focus on identifying the optimal dosage and delivery method, as well as elucidating the underlying molecular mechanisms.

Systemic and local inflammatory and immunological responses were greatly influenced by the Macrophage Migration Inhibitory Factor (MIF) protein. The pleiotropic cytokine (h-MIF) has two enzymatic activities, tautomerase, and oxidoreductase, in addition to its cytokine properties [166]. MIF is also found in several parasites, including *W.bancrofti*, the major lymphatic filarial parasite [28, 29]. Likewise, parasitic MIF (Wba-MIF2) also has two enzymatic functions [29]. There is currently sufficient data that conclude that h-MIF is crucial in autoimmune conditions such as diabetes mellitus (DM), ulcerative colitis, arthritis, and immunomodulation [275, 141, 276, 277]. Since parasite MIF homologs (Wba-MIF2) also possess enzymatic functions, it also plays a significant role in immunomodulation [8, 28, 29, 30, 278]; however, its molecular mechanism was not explored much. Therefore, we conducted this study to delineate the signaling mechanism involved in the immunomodulation by the Wba-MIF2 and compare it with that of the h-MIF. From our study, it is evident that Wba-MIF2 can activate signaling cascades. Our results identified that Wba-MIF2 can activate MEK-ERK and AKT signaling pathways, in a time-dependent manner. Based on this observation, we have selected 5- and 15 min time points for our further experiments.

Wba-MIF2 showed a vigorous indication of phosphorylated proteins across these two-time points. We have observed hyperphosphorylation in several proteins, including Ubiquitin-conjugating enzyme E2 E3 (UBE2E3), which is involved in various cellular activities, including DNA binding, cytoskeletal motoractivity [279], and immune system process [280, 281]. In our data SF3B2 was hyperphosphorylated at 15 min at serine 430, 434 and 435. Generally, this protein served as anti-inflammatory and brings immunomodulation in neurons [282]. Our findings indicated that Wba-MIF2 has the potential to shift the immune response in a T1DM mouse model from a pro-inflammatory to an anti-inflammatory state.

Five weeks of Wba-MIF2 administration led to a significant reduction ($p<0.05$) in pro-inflammatory cytokines, including IL-1 β , IL-2, IL-6, TNF- α , and IFN- γ , in diabetic animals compared to the control group. Conversely, there was a significant increase ($p<0.05$) in anti-inflammatory cytokines, IL-4 and IL-10, in the same group. Gene expression analysis of pro-inflammatory and anti-inflammatory cytokines in splenocytes mirrored the corresponding cytokine concentrations in the animals' blood. Treatment with Wba-MIF2 led to a significant upregulation of IL-4 and IL-10 gene expression in spleen tissue, while the expression of IL-1 β , IL-2, IL-6, IFN- γ , and TNF- α genes were markedly downregulated.

RPLP2 is crucial for the elongation phase of protein synthesis. Increasing or reducing RPLP2 expression can change the pace of protein translation, which in turn affects how quickly cells proliferate [283]. Our mass spectrometry-based phosphoproteomics data revealed that Wba-MIF2 promotes phosphorylation of RPLP2 at serine 303/306. It may be possible that these phosphorylation sites of RPLP2 may induce Wba-MIF2 mediated cell proliferation. In some cell types, PTBP1 mediates several biological activities, including immune cell activation, neuronal cell

development, and differentiation. In order to control the expression of CD40 ligand (CD40L) and interleukin-2 (IL-2), PTBP1 regulates the cluster of differentiation 4 (CD4) T-cell activation by activating the nuclear factor-B (NF-B) and phospholipase C-1 (PLC-1)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathways [284]. In our results we have identified hyperphosphorylation of PTBP1 at serine 141 and 142. It suggested that this protein may be involved in Wba-MIF2 mediated immunomodulation.

Our gene ontology and pathway enrichment results demonstrated that most of the enriched phosphoproteins are involved in cytoplasmic translation, translation, mRNA splicing, and RNA processing. Our molecular functional enrichment analysis showed that many phosphoproteins have protein binding, RNA binding and DNA binding properties. Our pathway enrichment results showed enrichment of several key pathways including spliceosomes, T cell receptor signaling pathway, Th17 differentiation pathway, IL-17 signaling pathway and insulin signaling pathway. Based on these observations it is evident that Wb-MIF2 activates signaling pathway via modulating RNA binding proteins and spliceosome machinery and may bring immunomodulation.

We further carried out several analyses to gain deeper insights into the Wba-MIF2 mediated signaling mechanism in macrophages. Our transcription factor enrichment analysis identified several downstream transcription factors involved in transcription of genes involved in various cellular events. CREB1 having diverse cellular functions including proliferation, survival, and differentiation of the cells. Immediate early genes (IEGs) transcriptional induction is one of the crucial functions of CREB as a transcription factor and master regulator of anti-inflammatory immune responses. These genes contain nuclear receptors, cytokines, chemokines, growth

factors, and transcription factors [285]. Myc was another transcription factor which was enriched in our study. Myc controls a wide range of genes in both innate and adaptive immune cells, regulating their activation, proliferation, polarisation, and subsequent functional events that these cells generate [286]. ATF2 is another transcription factor, which upon activation stimulated transcription of several genes involved in immune responses and immunomodulation. It regulates the expression of cell adhesion molecules, proinflammatory cytokines, and chemokines [287].

Our analysis of Wba-MIF2 mediated dysregulated phosphoproteins identified several upstream kinases including CDK4, CDK1, and DNAPK. DNA-dependent protein kinase catalytic subunit (DNAPK or PRKDC) is a kinase that is a component of the DNA-dependent protein kinase (DNA-PK) complex, which is essential for recombination and the repair of DNA double-strand breaks. It is also known to promote central T-cell tolerance [288]. CDK1 and CDK4 involved in the development of innate immune system cells and the maintenance of balanced innate immunological activity. Type I IFN (IFN-) secretion was suppressed in monocytes thus modulating immune system. Macrophage differentiation and activity are dependent upon CDKs [289]. We also compared the immunomodulatory properties of Wba-MIF2 with h-MIF as h-MIF is a well established molecule to bring about immunomodulation [141]. Our results showed that both MIF bring about immunomodulation by the same molecular mechanism and involvement of same signaling pathways. However, further studies are required to understand how the role of a parasite MIF and human MIF differs in immunomodulation.

Research has revealed the binding affinity of hMIF and insulin within the secretory granules of pancreatic β cells, leading to the modulation of insulin activity [290, 291]. These findings have also indicated that insulin production and secretion

remained comparable between *Mif*^{-/-} mice beta cells and wild type mice. However, when tested on HepG2 liver cells, insulin from *Mif*^{-/-} β cells exhibited contrasting effects compared to recombinant human insulin or that from WT cells. It failed to induce significant glucose uptake and Akt phosphorylation, a crucial element in the insulin signaling pathway. Consequently, the findings implied a reduced activity of insulin from *Mif*^{-/-} β cells in comparison to WT cells [290, 291]. Co-immunoprecipitation assays conducted on pancreatic islet lysates from WT mice corroborated a direct interaction between hMIF and insulin. Despite identical molecular weights, insulin from *Mif*^{-/-} β cells exhibited highest susceptibility to trypsin digestion, suggesting a distinct protein conformation from that of WT cells. Interestingly, incubating insulin with rMIF resulted in a noteworthy increase in the ratio of insulin hexamers, monomers, and dimers compared to the control, further supporting involvement of hMIF in protein folding and conformational processes. This underscores the idea of chaperone-like activity of MIF [290, 291]. Wba-MIF-2 also showing oxidoreductase activity with human insulin and this concept creates a research interest for identification of the motif(s) those are responsible for its oxidoreductase activity. In the present study we attempted to identify the region of Wba-MIF-2 which contributes to major interaction with the human insulin. In our study mmpbsa calculation was performed between the Insulin and the Peptide complex during the simulation period and obtained energy values as follows: van der Waals energy of -65.849 $\pm$ 0.682 kJ/mol, Electrostatic energy of -120.057 $\pm$ 1.245 kJ/mol, Polar solvation energy of 135.511 $\pm$ 1.386 kJ/mol, SASA energy of -8.993 $\pm$ 0.094 kJ/mol and Binding energy of -59.439 $\pm$ 0.967 kJ/mol. From all these calculations, it could be inferred that the derived peptide-Insulin complex to feature significant binding affinity that is comparable with Wba-MIF2-Insulin complex during the simulation period. The per residue contribution energy based on major

interacting residues featured in DIMPLOT (Figure 38.C) alone are shown in Figure 39. E & F. The per residue contribution of Insulin Chain-Bis L30 (-2.24kJ/mol), E37 (-8.13 kJ/mol), E45 (-10.46kJ/mol) and for Peptide Chain-C are S31 (-16.61 kJ/mol), V33 (-2.35 kJ/mol), P34(-7.02 kJ/mol), A35 (-2.80 kJ/mol), S36 (-1.51 kJ/mol), R37(-28.39 kJ/mol), F38 (-1.1 kJ/mol), P39 (-1.12 kJ/mol) (Figure 39.E & F).

The key residues of the derived peptide were observed to contribute good interaction energy during the simulation period and showed lesser fluctuation in the RMSF graph. Based on all these inferences we can conclude that the identified peptide (“**SNVPASRFPS**”) shall form stable interactions with the insulin and could sustain stability during physiologically simulated conditions. hMIF have two enzymatic functions. One of these functions involves acting as a thiol-protein oxidoreductase (TPOR). This activity relies on a specific amino acid sequence within MIF called a CALC motif, consisting of the residue’s cysteine-57, alanine, leucine, and cysteine-60 [147]. Crystallographic studies suggested a static structure for MIF, but research indicated that the cysteine residues can form a reversible bond within the molecule. This internal disulfide bridge is believed to be MIF's catalytic center [292, 153]. In line, an identified peptide of Wba-MIF-2 has also shown *in-vitro* oxidoreductase activity against human insulin with different dose. Wba-MIF2 peptide has shown a significant activity with human insulin and was found similar to wild type Wba-MIF2 (Figure 43). On cumulative analysis of all these results, it can be inferred that Wba-MIF-2 derived peptide can potentially interact with insulin and form a stable complex with optimal intermolecular interactions. Certain immune process mediated by MIF, like the activation of T cell, monocytes, and macrophages, depend on a mechanism involving cysteine and cellular redox balance. Studies reported that mutations within the CALC motif, particularly at Cys60, have a

significant impact on MIF's protein folding structure. This region coincides with the JAB1-binding site [293], a feature found in many, though not all, vertebrate homologs [294]. Moreover, other sequence-based predictions also inferred that this peptide to be non-allergenic, non-hemolytic and less cell penetrative, thereby favoring binding with insulin in the extracellular milieu. Though in-vivo study and mechanistic study of the insulin treated with this peptide is under proposal. This novel peptide may help insulin folding and sustain releases action. This could be a new idea for the pharmaceutical aspect.

5. SUMMARY

Tropical diseases like lymphatic filariasis, onchocerciasis, schistosomiasis, and intestinal infections are heavily driven by helminthic parasites. These parasitic infections are characteristically chronic, prompting the parasites to develop intricate mechanisms for persisting within the host for extended periods [76]. Well-documented immunomodulatory mechanisms employed by helminths contribute to their long term persistence within the host during disease progression. Parasites employed a range of strategies to ensure their long-term survival, inhabiting areas with a weakened immune response, tightly regulating the production and release of excretory-secretory products and shifting surface antigenicity throughout their lifecycle stages. While the secretome of helminthic worms has revealed numerous parasite molecules with potential immunomodulatory roles, further investigation is needed to understand the specific effects of individual molecules. Among several prominent molecules includeing ALT, TGF- β , VALT and most importantly is homologues of MIFs [258]. The precise function of MIF homologues in these organisms remains elusive, although evidence suggested they may play a critical role in modulating the host's immune response, through differential macrophage activation and other immune cells [295]. MIF homologs from filarial parasites have emerged as potential modulators of the host immune response. The global prevalence of diabetes is escalating at a faster pace compared to heart disease and cancer, making it the most rapidly growing chronic condition [296]. Diabetes is a condition characterized by persistent high blood sugar levels. Generally, T1DM is an autoimmune disorder where the body's immune system attacks and destroys insulin-producing cells in the pancreas. T2DM is primarily a metabolic disorder resulting from either insufficient insulin production or the body's decreased responsiveness to insulin. A growing body

of research indicates that pro-inflammatory conditions contribute to the loss of functional β cell population, a common factor in the pathogenesis of both T1D and T2D [297, 298]. Central to both diseases, immune cells initiate and perpetuate harmful inflammatory responses that disrupted β cell activity [298]. Additionally, the interaction between immune cells especially macrophages and β cells drove the inflammatory process, ultimately leading to impaired β cell function and loss [299]. Consequently, selectively modulating the communication between immune and β cells may be a novel approach to preventing or halting the islet inflammation underlying both T1D and T2D [300, 301]. Conversely, this interaction can also promote immune tolerance and safeguard β cell mass and function [298]. Due to their strong influence on the mammalian immune system, helminths and their excreted/secreted compounds were studied as possible therapeutic interventions [298]. Our study explored the effects of Wba-MIF2, a protein secreted by the parasite *Wuchereria bancrofti*, on the immune system of mice with type 1 and type 2 diabetes. The research aimed to understand how Wba-MIF2 influences immune responses and its potential as a therapeutic agent for diabetes were summarised in multiple aspects:

➤ **Immune Modulation**

The study demonstrated that Wba-MIF2 has a notable impact on the immune system of diabetic mice. By reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines, Wba-MIF2 modulated the immune response. This dual action is crucial because chronic inflammation is a major issue in diabetes, and contributing to various complications. By shifting the balance towards an anti-inflammatory state, Wba-MIF2 could help mitigate some of the inflammatory responses seen in diabetes.

➤ **Signaling Mechanisms**

The activation of the MEK-ERK and AKT pathways by Wba-MIF2 is particularly important. These pathways are involved in critical cellular processes such as proliferation and survival. In the context of diabetes, where cellular health and function are often compromised, enhancing these pathways could support the maintenance and repair of tissues. This aspect of Wba-MIF2's action adds another layer of potential therapeutic benefits, as it could help sustain cellular functions and reduce the progression of diabetic complications.

➤ **Insulin Interaction**

One of the most intriguing findings of the study is the interaction between Wba-MIF2 and human insulin. The oxidoreductase activity of Wba-MIF2 might show that proper folding and stability of insulin molecules is connected with this activity. Proper insulin function is crucial for regulating blood glucose levels, and any factor that enhances insulin stability and activity can be beneficial in managing diabetes. This interaction highlights a possible direct effect of Wba-MIF2 on insulin metabolism, which could translate into better glycemic control in diabetic patients.

➤ **Implications for Diabetes Therapy**

The findings from this study pointing towards Wba-MIF2, this may be as a promising future candidate for diabetes therapy. Its ability to modulate immune responses and interact with insulin opens new avenues for treatment strategies. Specifically, therapies based on Wba-MIF2 could potentially reduce the inflammatory state associated with diabetes and enhance insulin function, addressing two major aspects of the disease.

Key Findings of the study

1. Reduction in Pro-Inflammatory Cytokines:

Wba-MIF2 treatment led to a significant decrease in levels of pro-inflammatory cytokines in both type 1 and type 2 diabetic mice. These cytokines are typically associated with inflammation, which may be responsible for the diabetic symptoms.

2. Increase in Anti-Inflammatory Cytokines:

The treatment also resulted in higher levels of anti-inflammatory cytokines. These cytokines help reduce inflammation and may contribute to better regulation of the immune response in diabetic conditions.

3. Activation of Signaling Pathways:

Wba-MIF2 was found to activate several crucial signaling pathways, including the MEK-ERK and AKT pathways. These pathways are integral to cell proliferation and survival, suggesting that Wba-MIF2 may help in maintaining cellular health in diabetic conditions.

4. Interaction with Insulin:

Wba-MIF2 interacts with human insulin and possesses oxidoreductase activity, indicating a potential role in insulin folding and stability. This interaction could be significant for improving insulin function and regulation in diabetes.

Future perspectives

1. While the results are promising, further research is required to fully understand the mechanisms by which Wba-MIF2 exerts its effects.
2. Detailed studies on its interaction with insulin and the specific pathways involved in its immunomodulatory action are necessary.
3. Additionally, translating these findings from animal models to human patients will be required an extensive clinical trials to ensure safety and efficacy.

Conclusion

This study sheds light on the potential of parasitic Wba-MIF2 cytokine as a therapeutic agent for inflammation and diabetes. By reducing inflammation and possibly enhancing insulin function, Wba-MIF2 offers a novel approach to managing both type 1 and type 2 diabetes. The complex interactions between parasitic proteins, the immune system, and insulin metabolism present a unique opportunity for developing new treatments that could significantly improve the quality of life for diabetic patients. Further research will be crucial in translating these findings into viable clinical therapies

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301. Cnop M, Welsh N, Jonas J-C, Jörens A, Lenzen S, Eizirik DL. Mechanisms of pancreatic β -cell death in type 1 and type 2 diabetes. *Diabetes.* 2005;54(suppl_2):S97–107.

ANNEXURES

ANNEXURE-I

icmr NITM
ICMR- National Institute of Traditional Medicine, Belagavi

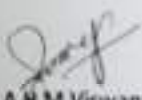
Institutional Animal Ethics Committee
Reg. No: 1388/GO/Re/5/10/CPCSEA
Resolution No: IAEC/ICMR-NITM BGM/ Sept 2019/4 Date: 08.09.2019

CERTIFICATE

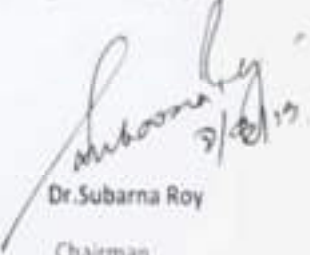
This is to certify that the research project proposed by Dr. S. L. Hoti entitled "Immunomodulation by parasitic Macrophage Migration Inhibitory Factor (MIF) in Type 1 and Type 2 diabetic animal model" has been approved by IAEC. The approval granted for one year with following number of animals:

Species	Gender	Number of animals
Mice (Balb/C)	Male	42
Mice (C57BL/6)	Male	35

The investigator must report the outcome of the experiment to IAEC/CPCSEA. The approved number/species/gender of animals should not be changed without prior permission of CPCSEA/IAEC.


Dr. A H M Viswanath Swamy
 CPCSEA Main Nominee


Dr. Banappa S Unger
 Member Secretary


Dr. Subarna Roy
 Chairman


 23/9/19



ANNEXURE-II

HIGH FAT DIET FROM RESEARCH DIET

[Home](#)

Search Results

D12451

Rodent Diet With 45 kcal% Fat

Intended species: Rodent (Rat/Mouse) **Irradiated:** No

^ **Ingredients**

- Casein, Lactic, 30 Mesh
- Cystine, L
- Sucrose, Fine Granulated
- Lodex 10
- Starch, Corn
- Solka Floc, FCC200
- Lard
- Soybean Oil, USP
- S10026B
- Choline Bitartrate
- V10001C
- Dye, Red FD&C #40, Alum. Lake 35-42%

D12450H

Rodent Diet With 10 kcal% Fat (Matching Sucrose to D12451)

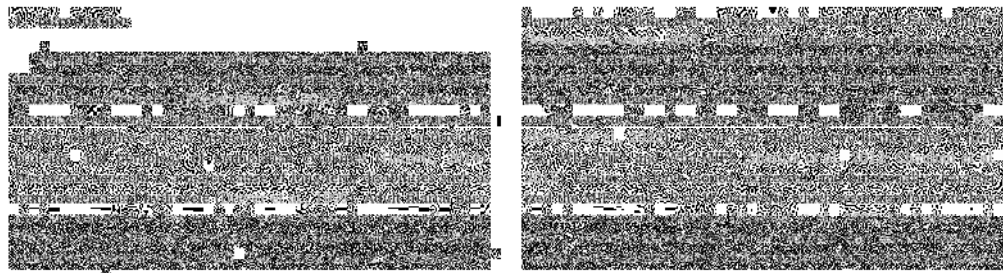
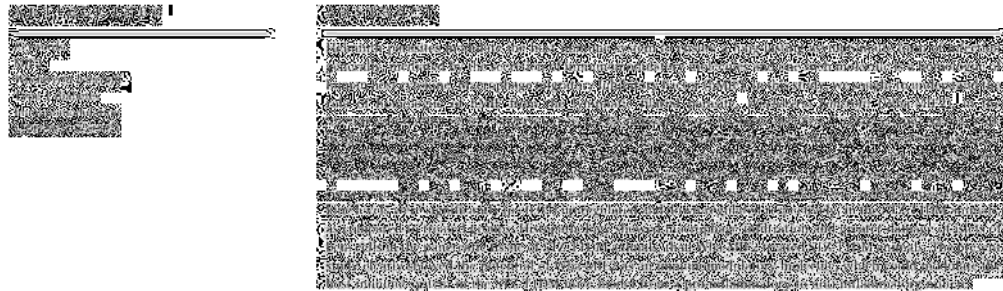
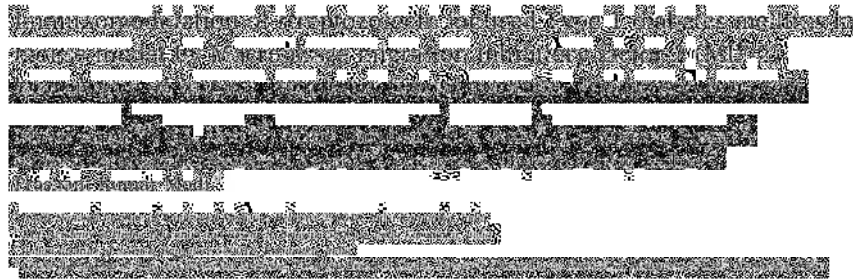
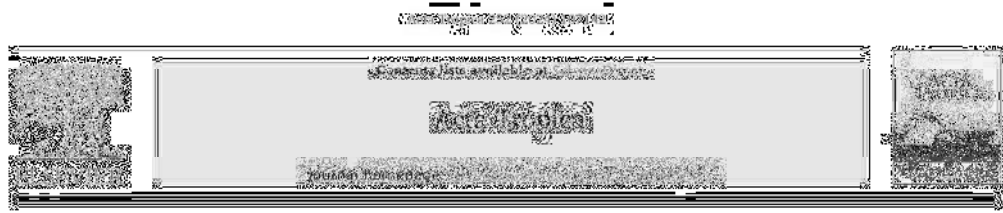
Intended species: Rodent (Rat/Mouse) **Irradiated:** No

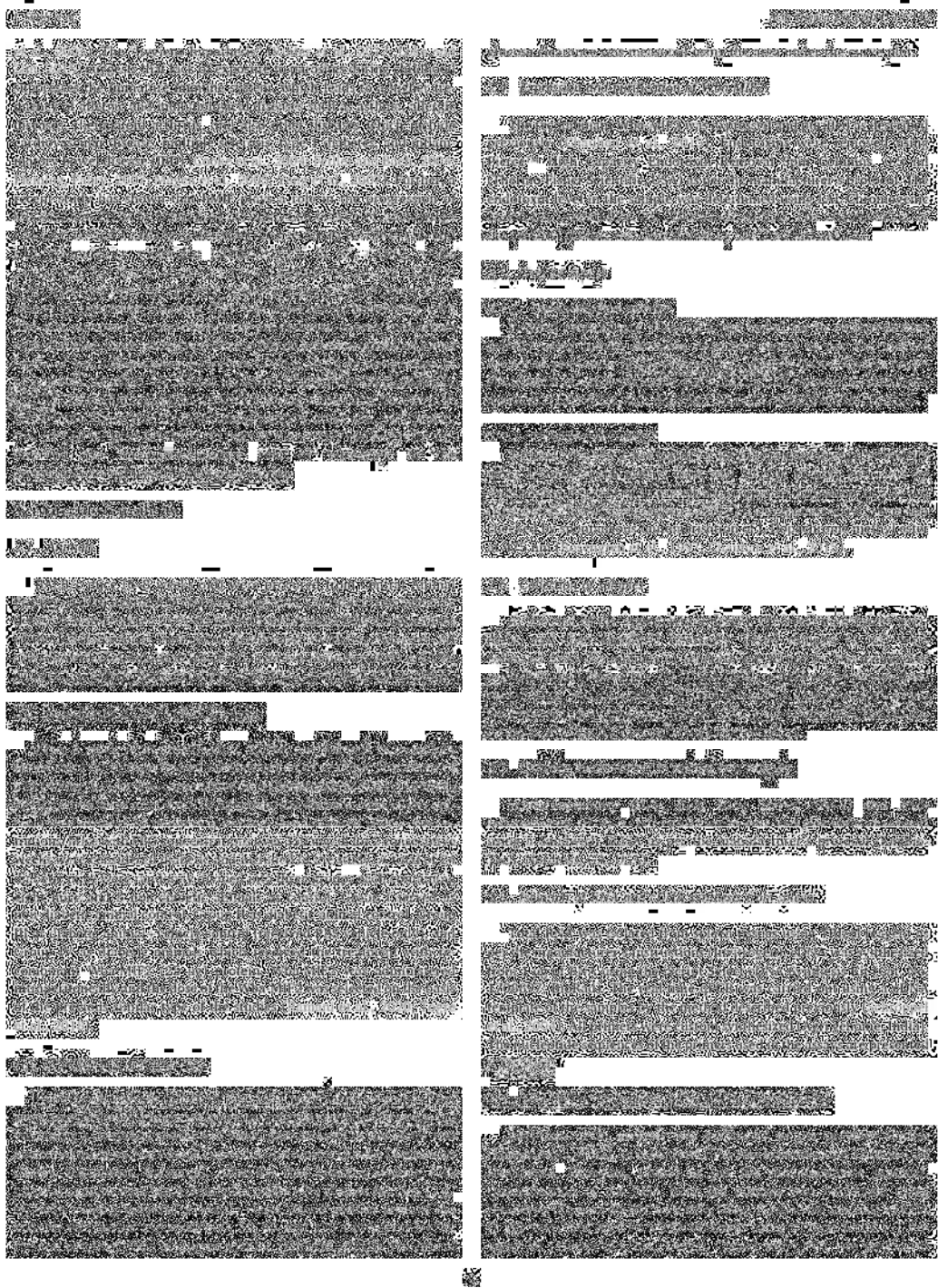
^ **Ingredients**

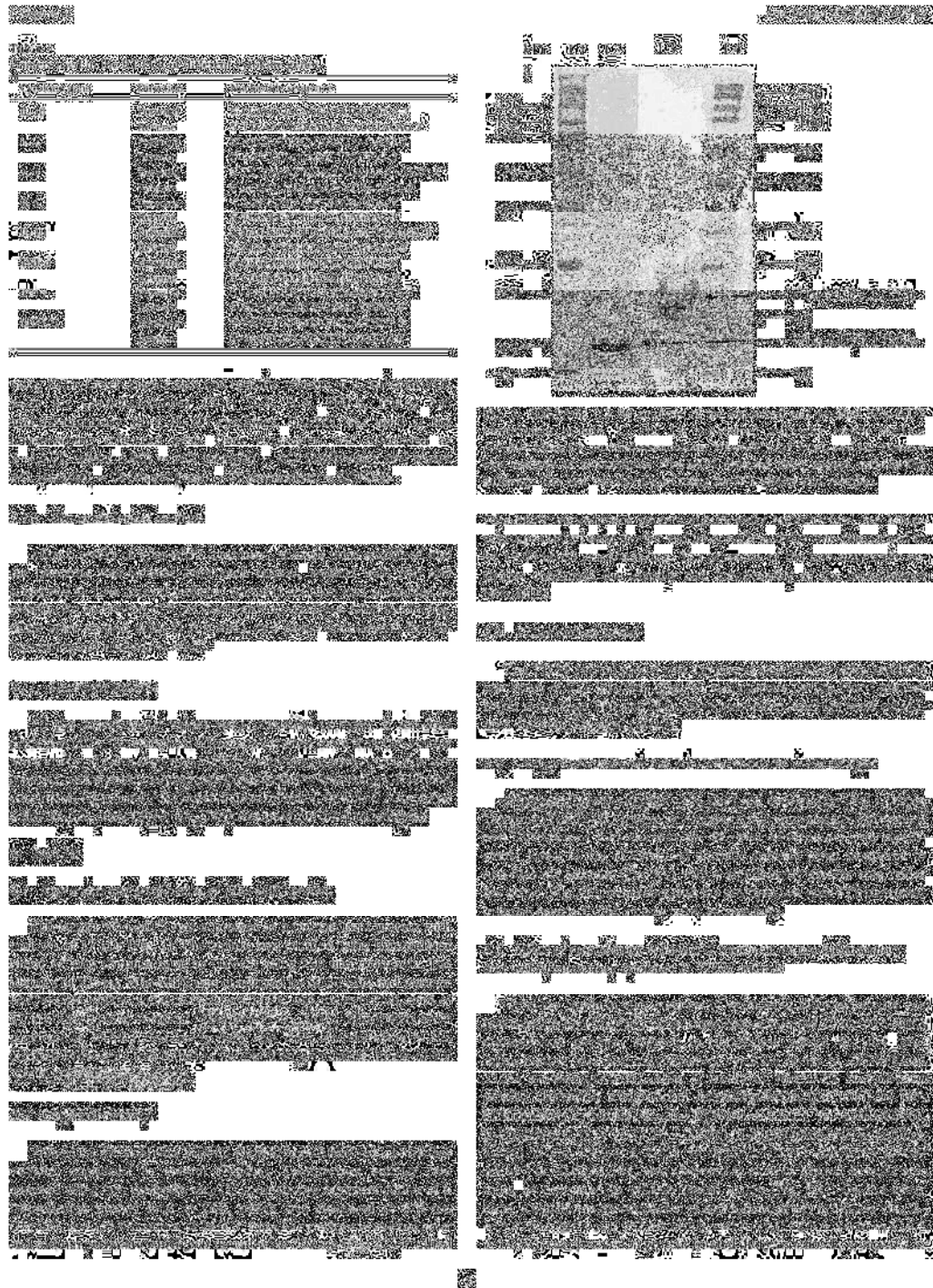
Annexure-III

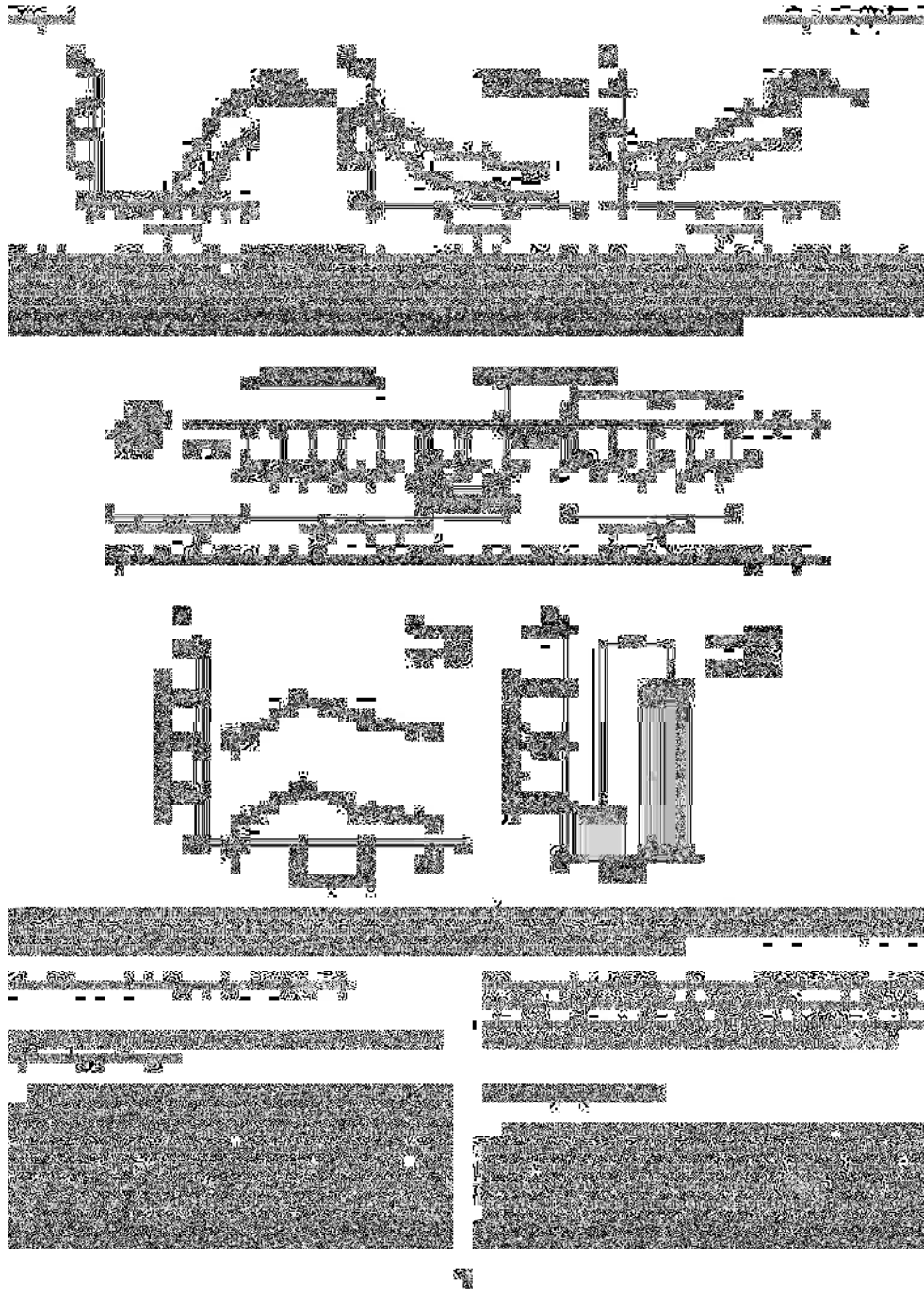
Publication Details

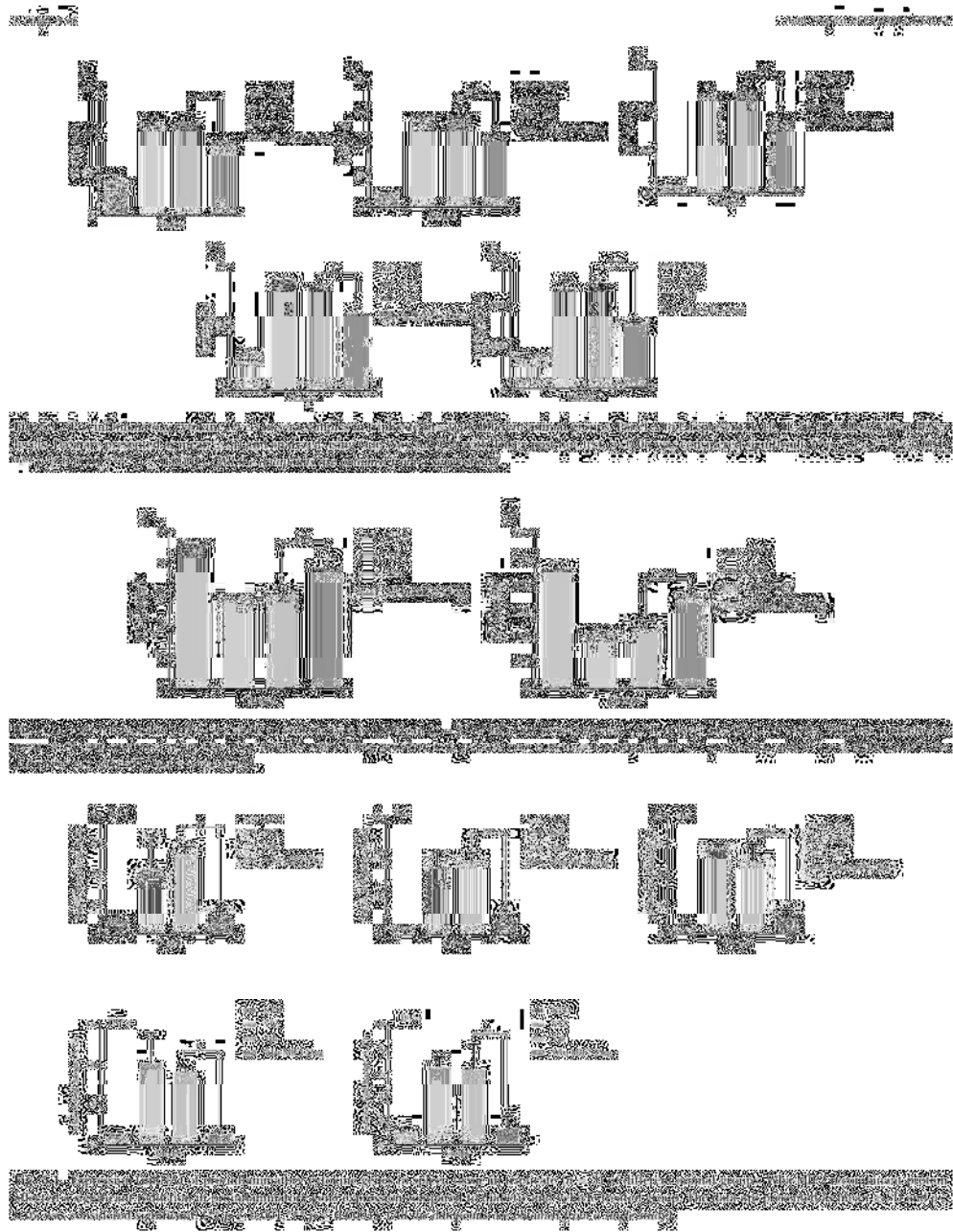
Sr. No	Publication Title	Journal Name	Impact factor
1	Immunomodulation of streptozotocin induced Type 1 diabetes mellitus in mouse model by Macrophage migration inhibitory factor-2 (MIF-2) homologue of human lymphatic filarial parasite, <i>Wuchereria bancrofti</i>	Acta Tropica, doi: 10.1016/j.actatropica.2024.107142	2.1
2	Unpacking Immune Modulation as a Site of Therapeutics Innovation for Nematode Parasite <i>Wuchereria bancrofti</i> : A Temporal Quantitative Phosphoproteomics Profiling of Macrophage Migration Inhibitory Factor 2	OMICS: A Journal of Integrative Biology, doi: 10.1089/omi.2024.0002.	2.2

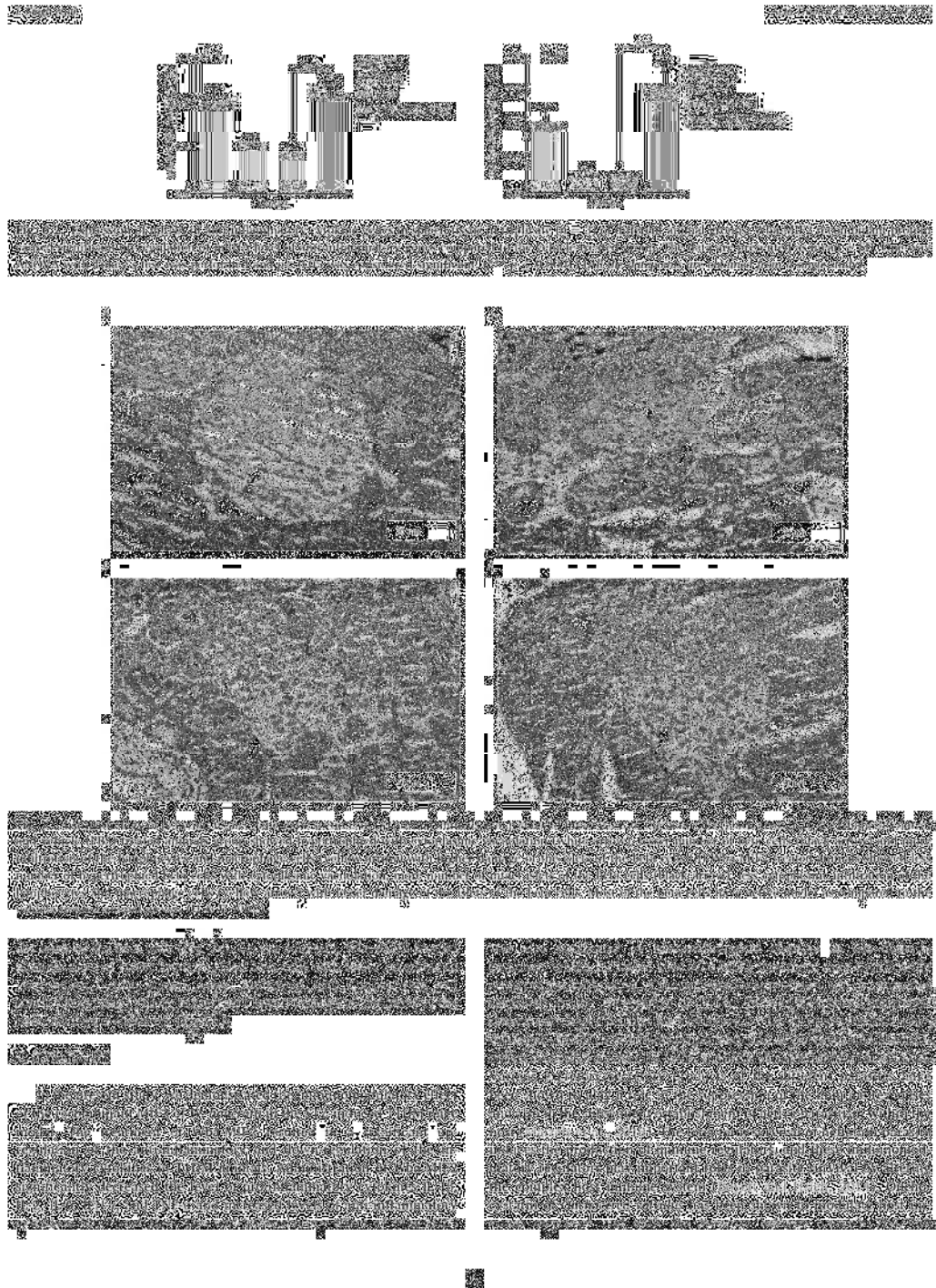


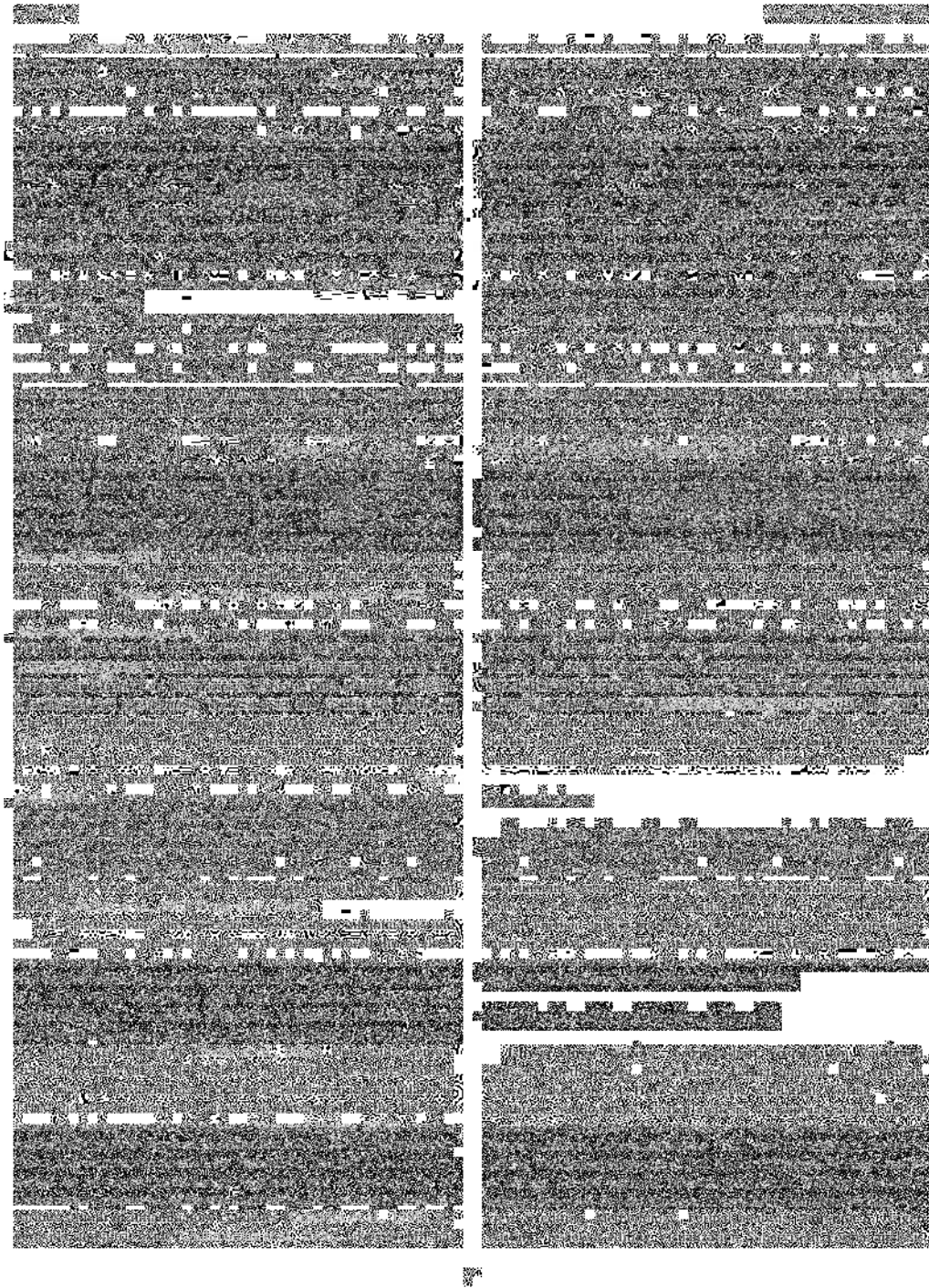












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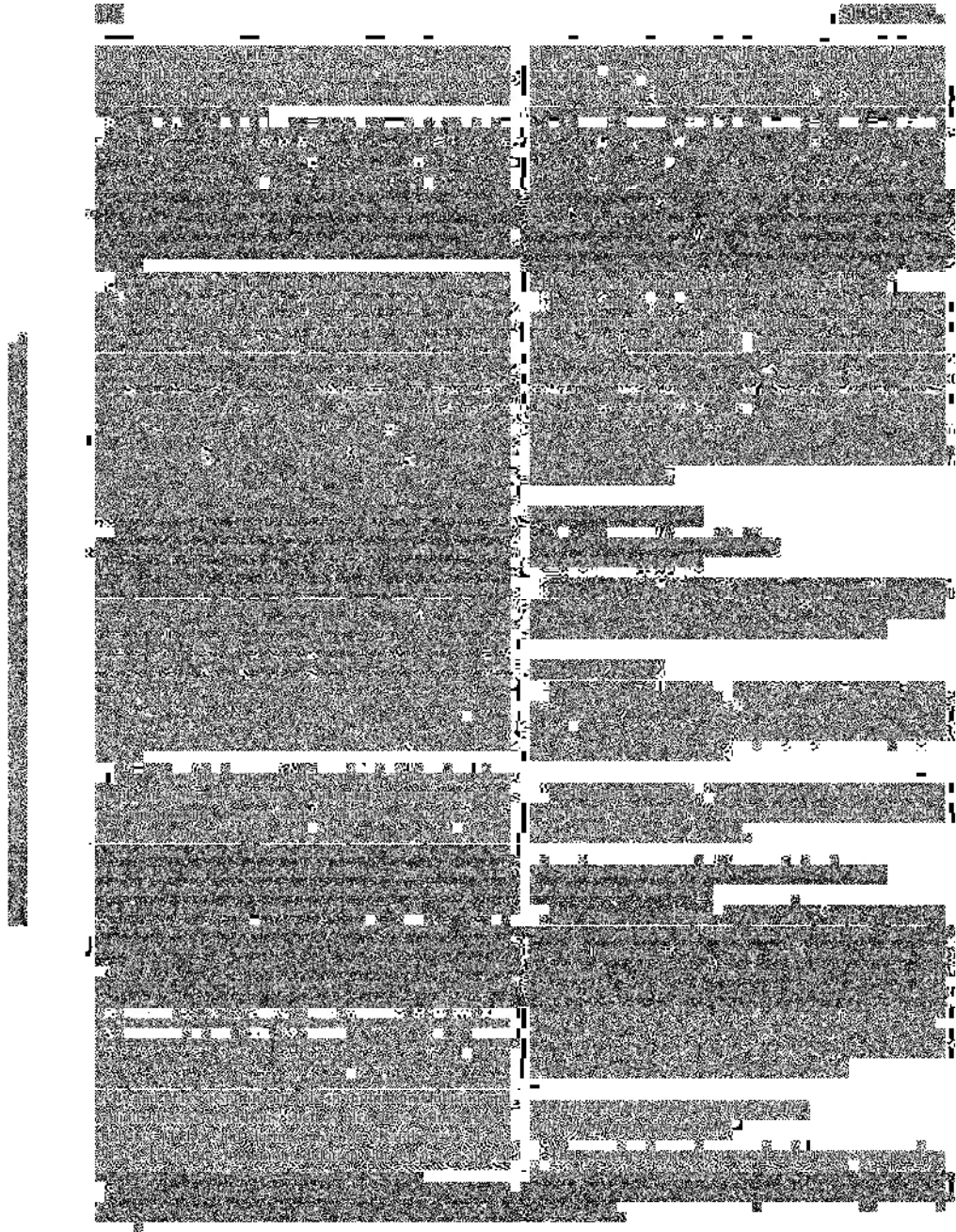
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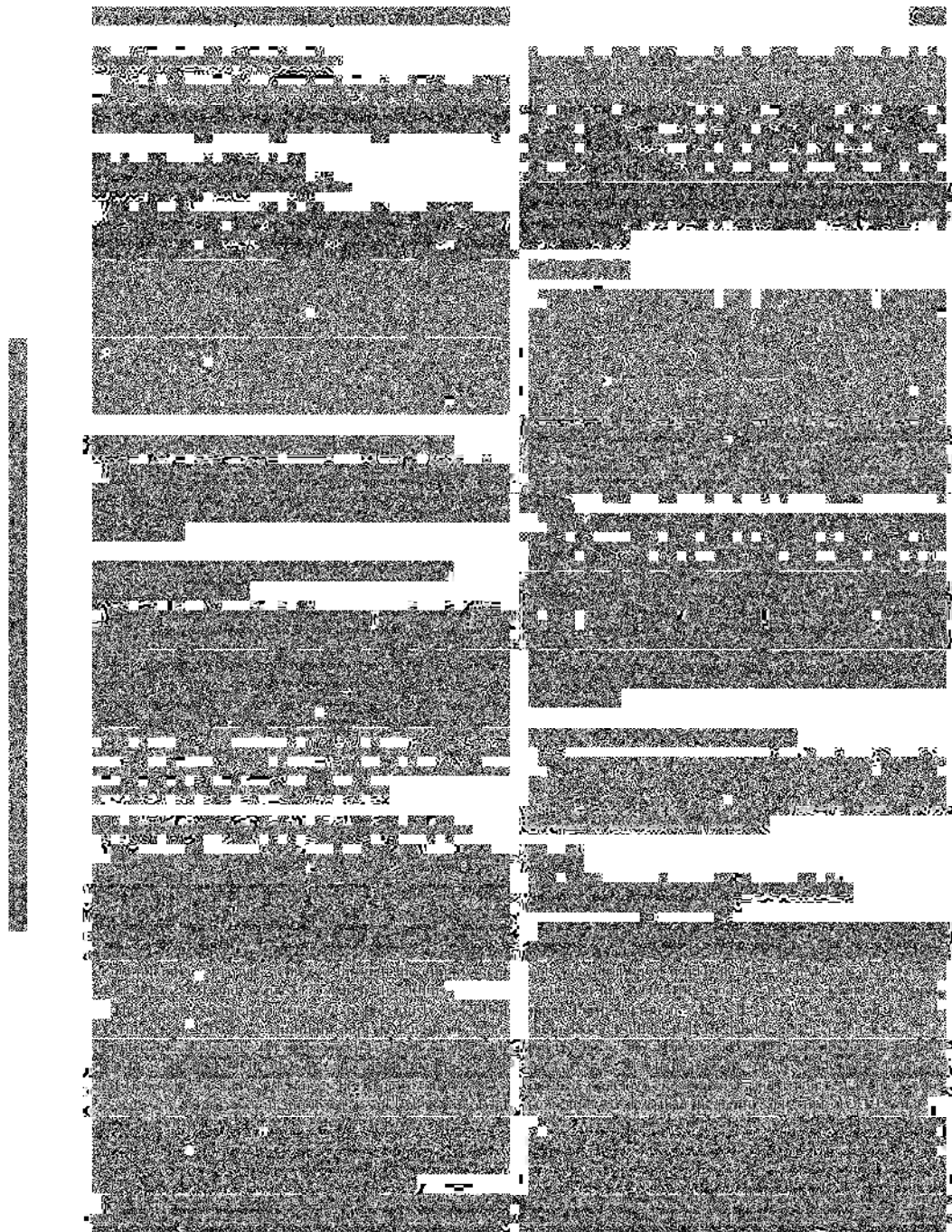
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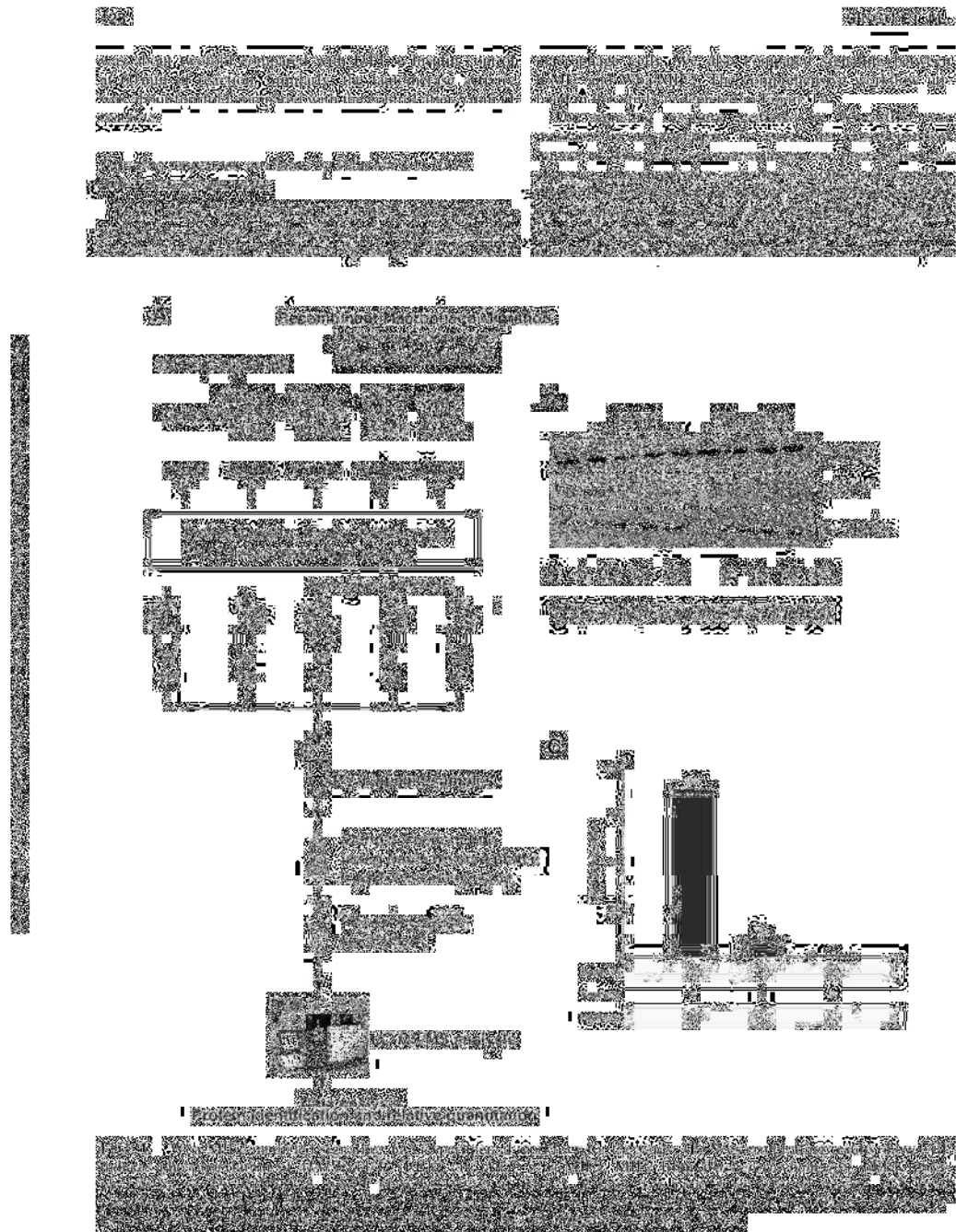
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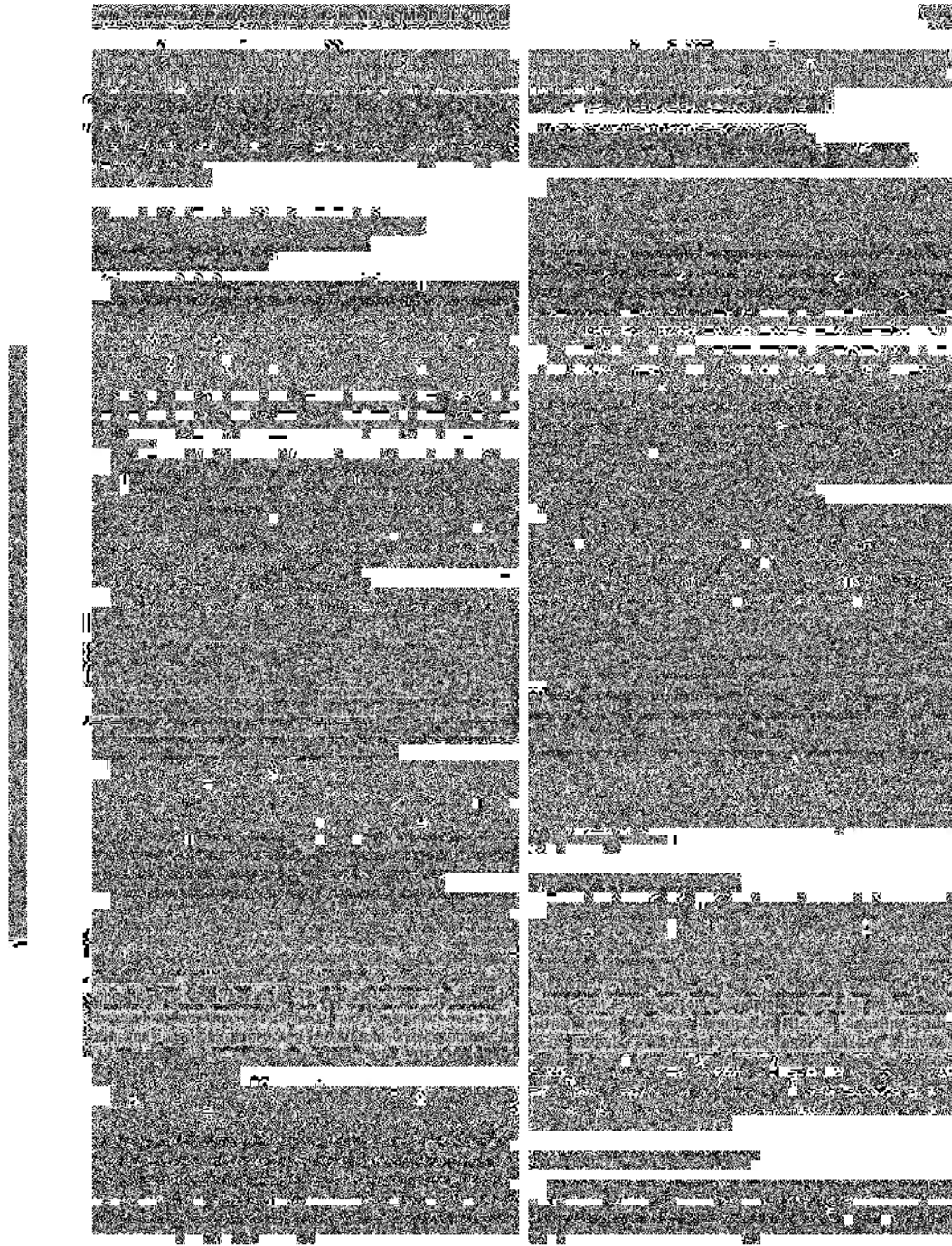
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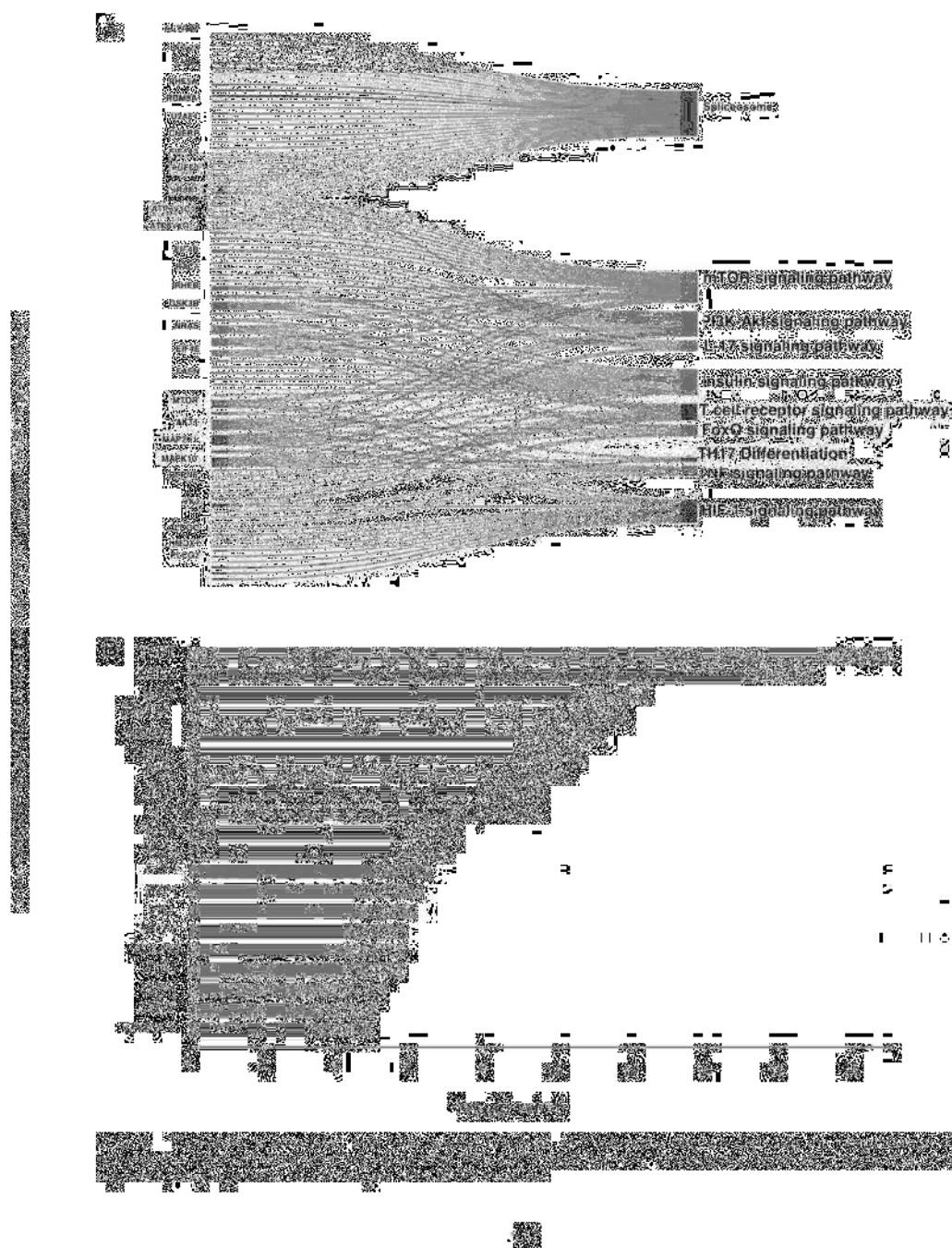


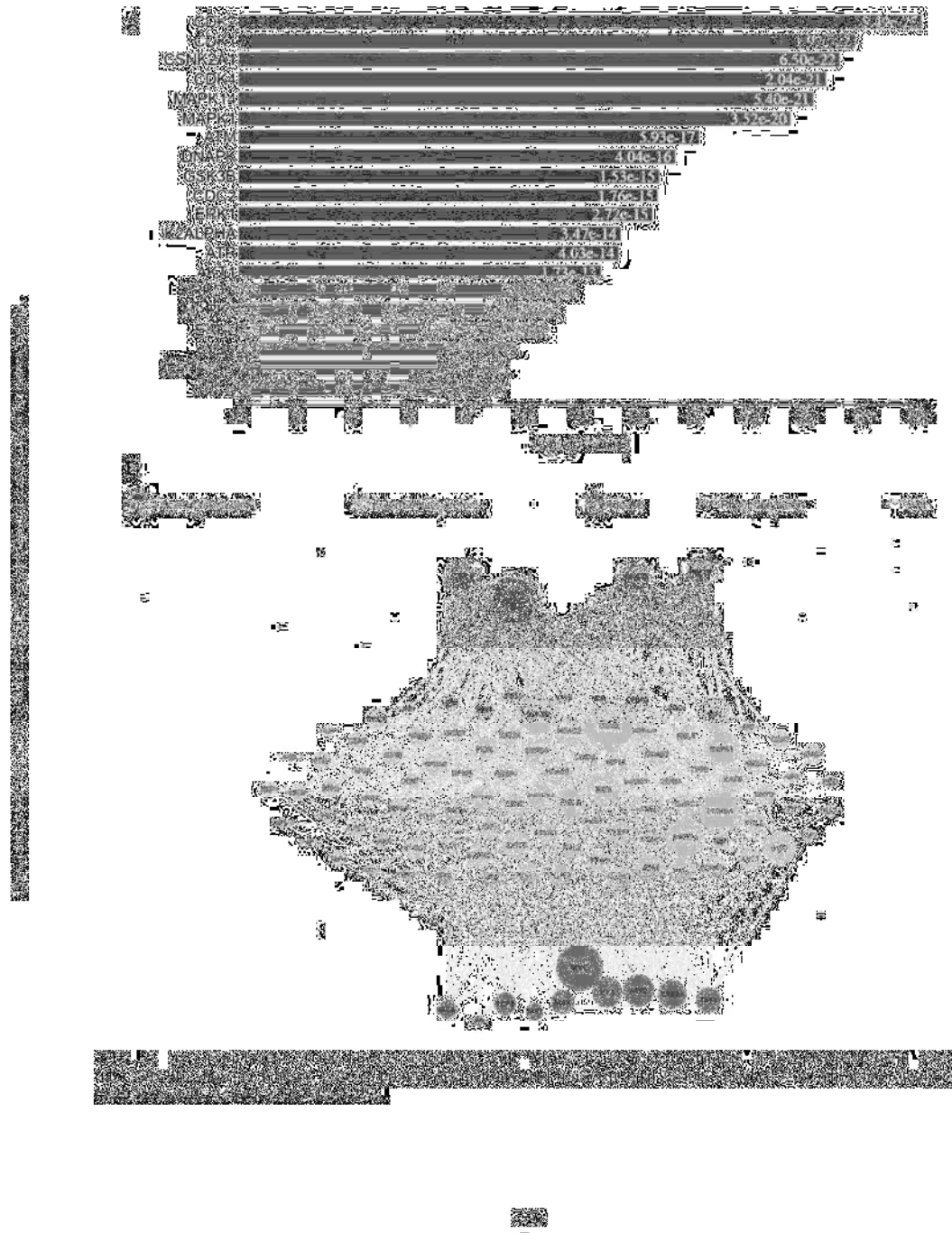


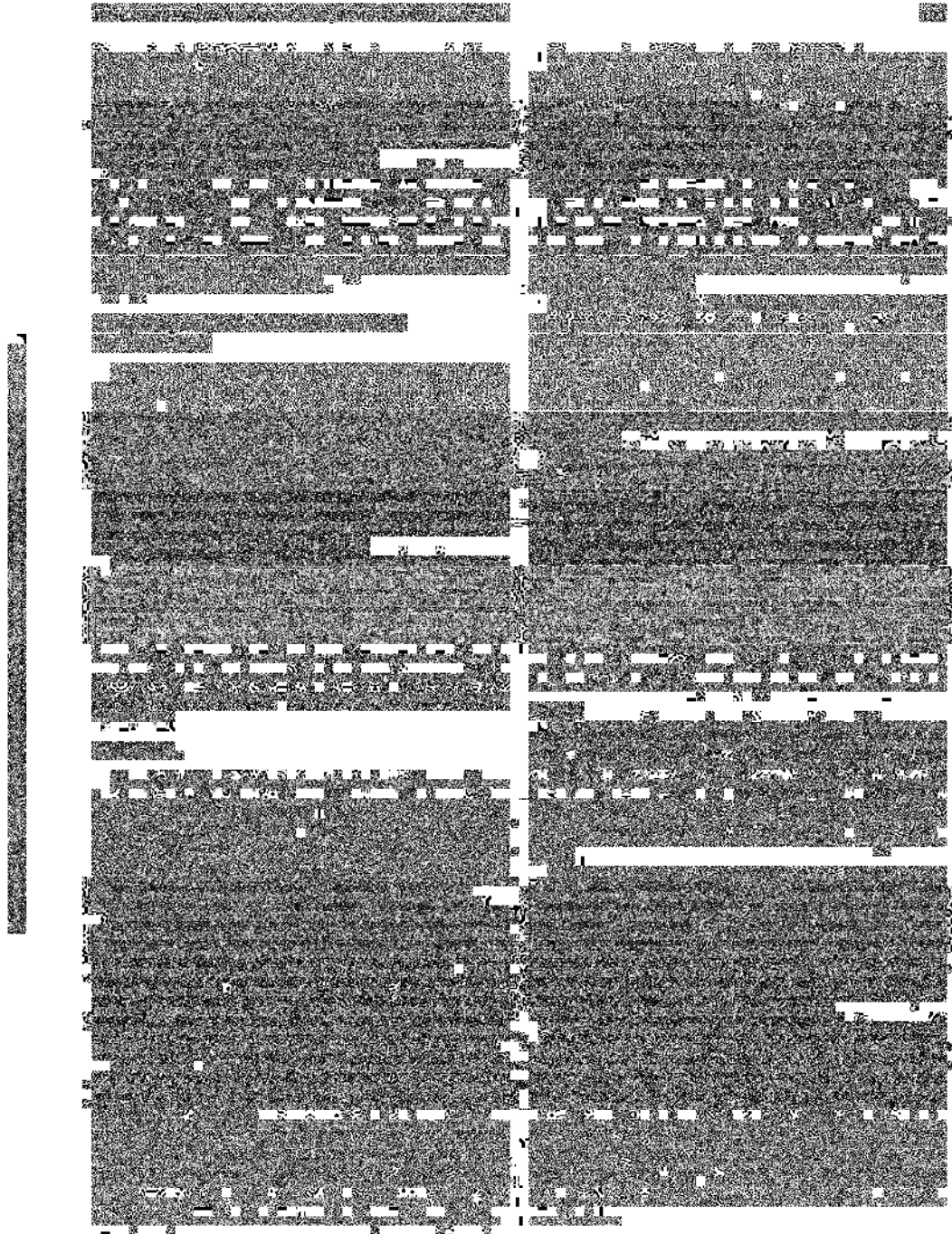


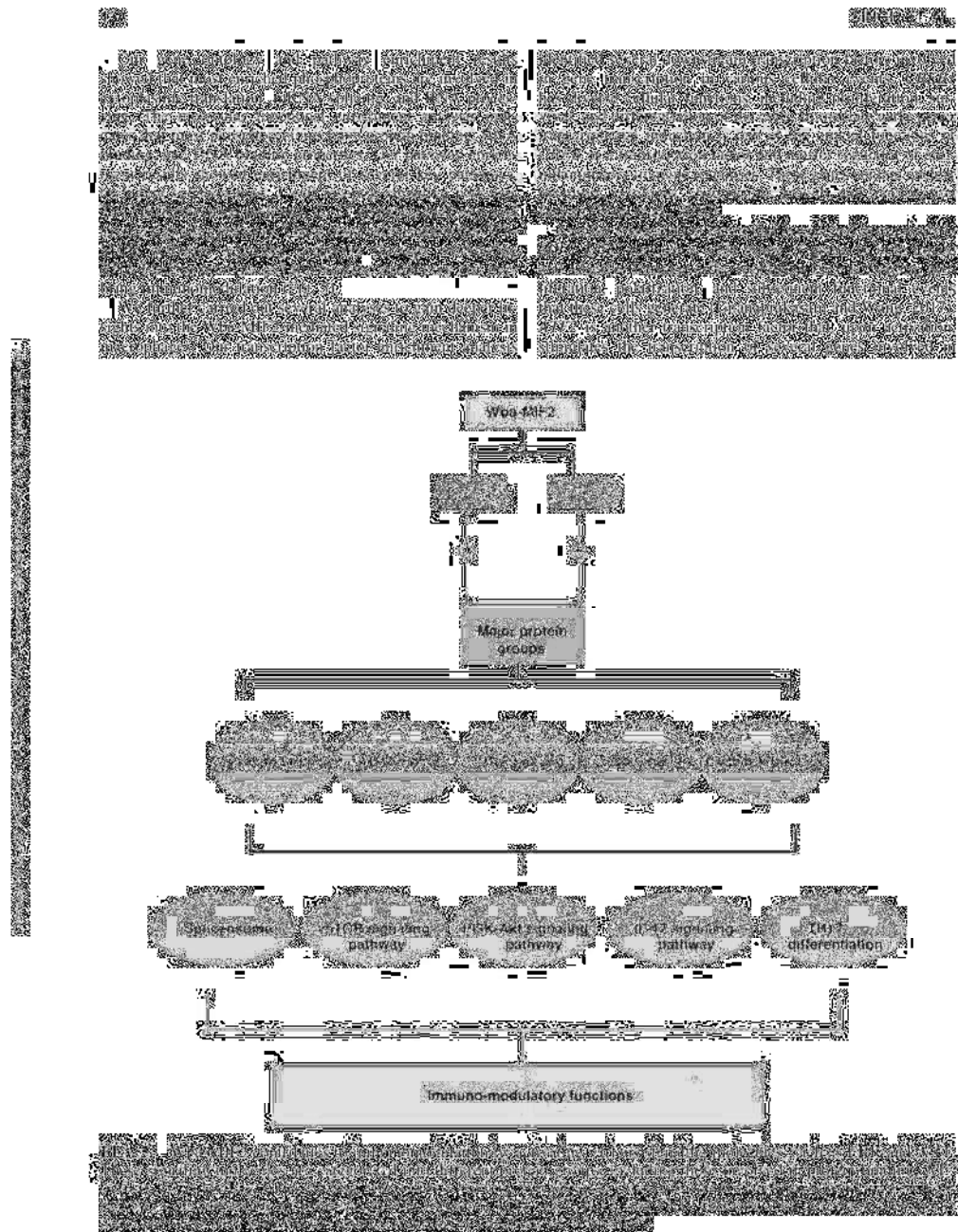


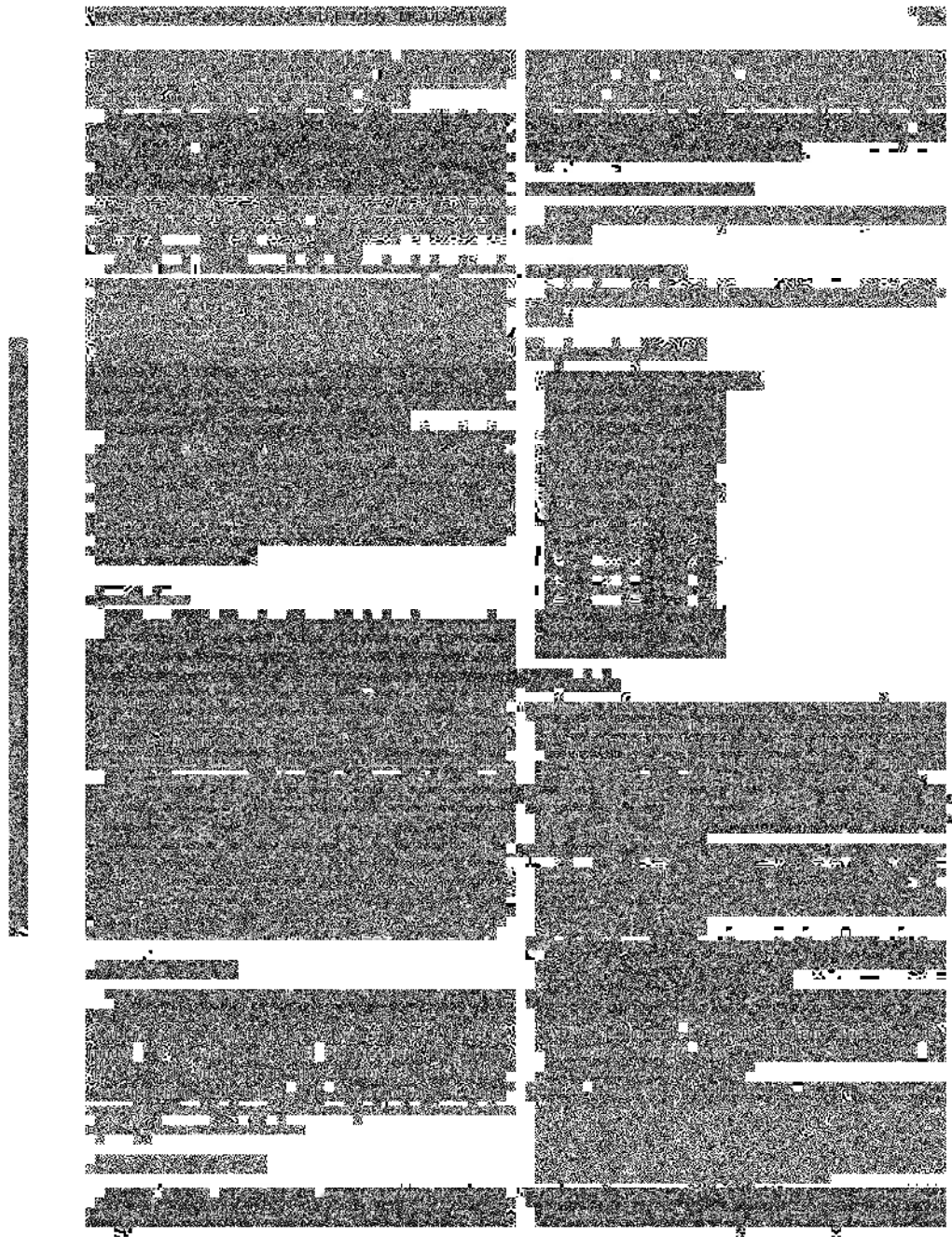


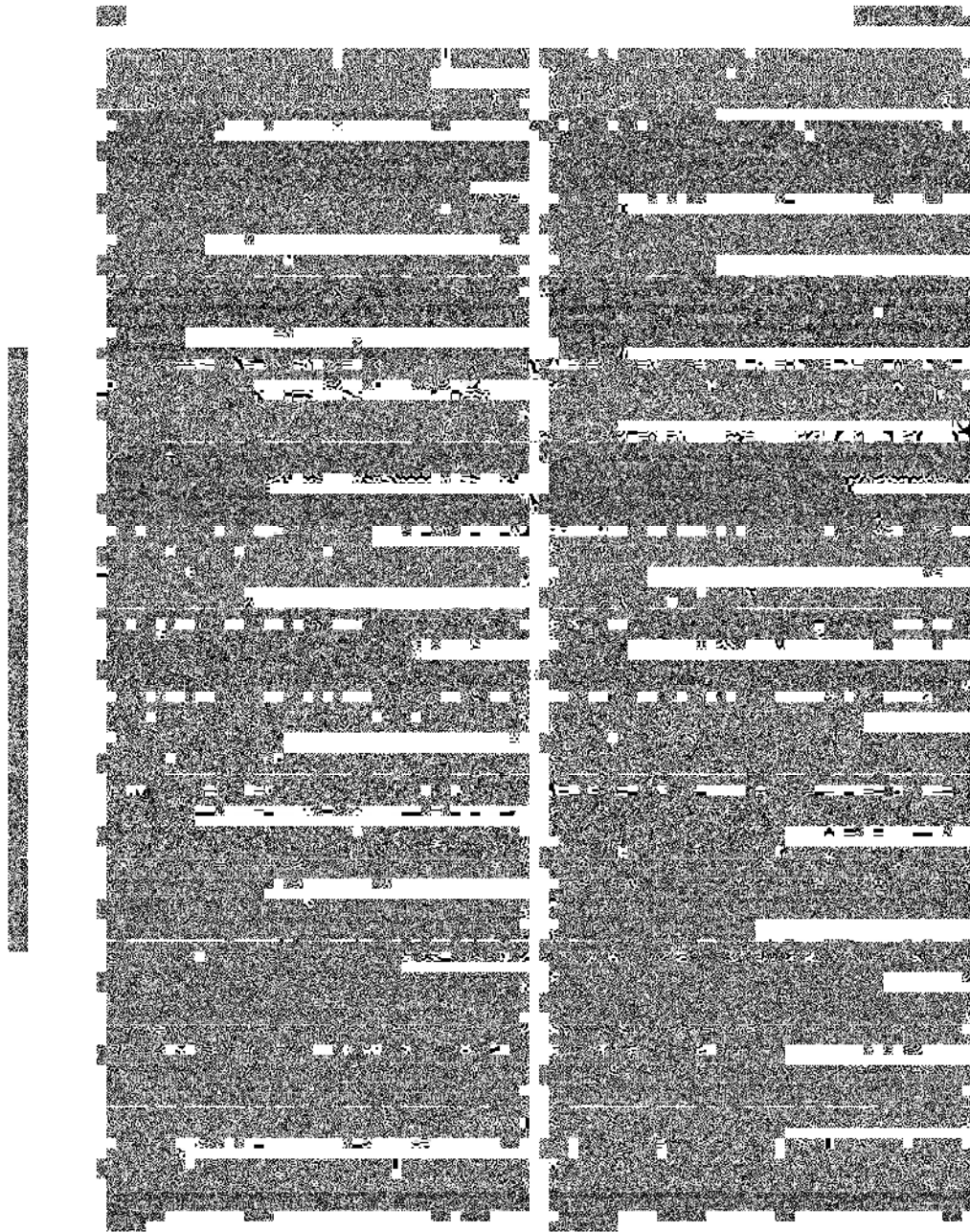


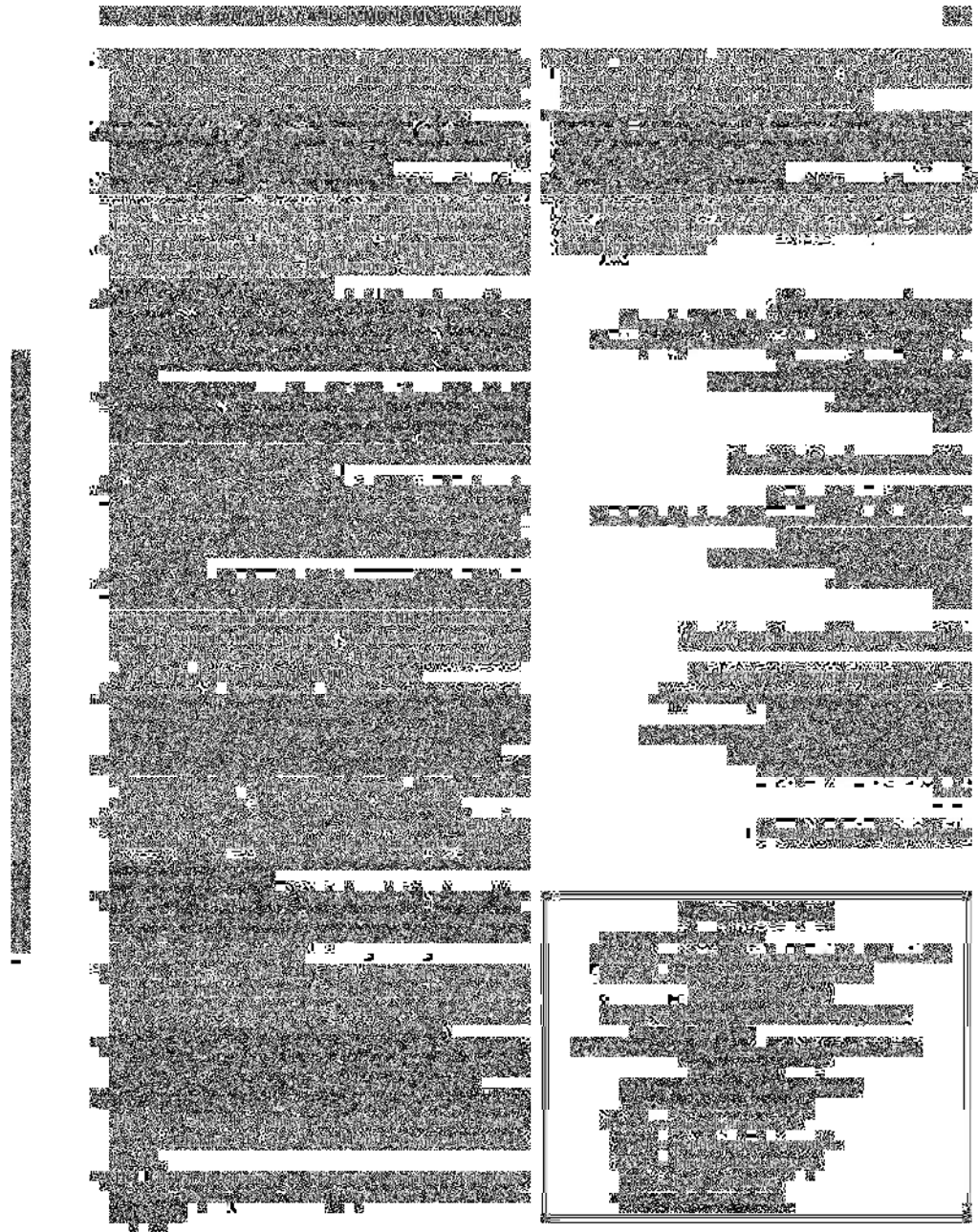






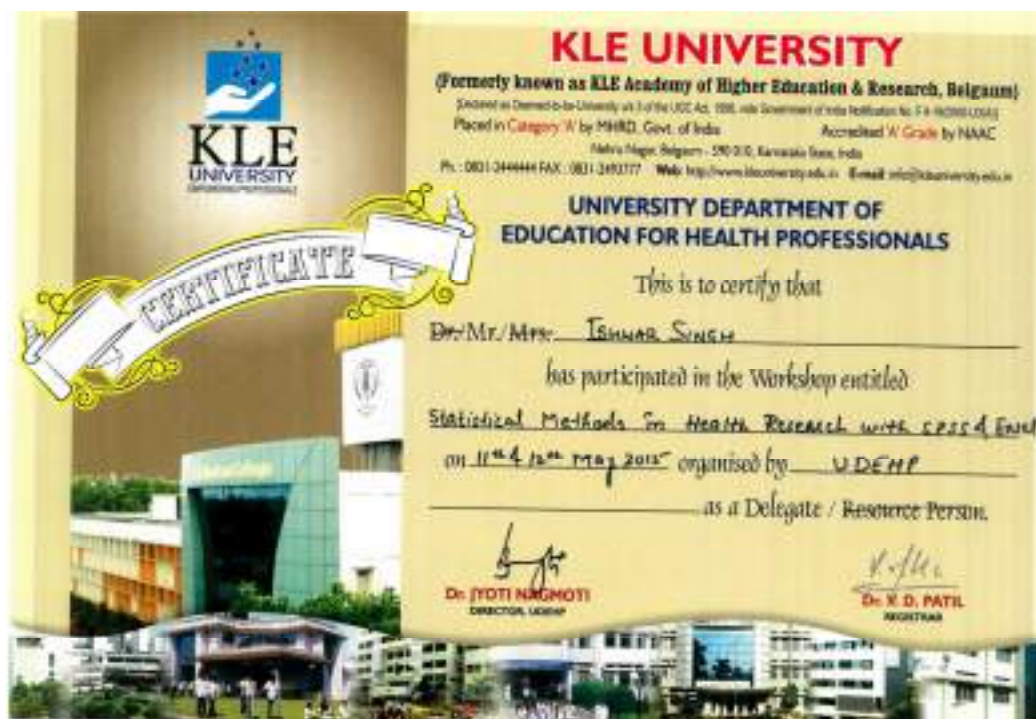






Annexure -IV







**CENTRAL ANIMAL FACILITY
INDIAN INSTITUTE OF SCIENCE
BANGALORE - 560 012**

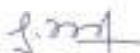


CERTIFICATE

This is to certify that **Mr. Ishwar Singh**, Dept. of Microbiology and Cell Biology, Indian Institute of Science, Bangalore participated in the orientation course on "Laboratory Animal Care and Use" held between August 28 - September 01, 2017 at the Central Animal Facility, Indian Institute of Science, Bangalore.


(Dr. S.G. Ramachandra)
Course Coordinator


(Prof. K.N. Balaji)
Convenor


(Prof. Kumar Somasundaram)
Chairman