Analysis of the Functional Role of MicroRNAs in Regulating Macrophage Mediated Inflammatory Responses

A Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Analysis of the Functional Role of MicroRNAs in Regulating Macrophage Mediated Inflammatory Responses"** has been carried out by me under the supervision of **Dr. Kishore Parsa (Guide),** Principal Research Scientist at Dr. Reddy's Institute of Life Sciences, University of Hyderabad. I am registered for Ph.D degree in the Department of Biochemistry, School of Life Sciences, University of Hyderabad and this thesis has not been submitted for any degree or diploma at any other University. I hereby agree that my thesis can be deposited in Shodhganga/INFLIBNET. A report on plagiarism statistics from the University Librarian is enclosed.

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CERTIFICATE

This is to certify that **Ms. Malathi Talari** has carried out the research work embodied in the present thesis entitled **"Analysis of the Functional Role of MicroRNAs in Regulating Macrophage Mediated Inflammatory Responses"** being submitted to the Department of Biochemistry, School of Life Sciences, University of Hyderabad for the degree of Doctor of Philosophy, under my supervision in the Department of Biology, Dr. Reddy's Institute of Life Sciences, Hyderabad.

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ABSTRACT

Unrestrained inflammation frequently observed in chronic inflammatory diseases such as insulin resistance, Type 2 Diabetes (T2D) and several types of cancers is prominently due to imbalances in different activation states of macrophages. Delineation of the regulatory mechanisms underlying macrophage polarization may help us to better understand the pathophysiological basis of inflammation linked diseases. MicroRNAs are post-transcriptional regulatory molecules that drive distinct biological processes such as proliferation, cell survival, differentiation and inflammation. However, the functional role of microRNAs in inflammation induced insulin resistance is poorly studied. Thus, there is a necessity to study the involvement of microRNAs in inflammation induced insulin resistance (IR), as IR is the leading cause of diabetes. With this objective we sought to understand the regulatory role of microRNAs in macrophage polarization and insulin resistance.

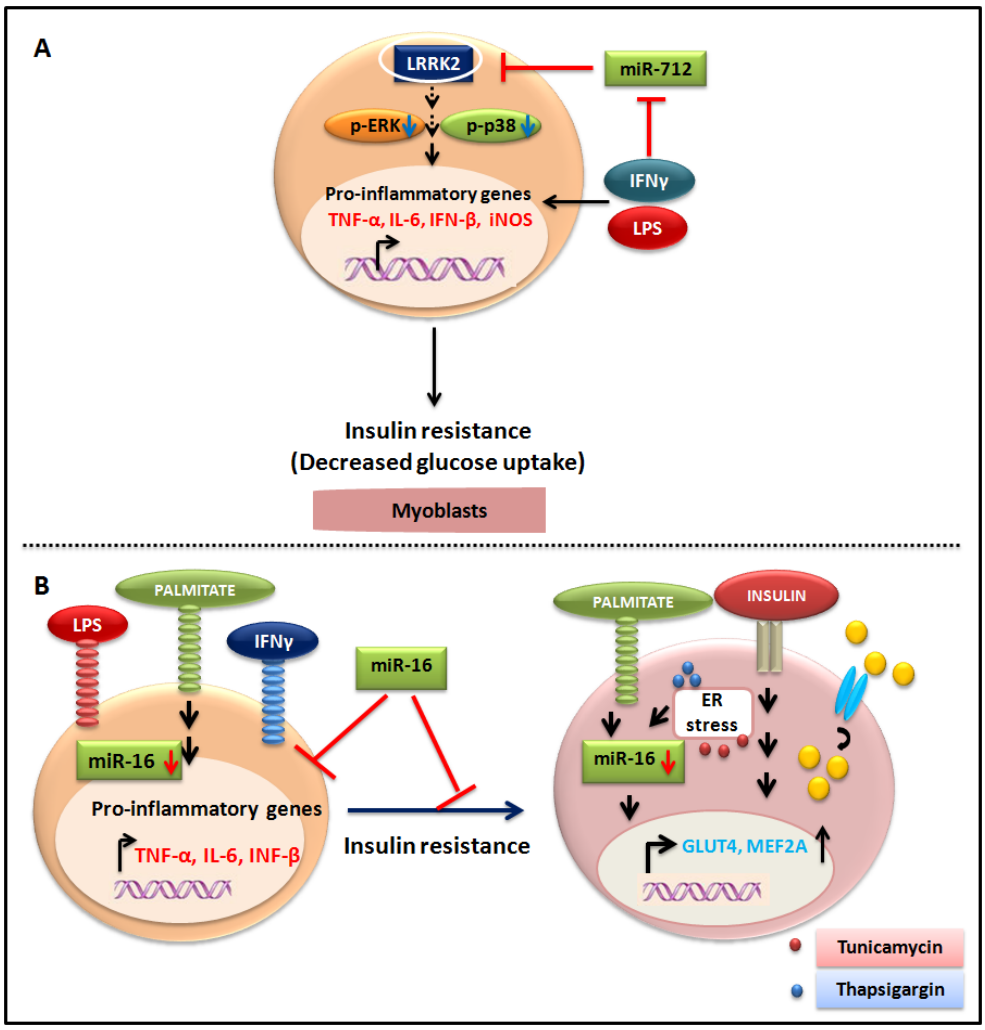
Our microRNA microarray data revealed several microRNAs that were differentially regulated in polarized macrophages. Among them, we investigated the functional roles of two microRNAs: miR-712 and miR-16 whose expression levels were down-regulated in macrophages exposed to pro-inflammatory stimuli such as LPS+IFNγ and palmitate. Additionally, we have observed that miR-16 expression is also down-regulated in palmitate exposed myoblasts, ER stress inducer (tunicamycin and thapsigargin) treated myoblasts and insulin responsive tissues of high sucrose diet (HSD) induced insulin resistant rats. On the other hand unlike in macrophages, miR-712 expression levels were observed to be increased in myoblasts upon exposure to stress inducers such as palmitate,

tunicamycin and thapsigargin pointing to potential cell specific effects. Investigating the direct role of miR-712 in skeletal muscle may help us in better understanding of insulin mediated effects in physiology and disease.

We next noted that ectopic expression of miR-712 and miR-16 in macrophages reduced the production of pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-β which in turn led to improved insulin sensitivity in insulin resistant (IR) skeletal myoblasts suggesting reduced paracrine inhibitory effects of LPS+IFNγ polarized macrophages on skeletal myoblasts insulin sensitivity. In addition we observed that forced expression of miR-16 directly in myoblasts augmented insulin stimulated glucose uptake via up-regulation of two key players: GLUT4 and MEF2A that are involved in insulin stimulated glucose uptake.

Mechanistic analysis revealed LRRK2 (a serine / threonine protein kinase associated with inflammatory diseases such as Crohn's and Parkinson's disease) as the target of miR-712. Further over-expression of miR-712 resulted in reduced phosphorylation of p38 and ERK1/2, key players involved in inflammatory gene expression suggesting that miR-712 is positioned to control macrophage mediated pro-inflammatory responses.

Collectively, our data demonstrates the pivotal roles of microRNAs miR-712 and miR-16 in alleviating inflammation induced insulin resistance for the first time. Exploring the pathophysiological roles of these microRNAs may further help us in understanding the progression and treatment of T2D.



MicroRNA-712 and miR-16 dampen macrophage mediated pro-inflammatory responses and improve insulin mediated glucose uptake in myoblasts. (A) miR-712 expression is down-regulated in LPS +IFNγ polarized macrophages. Ectopic expression of miR-712 attenuated macrophage pro-inflammatory responses and their paracrine inhibitory effects on myoblast insulin sensitivity. Mechanistic analysis showed that miR-712 directly targets LRRK2 resulting in decreased phosphorylation of p38 and ERK1/2 in macrophages. (B) miR-16 expression is down-regulated in LPS+IFNγ and palmitate stimulated macrophages and in palmitate, tunicamycin and thapsigargin treated myoblasts. Importantly, forced expression of miR-16 into macrophages improved myoblast insulin sensitivity by enhancing GLUT4 and MEF2A expression levels.

Dedicated to

My Dearest Family

and

My Beloved Husband

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ABBREVIATIONS

AAP	Amyloid precursor protein
ACSL	Acyl co-A synthetase long chain family
AD	Alzheimer's disease
ADORA	Adenosine receptors
ADAR	Adenosine deaminase acting on RNAs
AGPS	Alkyl dihydroxy acetone phosphate synthase
AID	Activation induced cytidine deaminase
AKT/PKB	Protein kinase B
ALOX	Arachidonate lipoxygenase
AP-1	Activator protein 1
APCs	Antigen presenting cells
APO E	Apolipoprotein E
ARE	AU rich elements
ARG-1	Arginase 1
BCL	B cell lymphoma
BIM/BCL2L11	BCL2 like protein 11
BMDMs	Bone marrow derived macrophages
BMP	Bone morphogenetic proteins
BSA	Bovine serum albumin
BTBD3	BTB (POZ) domain containing 3
BTK	Bruton's tyrosine kinase
C1q, C3b	Complement proteins
CADM	Cell adhesion molecule
CCDN1	Cyclin D1
CCL	C–C motif ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
CD	Crohn's disease
CDK	Cyclin dependent kinase
CDS	Coding sequence
CDP	Common dendritic cell progenitor
C/EBP	CCAAT-enhancer binding proteins
CHN	Chimerin
CISH	Cytokine inducible SH2 containing proteins
CLDN	Claudin
CLL	Chronic lymphocytic leukemia

CLTC	Clathrin heavy chain
CMP	Common myeloid progenitors
COL	Collagens
COPD	Chronic obstructive pulmonary disease
COX	Cycloxygenase
CR	Complement receptors
CREB	cAMP response element binding protein
CSFR	Colony stimulating factor receptor
CTGF	Connective tissue growth factor
CTPS	CTP synthase
CXCL	C–X–C motif ligand
DAMPs	Danger-associated molecular patterns
DC-SIGN/CD209	Dendritic cell specific intercellular adhesion molecule-3-grabbing-non integrin
DLEU	Deleted in leukemia
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNM	Dynamin protein
DUSP	Dual specificity phosphatase
EC	Endothelial cells
ECM	Extracellular matrix
EMP	Erythro-myeloid progenitor
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
ETLK	Ethanolamine kinase
F13A1	Fibrinoligase
FABP	Fatty acid binding protein
FBS	Fetal bovine serum
FcR	Fragment of crystallization receptor
FFA	Free fatty acids
FGF	Fibroblast growth factor
FIZZ1/Relmα	Resistin like molecule
FN	Fibronectin
FOXO	Fork head box o
FRK	Fyn related Src family tyrosine kinase
GC	Glucocorticoids
GBP 1	Guanylate-binding protein 1
GDF	Growth differentiation factor
GJ	Gap junction protein

GLUT	Glucose transporter
GM-CSF	Granulocyte-macrophage Colony Stimulating Factor
GMP	Granulocyte myeloid progenitor
GNAS	Guanine nucleotide binding protein
GPX	Glutathione peroxidise
GSK-3beta	Glycogen synthase kinase-3 beta
GXYLT	glucoside xylosyltransferase
HDAC	Histone deacetylases
HER/Neu	Human epidermal growth factor receptor
HIF	Hypoxia inducible factor
HNRNPH	heterogeneous nuclear ribonucleoprotein H
HS	Horse serum
HSCs	Hematopoietic stem cells
HSD	High sucrose diet
HSP	Heat shock protein
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IFNAR	Interferon-alpha receptor
Ig	Immunoglobulin
IGFBP	Insulin like growth factor binding protein
IKK	Ikappa B kinase
IL	Interleukin
iNOS/NOS2	Inducible nitric oxide synthase
INPP	Inositol poly phosphate phosphatise
IP-10	IFN-γ-Inducible Protein 10
IRF	Interferon regulatory factor
IRS	Insulin receptor substrate
IRAK	Interleukin receptor associated kinase
ITS	Internal transcribed spacer
ITGA	Integrin subunit alpha
JAK	Janus tyrosine kinase
JNK	C-Jun N-terminal kinase
JMJD3	Jumonji domain containing 3
KLF	Kruppel like factors
KYNU	Kynureninase
LDL	Low density lipids
LITAF	LPS induced TNF factor
LMNA	Lamin protein A

LXR	Liver X receptor
Ly6c1	Lymphocyte antigen 6 complex, locus 1
LYSMD3	LysM, putative peptidoglycan-binding, domain containing 3
MAPK/MPK	Mitogen activated protein kinase
MARCKS	Myristoylated alanine rich protein kinase C substrate
MARCO	Macrophage receptor with collagenous structure
MCL	Myeloid cell leukemia
M-CSF	Macrophage/monocyte colony stimulating factor
MD-2	Myeloid differentiation protein-2
MDM	Monocyte derived macrophages
MDP	macrophage or dendritic cell progenitor
MEF	Myocyte enhancer factor
MERTK	Tyrosine protein kinase mer
MGL	Macrophage galactose lectin
MHC	Major histocompatibility complex
MiRNA/miR	MicroRNA
MLL	myeloid/lymphoid or mixed-lineage leukemia 1
MMP	Matrix metalloproteinase
MMR/MR	Macrophage mannose receptor
MOMP	Major outer membrane protein
MPS	Mononuclear phagocytic system
mRNA	Messenger RNA
MSK	Mitogen and stress activated
MyD88sh	Myeloid differentiation factor 88 short
NCAM	Neural adhesion cell molecule
NCOR	Nuclear receptor co-repressor
NFAT	Nuclear factor of activated T cells
NFIL	Nuclear factor interleukin
NFκB	Nuclear factor κb
NFκBiz	Nuclear factor κb inhibitor zeta
NIK	Nuclear factor κb inducible kinase
NLR	NOD (nucleotide-binding oligomerization-domain protein)-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
NSF	N-ethylmaleimide sensitive factor
PAMPs	Pattern/ pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction

PDCD	Programmed cell death protein
PDK	Pyruvate dehydrogenase kinase
PHB	Prohibitin
PHLPP	PH (pleckstrin homology) domain Leucine-rich repeat Protein Phosphatase
PIAS	Protein inhibitor of activated STAT
PI3K	Phosphatidylinositol 3 kinase
PKC	Protein kinase C
PKNOX	PBX/Knotted 1 homeobox
PPARγ	Peroxisome proliferator activated Receptor γ
PPP1R3	Protein phosphatase 1 regulatory subunit 3
PRR	Pattern-recognition receptor
PTEN	Phosphatase and tensin homologue
P2YR	Purinergic G protein coupled receptors
RBC	Red blood corpuscles
RBL	Retinoblastoma like protein
REC	Retinal endothelial cells
RES	Reticuloendothelial system
RGS	Regulator G protein signalling
RHOQ	Rho related GTP binding protein Q
RISC	RNA inducible silencing complex
RNA	Ribonucleic acid
RNAa	RNA activation
RNAi	RNA interference
RNase	Ribonucleases
RNGTT	RNA guanylyl transferase and 5’ phosphatise
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SBNO	Strawberry notch homolog
SGMS	Sphingomyelin synthase
SHIP	(SH2)-containing inositol phosphatise
SHP	Src homology region 2 domain containing tyrosine phosphatise
SIGNR3	Specific intracellular adhesion molecule-3 grabbing non-integrin homolog-related 3
SMAD	Small mothers against decapentaplegic
SMC	Structural maintenance of chromosomal proteins
SNP	Single nucleotide polymorphism
SNORNA	Small nucleolar RNA
SOCS	Suppressor of cytokine signalling

SOS	Son of sevenless
SORBS	Sorbin and SH3 domain containing protein
SR	Scavenger receptor
SREBP	Sterol regulatory element binding protein
SRSF	serine/arginine-rich splicing factor
ST2/ IL-1RL1	Interleukin receptor like
STAB	Stabilin
STAT	Signal transducer and activator of transcription
STK	Serine threonine kinase
TAB	TAK binding protein
TAM	Tumour associated macrophage
TAOK	Serine threonine protein kinase
TCR	T cell receptor
TGF-β	Transforming growth factor-β
TGM	Transglutaminase
Th	Helper T cell
TIR	Toll or interleukin receptors
TIRAP	Toll interleukin 1 receptor domain containing adaptor protein
TLR	Toll-like receptor
TM9SF2	Transmembrane 9 superfamily member 2
TNF-α	Tumour Necrosis Factor-α
TP53	Tumour protein 53
TRAF	TNF receptor associated factor
Treg	Regulatory T cell
TRIF	TIR-domain-containing adapter-inducing IFN-β
tRNA	Transfer RNA
T2D	Type 2 diabetes
TSC-mTOR	Tuberous sclerosis complex-mammalian target of rapamycin
UC	Ulcerative colitis
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WBC	White blood corpuscles
WT	Wilm's tumour
YM-1/CHIL3	Chitinase-like 3

Chapter 1

Introduction and review of literature

Macrophages are tissue differentiated cells that perform a variety of homeostatic and immune regulatory functions. They differentiate from peripheral blood monocytes that migrate across various tissues to carry out diverse physiological functions. Macrophages primarily balance cellular homeostasis by clearing extraneous materials like cellular debris and '*effete*' or apoptotic cells. They are also chiefly involved in remodelling of damaged tissue by forming ECM (extracellular matrix) and supply of adequate amount of blood and oxygen to the damaged site [1, 2]. In addition to these functions macrophages also perform a crucial process of recycling the functional material that becomes a remarkable metabolic contribution without which the host would not survive. Apart from these '*janitorial*' functions they also execute '*effector*' immunity functions to provide defence against malicious particulates thus forming a barrier across the cell surface. Any dys-regulation in their performances would lead to fatal inflammatory diseases [1, 3-6]. In the coming sections we shall comprehensively discuss the diverse roles of macrophages, their pathophysiological and regulatory aspects.

1.1. Macrophages and their regulatory role in inflammation and diseases

Macrophages are tissue differentiated monocytes that perform a variety of homeostatic and immune-regulatory functions. They respond to surrounding milieu and can alter their phenotype and cellular repertoire to suit their functional requirements. Such a plastic behavior of macrophages is termed as macrophage *polarization*. Tight balance between different activated/polarized states of macrophages is essential to maintain immune homeostasis and dys-regulation of their functionality may lead to fatal inflammatory related diseases. The macrophage differentiation, proliferation, activation and pathophysiological roles will help us in understanding the disease mechanism.

1.1.1 Macrophage development and differentiation

Macrophages belong to mononuclear phagocytic system (MPS) which is the product of a regulated process of differentiation [7]. Initially all the immune cells originate from a committed lineage of hematopoietic stem cells (HSC) in the bone marrow which subsequently undergo a series of differentiation steps to give rise to myeloid progenitor cells. These cells act as common myeloid progenitors (CMP) for a variety of cell types including neutrophils, eosinophils, basophils, monocytes, dendritic cells and mast cells depending on the growth factor they receive (Figure 1.1). For instance, in response to macrophage colony stimulating factor (M-CSF), myeloid progenitor cells commit to form a monocytic lineage for which they primarily differentiate into monoblasts followed by pro-monocytes where they exit the bone marrow and enter into the blood stream to finally differentiate into monocytes. Ly6C ^{hi} monocyte (lymphocyte antigen 6 complex, locus C1) populations are shown to actively extravasate into various tissues to differentiate into macrophages either in steady state or in response to an injury or infection to perform

prodigious functions such as tissue remodelling, clearance of pathogens, and maintenance of cellular homeostasis [8-10]. Two theories were proposed to explain macrophage development and differentiation; firstly macrophages were believed to originate from reticuloendothelial system (RES). This theory was widely accepted for about half a century, until van Furth et al., in 1972 proposed macrophage generation from mononuclear phagocytic system (MPS) [7]. According to them all the macrophages are derived from monocytes and not from endothelial cells, reticulum cells or fibroblasts of RES and they believed that there are two major subpopulations of macrophages; i) tissue resident macrophages which were identified to extravasate in normal steady state or unstimulated cells and were grouped into major family of histiocytes or tissues macrophages and ii) exudate macrophages which were differentiated from increased populations of monocytes during inflammatory stimuli. However many recent evidence indicated that monocytes do not significantly contribute to tissue macrophages rather majority of them are already formed during embryonic development and are maintained in adults by their self renewal property [8, 11]. According to this, erythro-myeloid progenitor cells (EMP) in the yolk sac migrate and colonize in the foetal liver during very early stages of embryonic development. Additionally it was reported that macrophages can be directly formed in the yolk sac in Myb (transcription factor) independent manner while HSCs in the foetal liver produce macrophages in Myb dependent fashion. These cells subsequently enter into the developing tissues to form tissue resident macrophages (Figure 1.2) [8, 12].

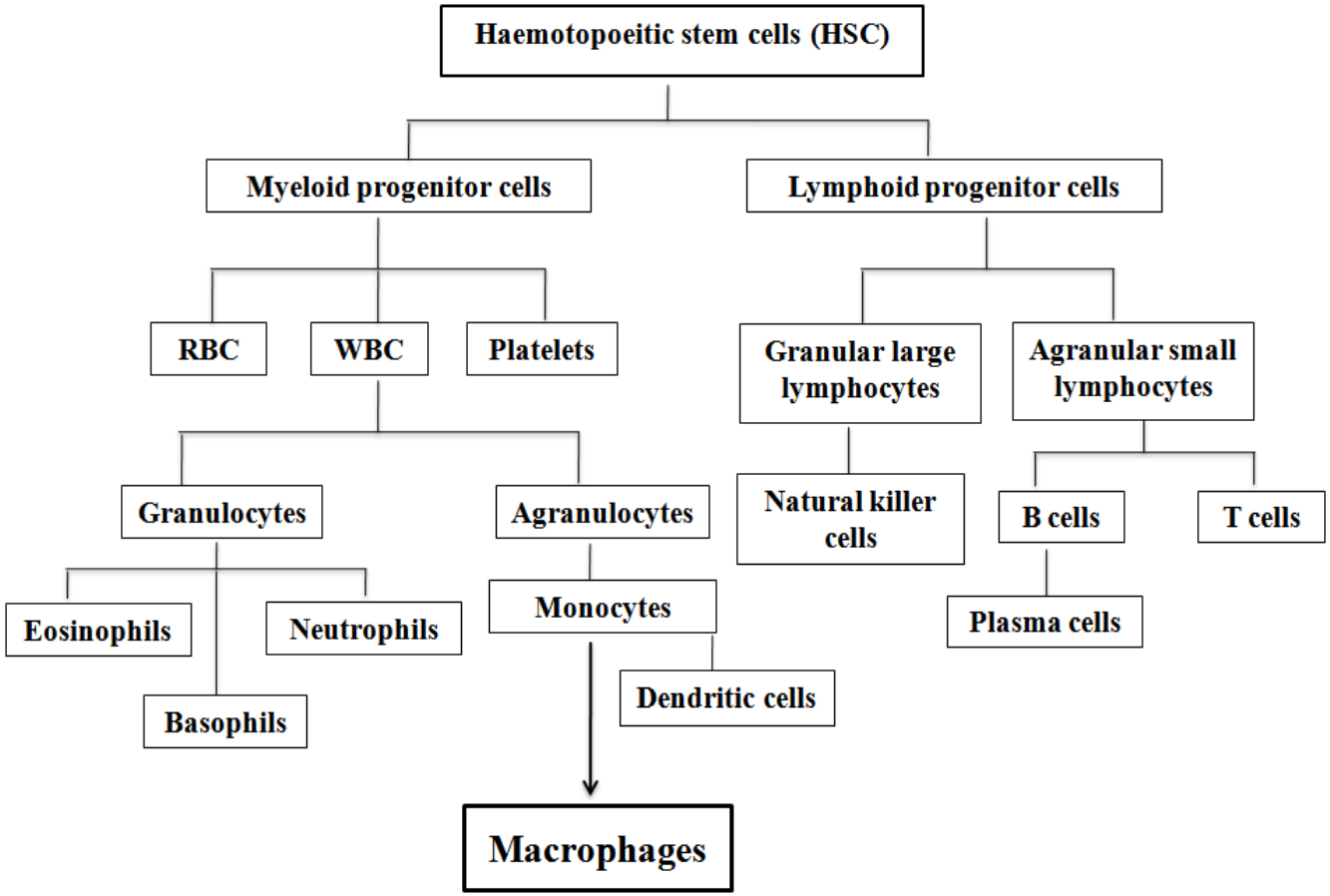


Figure 1.1. Macrophage development from hematopoetic stem cells. Macrophages are differentiated from monocytes via myeloid progenitor cells. They constitute a large group of agranulocytes which are evolved to perform diverse functions in the tissues.

1.1.2. Macrophage tissue distribution

Macrophages are virtually present in all the tissues and are named depending on their anatomical locations and functions. Specialized tissue resident macrophages in the bone are termed as osteoclasts. They are responsible for controlling bone resorption and thus regulate calcium homeostasis. Similarly macrophages are termed as langerhans (epithelial cells), kupffer cells (liver), microglia (brain), intraoccular (eyes) and alveolar macrophages (lungs) depending on the tissue located [1, 8, 11]. These macrophages are involved in performing tissue surveillance against pathogens and control of inflammation. They are also distributed across various regions of a secondary lymphoid organ, spleen (splenic macrophages) and are further named based on their zonal location. For instance they are termed as white-pulp macrophages, red pulp macrophages, marginal zone macrophages and metallophilic macrophages depending on their splenic location. Such macrophages are majorly involved in bridging the innate and adaptive immune responses to counteract blood borne particulates. More importantly, red pulp macrophages are critical in maintaining blood homeostasis by phagocytosing the senescent and injured erythrocytes followed by iron recycling from effete RBCs (Red blood cells) and kupffer cells are shown to be important for iron and cholesterol recycling. Similarly peritoneal macrophages in the peritoneum are shown to promote IgA secretion from peritoneal B cells which aid in immune surveillance. All the above mentioned populations of macrophages are reported to be formed during embryonic development while adult macrophages in the intestinal lamina propria, gut muscularis and mammary glands are shown to be differentiated from Ly6C ^{hi} monocytes . On the other

hand, bone osteoclasts are shown to be differentiated during both embryogenesis and adult stage as they are very crucial for bone formation (Figure 1.2) [8-10].

1.1.3. Proliferation and maintenance of macrophages

According to MPS concept macrophages were shown to be non-proliferative with short life span and poor self renewal capacity. In contrast to this researchers have repeatedly showed that tissue macrophages in severely monocytopenic (lack of monocytes) mouse models could survive for long time by their self renewal capacity and displayed slight increase in their cell numbers without the supply of blood monocytes signifying the fact that tissue resident macrophages proliferate [11, 13]. Unlike MPS concept which demonstrated that kupffer cells have short life span of about 4 days, several independent studies have reported that tissue macrophages like kupffer cells have a longer life span of about 4 months to 14 months. Thus macrophages colonize tissues not only by differentiation but also through local proliferation [14]. Although monocyte infiltration leads to macrophage development, during inflammatory conditions such as atherosclerosis it is observed that the disease development is mainly due to macrophage proliferation rather than monocyte influx [15]. Although many macrophages reside in the tissues, most of them die due to apoptosis and those which receive survival signals will proliferate and function [16].

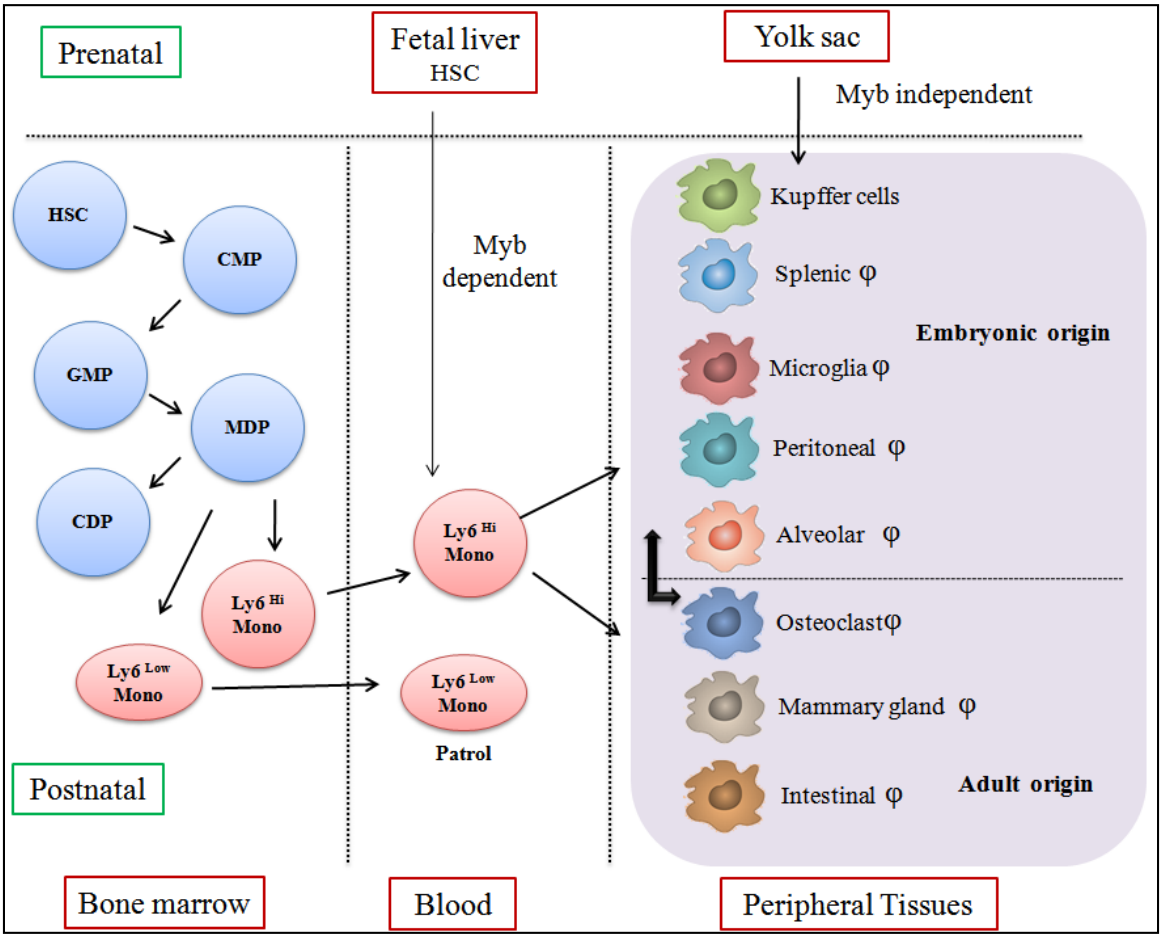


Figure 1.2. Macrophage differentiation and tissue distribution in embryonic and adult cells. In embryonic cells, macrophages differentiate directly from yolk sac in Myb independent fashion while HSCs in the foetal liver produce monocytes which later differentiate into macrophages in Myb dependent manner. Further HSCs in the bone marrow gradually differentiate into macrophages in both pre and postnatal stages. The macrophages which are formed in early stages get distributed across various peripheral tissues and are characterized by self-renewal and longevity. On the contrary macrophages developed during adult stages are primarily involved in regulating inflammation and usually have short life span. φ is the representation for macrophages, CMP-common myeloid progenitors, GMP-Granulocyte myeloid progenitor, MDP-macrophage or dendritic cell progenitor, CDP-common dendritic cell progenitor.

1.1.4. Macrophage polarization

Macrophages are constantly *activated* in a cell by a variety of factors secreted around them and hence they can alter their physiology and phenotype in response to surrounding environment. This plastic behaviour of macrophages where they can skew from one phenotype to the other is termed as macrophage *polarization* or *activation* [1, 17] (Figure 1.3B). Activated macrophages were classified in several ways based on their functions or stimuli they receive. Originally, depending on their functions they were categorized into classical and alternatively activated macrophages. Macrophages activated by LPS and IFNγ were termed as classically activated macrophages and macrophage gene expression induced by IL-4 were named as alternately activated macrophages where the former was believed to be involved in pathogen clearance while the later in resolution of inflammation and tissue repair (Figure 1.3A) [1].

Later in the year 2000 Mills *et al.* proposed another classification as M1 and M2 depending on the arginine metabolism and were named based on their pro-inflammatory and anti-inflammatory nature respectively [18]. Classically activated or M1 macrophages displayed enhanced microbicidal capacity (against bacteria, protozoa, virus) due to secretion of pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-12, IL-23 increased ROS production etc., and were found to be activated during treatment of IFN-γ alone or in combination with TNF-α or bacterial endotoxin LPS (Th1 responses). This kind of macrophage activation required priming with low doses of IFNγ soon after the transmigration into tissue. Similarly alternatively activated or IL-4 and IL-13 stimulated macrophages (Th2 responses) were observed to perform other immune regulatory functions such as resolution of inflammation, tissue repair and wound healing, combating

parasitic growth etc., mainly due to secretion of several factors such as IL-10, collagen, Arg-1 (arginase-1), MR (mannose receptors), VEGF (vascular endothelial growth factor), TGF-β (transforming growth factor-β), FGF (fibroblast growth factor), M-CSF, complement components etc., and thereafter were named as alternatively activated or M2 macrophages. Basically M1 and M2 activation mirror Th1 and Th2 responses respectively one being pro-inflammatory while the other being anti-inflammatory in nature (Figure 1.3B) [1, 18-20].

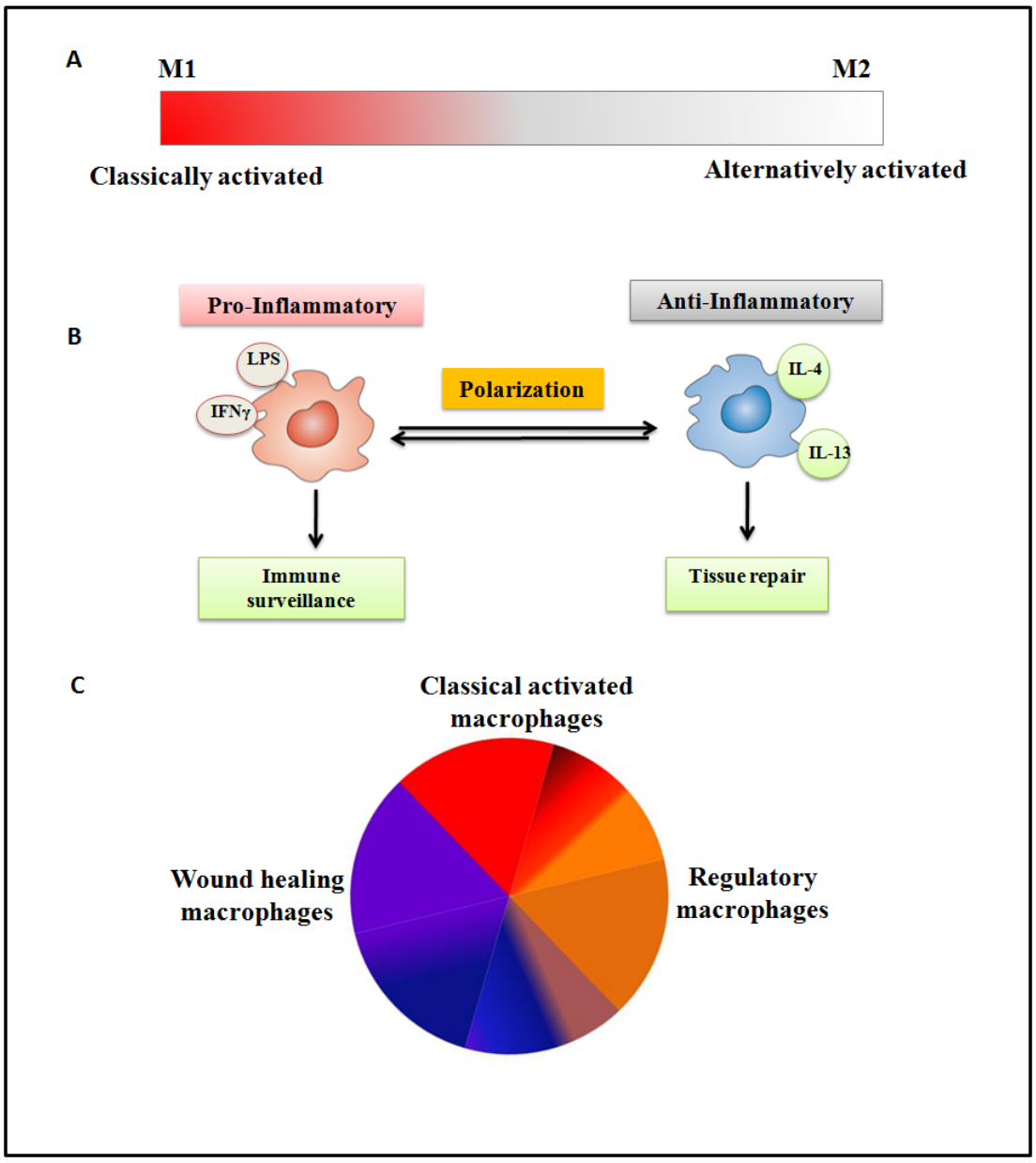


Figure 1.3. Prototypical classification of activated macrophages. Depending on the environmental cues, macrophages skew from one state to the other by a process termed as polarization. (A-B) Macrophages are broadly classified into M1 and M2 based on their pro-and anti-inflammatory natures respectively. (C) Subsequent nomenclature classified macrophages into classical, wound healing and regulatory. Figure A and C are adapted from Nature reviews immunology, 2008. 8(12): p. 958-969.

This classification was considered for long time until in the year 2004 Mantovani *et al.* further classified M2 macrophage into subtypes as M2a, M2b and M2c depending on the stimuli and distinct transcriptional profile during their activation. M2a macrophages were activated in presence of IL-4, IL-13, fungal and helminth infections, M2b by IL-1 receptor ligands, immune complexes and LPS, M2c by IL-10, TGF-β and glucocorticoids and lastly M2d by adenosine and IL-6 stimuli (Figure 1.4). M2a macrophages are also termed as alternative macrophages and were generally considered as wound healing macrophages because of their enhanced property to produce Ym1, while M2b were also known as type 2 macrophages and they comprised of mixed features of both M1 and M2 and were involved in mediating efficient immune surveillance by FcR induced effector functions [21]. M2C macrophages are well known as deactivated macrophages and as their name suggests they are involved in dampening M1 macrophage activation [22]. The M1/M2 classification was still inadequate to explain the role of tumour associated macrophages (TAMs) and thus over time macrophages were re-classified into classically activated, wound healing and regulatory macrophages depending on their functions and TAMs were grouped as regulatory macrophages (Figure 1.3C) [1]. In the recent times due to heterogeneity in M2 phenotypes and functions, TAMs are now considered as a discrete group which are formed in tumour microenvironment in presence of MCSF and VEGF (Table 1.1) [23]. Recently another category has been identified as M2d macrophages which were activated distinctly by IL-6 or adenosine receptors agonists in presence of TLR ligands. These subtypes are recently considered as a subset of TAMs as they are found to promote tumour development and metastasis [24, 25]. Currently M2 macrophages because of their heterogeneity are classified into several types (Figure 1.4) and each macrophage subset exhibits unique cytokine and chemokine expresson profile and characteristics of each phenotype is listed in table 1.1. Further certain macrophages displayed characteristics of both M1 and M2 expression pattern but did not fit into either of the groups and hence were individually grouped as atypical macrophages. They polarize in presence of IL-33 or mycobacterial infections, similarly tissue resident macrophages which were believed to originate in presence of IL-4, IL-13, C1q, C3b etc are now considered as a separate category [25].

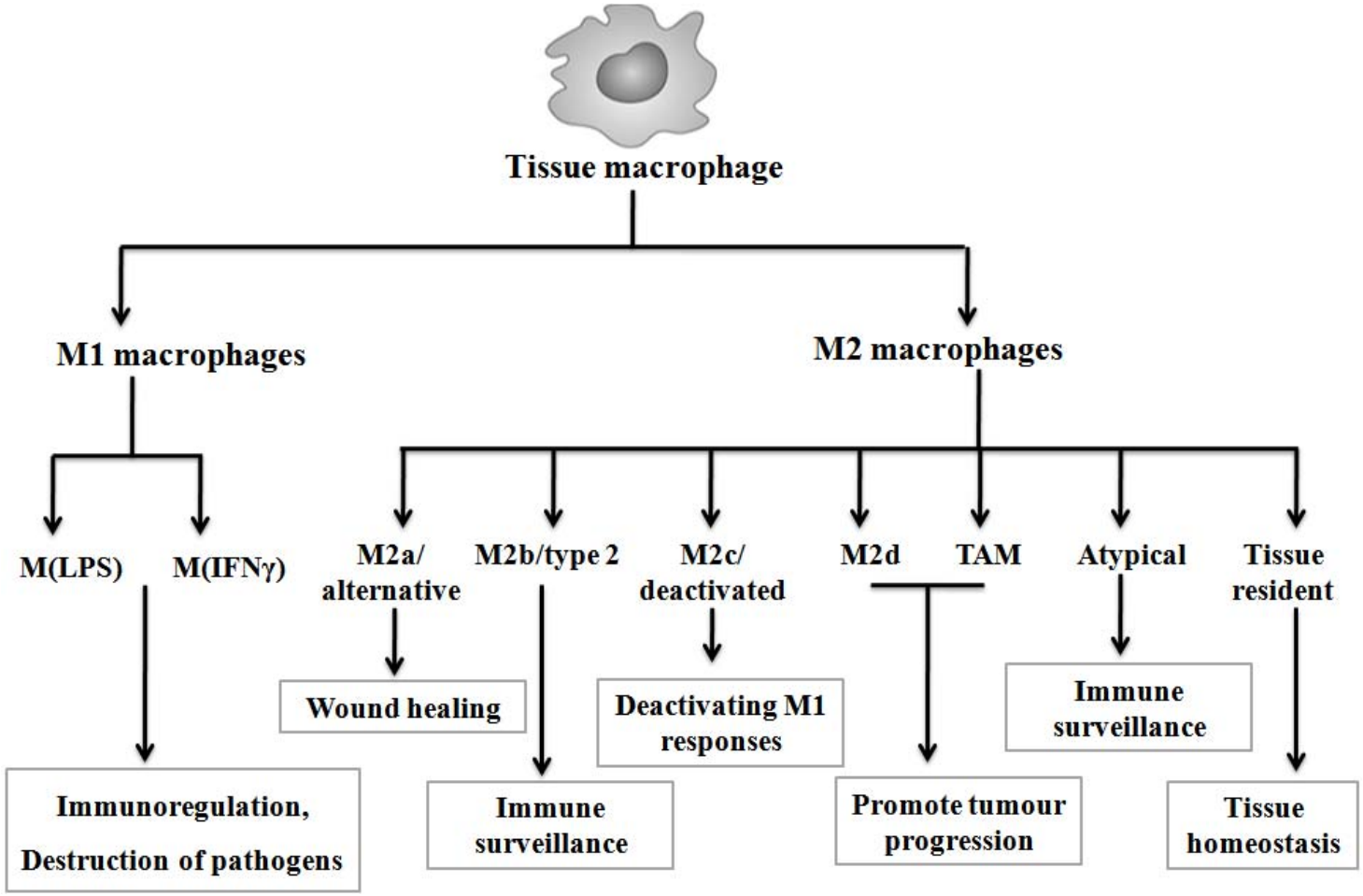
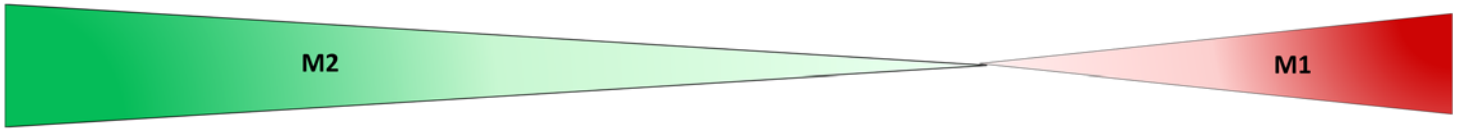


Figure 1.4. Heterogeneity in M2 macrophage populations and functions. M2 macrophages are recently classified into several subsets M2a, M2b, M2c, M2d, TAMs, atypical and tissue resident macrophages depending on the stimuli and functions.

Table 1.1. Characteristic features of polarized macrophages

Parameters	M1	M2a	M2b	M2c	M2d	Tissue resident	Atypical	TAM
Stimuli	LPS, IFNγ, GM-CSF	IL-4, IL-13, Fungal and helminth infections	Immune complexes+ LPS, IL-1r ligands	IL-10, TGF-β, Glucocorticoids	IL-6, Adenosine, Tumour derived factors	IL-4, IL-13, IL-13, C1q, C3b	Mycobacterial infection, IL-33	Tumour microenvironment, MCSF
Marker expression	CD68, CD86, CD80, MHCII, IL-1R, TLR2, TLR4, iNOS, SOCS3	CD163, MHCII, SR, MMR/CD206, CD200R, TGM2, DecoyR, IL-1R II, Ym1/2, Fizz-1, Arg-1	CD86, MHCII	CD206, CD163, TLR1, TLR8, MERTK	VEGF-A	Arginase-1, CD163, CD206 MGL-1, CD209 neuropeptides, growth factors, catecholamines	Ym1, CD163, NOS2, CD206, MGL-1	CD163, CD68, CD206, VEGF-A, Dectin-1, NOS2, MGL-1
Cytokine secretion	TNFα. IL-1β, IL-6, IL-12, IL-23,	IL-10, TGF-β, IL-1ra	IL-1, IL-6, IL-10, TNF-α	IL-10, TGF-β	IL-10, IL-12, TNF-α, TGF-β	IL-10	IL-12, IL-6 TNF-α, IL-10	IL-10
Chemokine secretion	CCL10,CCL11, CCL5, CCL8, CCL9, CCL2, CCL3, CCL4, CXCL5,CXCL9, CXCL10	CCL17, CCL22, CCL24	CCL1	CCR2	CCL5, CXCL10, CXCL16		CCL18	CCL2, CCL17, CCL22

Recently another set of classification emerged categorizing M1 population into GM-CSF-1 macrophages and M2 population into M-CSF-1 macrophages depending on the factors responsible for their origin [26, 27]. But till now there is no clear explanation to suggest that these phenotypes belong to a particular category because depending on the environmental cues macrophages can transit flexibly from one phenotype to the other. Thus very recently to avoid the confusion across the authors in naming the activated macrophages and in order to attain the consensus in the nomenclature terminology, a new convention was framed for naming the activated macrophages. According to the authors macrophages should no longer be represented as M1 or M2 instead their nomenclature must be linked to their activation standards such as M (LPS), M (IFNγ), M (IL-4) and so forth (Figure 1.5). Moreover a spectrum was generated to denote two states of activated macrophages with a caution that the activated macrophages lie within the spectrum with no defined boundaries or with no sudden conceptual shifts in their behaviour. According to this new convention, it is advised not to categorize macrophages as regulatory phenotype because all the macrophages are regulatory to some extent to drive cellular functions [27].



		M (IL-4)	M (IC)	M (IL-10)	M (GC+TGF-β)	M (GC)	M(-)	M (LPS)	M (LPS+IFNγ)	M (IFNY)
Transcription factors, SOCS proteins	Mouse	pStat6 +++, pStat 1 -ve, Irf4, Socs2		pStat3+,Nfil3, Sbno2,Socs3			Baseline gene expression dependent on culture variables	pStat 1+, pStat6 -ve, Socs 1, Nfkbiz	pStat1+, pStat6 -ve, Socs1, Nfkbiz, Irf5	pStat1 +++,Socs1
	Human	IRF4, SOCS1*, GATA3*		SOCS3	ID3,RGS1,pSMAD2+			IRF5	pSTAT1 +++, IRF5, IRF I	pSTAT1+++, IRF5
Cytokines	Mouse		Il-10,Il-6	Il-10				Tnf,Il-6, Il-27	Tnf, Il-6, Il-27, Il-23a, Il-12a	
	Human							TNF, IL-6,IL-1B	TNF, IL-6, IL-1B, IL-12A, IL-12B, IL-23A	
Chemokines	Mouse	Ccl17, Ccl24,Ccl22	Cxcl13,Ccl 1,Ccl20							
	Human	CCL4*,CCL13* CCL17,CCL18						CXCL10,1L8	CCL5,CXCL9, CXCL10,CXCL11	CCL 18 -ve
Scavenger receptors	Mouse							Marco	Marco	
	Human	MRC1*, STAB1, MARCO -ve, CD163 -ve				CD163,STAB1, MARCO				
Matrix	Mouse									

	Human	FN, TGFB1, MMP1, MMP12,TG, F13A1*				F13A1+,Negative for markers in M(IL-4)		MMP9		
Amino acid metabolism	Mouse	Arg1 +++	Nos2					Arg1+, Nos2 +	Arg1+, Nos2 +++	Nos2+++,Ido1
	Human								IDO1, KYNU	IDO1, KYNU
Others	Mouse	Retnla, Chi3l3, Alox15	Retlna -ve	Il-4ra						
	Human	TGM2*, ADORA3, TGFBR2 -ve,ILI7RB, ALOX15*,CD200R*		IL-4RA	TGFBR2++,ALOX5AP,IL-17RB	TGFBR2++,ADORA3		PTX3	GBP,CCR7, CD40	

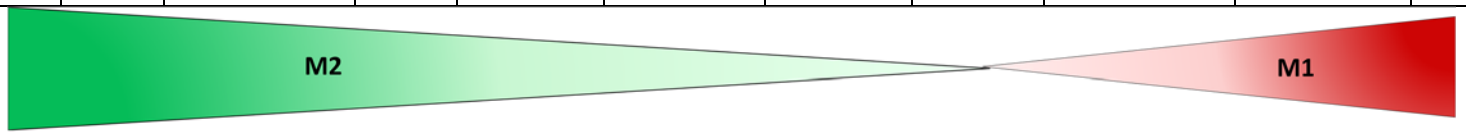


Figure 1.5. Representation of activated macrophages. Macrophage nomenclature terminology and their activation states would be hereafter denoted specifically as mentioned in the table. The figure is adapted from Immunity, 2014. 41(1): p. 14-20.

1.1.5. Macrophages and innate immune responses

Macrophages effectively eliminate pathogens by mediating innate immune responses which act immediately within hours of pathogen's appearance in the body. It initiates effective defence responses by distinct mechanisms evolved for pathogen recognition.

1.1.5.1. Pattern recognition receptors

In order to discriminate potential pathogens or damaged particles from self, macrophages of innate immune system have evolved pattern recognition receptors (PRR) to provide first line of defence. The ligands for these PRRs are termed as pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs) which recognize conserved motifs on pathogen associated molecules such as lipopolysaccharides, glycans, peptides, lipotechoic acids etc. or signals in response to stress, injury, respectively. These PRRs are highly conserved in higher eukaryotes and are specific towards their ligand recognition. PRRs can be classified based on their ligand specificity, location and functions [28, 29]. Based on their functions PRRs are majorly classified into signalling PRRs and endocytic PRRs. Signalling PRRs include membrane bound Toll-like receptors (TLRs) and cytoplasmic NOD-like receptors (NLRs) which trigger downstream signalling for executing their functions. Unlike signalling PRRs, endocytic PRRs do not rely on intracellular signals rather provide immediate first line of defence by promoting adherence and phagocytosis. Examples of such receptors include mannose (MR) and scavenger receptors (SR). There always exists a cross talk across diverse PRRs to combat infectious agents and thus concomitant activation of both endocytic and signalling receptors takes place in a cell.

TLRs form the most critical components of innate immunity in recognizing molecular patterns of the pathogens and till date 11 TLR families have been identified in humans. TLRs are transmembrane receptors and their signalling begins with the activation of their intracytoplasmic TIR domains (Toll-like receptors share homology with interleukin 1 receptors hence termed as **T**oll / **I**nterleukin **R**eceptors) which recruit specific TIR domain containing adaptors such as MyD88, TIRAP and TRIF. These adaptors in turn recruit other downstream molecules ultimately resulting in the synthesis of pro-inflammatory cytokines that are necessary for initiating TLR induced innate immune responses. The innate mechanisms in turn orchestrate adaptive immune responses which lead to the successful elimination of pathogen. MyD88 activation results in the induction of pro-inflammatory cytokines (eg; TNF-α, IL-6, IL-12, INF-β etc.,) by all the TLRs and these cytokines form the most essential components of the host defence but their excessive production can lead to extensive damage to the host hence macrophage activation is tightly regulated in a cell [30].

1.1.5.2. Phagocytosis

Macrophages are majorly involved in a process termed as *phagocytosis* which becomes the preliminary step to evoke both innate and adaptive immune responses. Phagocytosis is a phenomenon executed by phagocytic cells where they selectively uptake foreign particles through their surface receptors and take necessary steps to eliminate them from the resident tissues. Elie Metchnikoff was awarded Nobel Prize 100 years ago for describing phagocytosis as 'a key to immunity was to stimulate phagocytes'. Immune cells form a defined system to prevent any foreign attack and thus are categorized into phagocytic and non-phagocytic systems depending on their functions. Phagocytic cells

are further classified into professional and non-professional phagocytes depending on how effectively they phagocytose a foreign particle. The efficiency of the phagocytosis depends on the multitude of receptors expressed on their surfaces known as phagocytic receptors with which they initiate the mechanism and secondly based on nature of ligands binding onto them. Because of this kind of heterogeneity in ligand and receptor interactions the fate of the cell is undetermined and hence the process is described as a complex phenomenon. Phagocytosis initiates when they recognize foreign components such as bacterial endotoxins (LPS-lipopolysaccharides), carbohydrates (eg; mannose, fucose) or surface proteins (eg; MOMP-major outer membrane protein). The adhesion of these molecules is accompanied by actin polymerization at the interaction site which results in the internalization of these particles. Consequently phagosome maturation occurs by a series of fusion and fission events with the components of endolysosomal pathway [31].

The recognition mechanisms leading to phagocytosis can either *involve humoral components* mainly opsonins such as antibodies, complements, mannose binding and surfactant proteins which indirectly aid in phagocytosis or direct recognition of molecular patterns by *cellular receptors* such as Fc receptors (FcR), complement receptors (CR) or mannose receptors (MR). Scavenger receptors (SR) are a special class of receptors involved in engulfing apoptotic cells. Finally the fate of the cell is determined by the type and strength of interactions. For instance, FcR mediated phagocytosis can trigger the activation of inflammatory mediators such as reactive oxygen intermediates (ROS) and arachidonic acid metabolites (induce inflammation) whereas CR does not induce either of these classes. MR ligation also provokes pro-inflammatory responses such as cytokine

secretion of TNF-α, IL-6 and IL-12 similar to FcR mechanism however activate different pathways [32]. Apart from these functions macrophages perform homeostatic clearance without which the host cell would not survive. They are majorly involved in removing cellular debris that is generated during tissue remodelling and rapidly remove cells that have undergone apoptosis. These processes are carried out by surface receptors such as scavenger receptors, phosphatidyl serine receptors, thrombospondin receptors and complement receptors [33]. This type of phagocytic clearance is independent of other macrophage effector functions and becomes one of the most important operations performed by them on daily basis.

1.1.6. Pathophysiology of macrophages

Macrophages being versatile in their phenotypes execute diverse physiological functions and dys-regulation in their activation on the other hand can consequently trigger several pathological conditions.

1.1.6.1. Microbicidal functions

The pro-inflammatory cytokines secreted by M1 macrophages function to eliminate different types of microorganisms. This type of activation is observed when they encounter components like LPS (PAMPs or DAMPs) or cytokines such as IFNγ alone or in combination with TNF-α released from effector cells, natural killer cells (NK) and antigen presenting cells (APCs). Once PRRs on macrophages recognize their specific ligands these cells get activated and trigger downstream effector molecules such as pro-inflammatory cytokines, chemokines or ROS production. Additionally macrophages are also known to mediate anti-viral responses by inducing type I interferon signalling when

they encounter viruses. Further iNOS is shown to be one of the effective molecules involved in eliminating viruses by attenuating viral replication [34]. Macrophages are also shown to exhibit anti-fungal activity effectively. For instance they were reported to recognize Aspergillus conidia using non-specific receptor interactions involving mannosyl fucosyl receptors which aid in internalization and destruction of 90% of conidia [35].

In this way pro-inflammatory environment helps in destroying macrophage resident bacteria, viruses, fungi and intracellular parasites and thus M1 macrophages mediate resistance against a variety of microorganisms [3, 34-36]. This type of short term inflammation is termed as acute inflammation and is beneficial for host if it is properly regulated. If such macrophages are activated for long time, excess pro-inflammatory responses can lead to chronic inflammatory diseases like cardiovascular problems (eg; atherosclerosis, cardiomyopathy, etc.), metabolic diseases (type 2 diabetes, hepatosteatosis etc.), neurodegenerative disorders (Parkinson's and Alzheimer's disease) and several types of cancers (Figure 1.6) [37-40].

1.1.6.2. Anti-tumour responses

As macrophages are always associated with tissues, their activation is found to be altered in several types of cancers. These macrophages are termed as tumour associated macrophages (TAMs) and are found to be responsible for development and progression of several types of cancers. TAMs are linked to poor prognosis in breast cancers and ovarian cancers, glioma and lymphomas [41-44]. Several observations suggested that pro-inflammatory environment of M1 macrophages can destroy cancer cells thus

inducing anti-tumour responses. Based on these evidences several studies reported that polarizing macrophages from anti-inflammatory (M2) to pro-inflammatory (M1) phenotype reduced the severity of tumour development in several types of cancers [1]. For instance Xu et al. have showed that local delivery of IL-21 into breast cancer tissue have polarized TAMs away from M2 to tumour inhibiting M1 which rapidly induced Th1 responses leading to enhanced response to anti-Her2/neu antibody therapy [45].

1.1.6.3. Tumour associated macrophages (TAMs)

As tumours progress to grow, the surrounding microenvironment markedly influence tumour associated macrophages (TAMs) and subsequently there will be a gradual polarization of macrophages from pro-inflammatory M1 to anti-inflammatory M2 phenotype. Because of their pro-angiogenic and anti-inflammatory nature, TAMs suppress anti-tumour immunity possessed by tissue resident macrophages and thus promote cancer development and metastasis [4]. TAMs interact with a wide variety of cytokines, growth factors and chemokines and hence their roles vary in different types of tumours. Administration of IFNγ was shown to reverse pro-tumoural effects by inducing cytotoxic action against TAMs [46]. Several recent studies have shown that reprogramming of TAMs from anti-inflammatory to pro-inflammatory phenotype can reduce tumour burden in a variety of cancers [47, 48].

1.1.6.4. Neoplasia

The excess pro-inflammatory molecules secreted by the activated M1 macrophages can contribute to tumour initiation (neoplasia) primarily because of toxic free radicals generated by them which induce mutations in the genetic material DNA leading to

cancers [1]. This condition can be specially observed in patients experiencing chronic inflammatory diseases. For example patients suffering from inflammatory bowel syndromes like Crohn's disease are more prone to develop colorectal cancers; similarly long term pancreatitis can lead to pancreatic cancers [49, 50].

1.1.6.5. Chronic obstructive pulmonary disease (COPD)

COPD is becoming one of the most common respiratory diseases in the modern world due to increased air pollution. Cigarette smoking is identified as one of the causative factors to induce M1 polarization because it is known to contain trace amounts of LPS [51-53]. Oxidative stress due to generation of ROS and nitric oxide (NO) production is identified as one of the mechanisms in the progression of COPD. Along with iNOS, IL-1β, IL-6, IL-8, and TNF-α expression levels, are found to be significantly up-regulated in COPD which form the activation signature of M1 polarized macrophages [54, 55]. Additionally reduced phagocytic ability of macrophages is considered to be responsible for endurance of many infectious microorganisms in the lungs indicating that M2 macrophages are also significantly dys-regulated in COPD pathogenesis analogous to M1 macrophages. However M2 macrophages show mixed phenotype where they exhibit decreased efferocytosis but increased expression of DC-SIGN marker which is a C-type lectin required for recognition of mannose type of carbohydrates [56]. Thus COPD is an inflammatory damage to lungs where there is a reduced clearance of microorganisms and apoptotic bodies because of the dys-regulated macrophage activation states and the exact roles of these phenotypes need further elucidation.

1.1.6.6. Tissue repair and granuloma formation

One of the first signals released by basophils and mast cells during tissue injury is Th-2 induced IL-4 secretion. It is also secreted by them in response to chitin, a structural element of some fungi and parasites. IL-4 stimulates arginase expression in macrophages allowing them to promote polyamine precursors urea and ornithine required for collagen synthesis and cell proliferation, respectively [57, 58]. These macrophages produce increased amounts of chitinase like molecules referred to as YM-1 (CHI3L3) which aid in carbohydrate and matrix binding activity. They have an important feature of inducing the expression of pro-angiogenic factors such as VEGF, TGF-β and FGF that serve as mitogens and chemotactic agents required for wound healing and cell proliferation at the injured site [25]. Of all the three mentioned factors, VEGF is considered as a multifaceted candidate as it heals the wound by working on several aspects such as collagen deposition, epithelization, vasodilation and angiogenesis. Because of these properties researchers consider that VEGF alone may be effective in treating non-healing wounds in diabetic vascular diseases and several types of ulcers [59].

In response to parasitic infections these cells induce STAT6 expression and secretion of IgG and IgE antibodies which in turn stimulate mast cell degranulation thus aiding in worm expulsion [60]. On the other hand when M2 macrophages are activated for a prolonged time, it leads to conditions such as tissue fibrosis or granulomas as the parasites exploit the conditions favoured by these macrophages. For instance with the onset of egg deposition by some of the parasites, Th1 (IFNγ, TNF-α) induced macrophage inflammatory responses are down-regulated with subsequent activation of Th2 responses (IL-4, IL-13, IgG etc.). This results in continuous build-up of matrix

around the tissue leading to fibrosis. In this way parasites develop a strong barrier around them to survive by switching from M1 to M2 phenotype thus exploiting the host mechanisms [61]. Further these macrophages are well known to trigger allergic responses like asthma and other airway inflammatory diseases such as mucous hypersecretions and emphysema [56].

1.1.6.7. Atherosclerosis

Classically activated M1 macrophages are well known to be involved in the progression of atherosclerosis because excess free fatty acids in dyslipidemia can activate TLR2 and TLR4 responses which in turn trigger pro-inflammatory responses. This continuous activation leads to damage of cells which in turn trigger signals for inducing apoptosis and therefore recruit M2 macrophages [62, 63].

M2 macrophages are also shown to have an equivalent role in inducing atherosclerosis because of their scavenging nature. Lipid loaded macrophages (foam cells-macrophages which scavenge lipids) contribute to the plaque formation, thinning of fibrous cap and necrotic core all of which again trigger pro-inflammatory responses. The activated responses in turn signal for apoptosis because the vulnerable plaques get ruptured leading to thrombosis. This leads to clogging of arteries resulting in a reduced blood supply to the downstream tissues a condition known as ischemia. Once blood and oxygen levels stop reaching the tissues it leads to necrosis, a catastrophic condition termed as infarction where death of the tissue occurs in approximately five minutes. Thus macrophages exist in heterogenous populations in the atherosclerotic lesions and their balance dictates the severity of the disease. The exact roles of these macrophages in atheroma formation are poorly studied [64, 65].

We will understand better in the coming sections on how macrophage induced chronic inflammation can also lead to metabolic disorders like insulin resistance which in turn causes diabetes.

1.1.6.8. Inflammation and metabolism

Metabolism and immune responses are evolutionary conserved as they form the fundamental processes for the host survival. Thus nutrient and pathogen sensing systems are highly integrated and always dependent on each other. Dys-regulation of these processes leads to inflammation linked metabolic diseases such as obesity associated insulin resistance and type 2 diabetes, fatty liver changes, atherosclerosis, airway diseases, neurodegenerative diseases like dementia and various types of cancers [66, 67]. Currently these diseases constitute the greatest threat to human health globally. All the above stated diseases are chronic inflammatory conditions characterized by increased pro-inflammatory cytokines, acute phase reactants and other inflammatory factors signifying that inflammation and metabolic diseases are tightly linked. All the recent evidences suggest that the relationship between immunity and metabolism is markedly delicate and any significant changes in their behaviour are detrimental to the host cell. For instance long term inflammation can disrupt metabolic functions as seen in case of obesity induced type 2 diabetes. Similarly any agitation in the metabolic homeostasis can provoke aberrant immune responses as seen in malnutrition conditions [66-68]. In the present section we will more particularly discuss about the relationship between metabolism and inflammation in the context of obesity and type 2 diabetes.

Due to change in the diet and life style, the incidence of obesity is increased in the developing world during the recent decades and inflammation is reported to be a key process associated with it. This is evident from several reports which showed that TNF-α (potent pro-inflammatory cytokine) is abundantly expressed in adipose and muscle tissues of obese humans and when injected exogenously leads to insulin resistance [69]. In obese mouse models, lack of TNF-α noticeably improved insulin sensitivity and glucose homeostasis [70]. It is clear from several other studies that in addition to TNF-α, there are a wide variety of factors that are highly expressed in obese conditions and are common mediators of both immunity and metabolism. Some of them are IL-6, leptin, adiponectin, resistin, TGF-β, haptoglobin etc. [71, 72]. Additionally lipids also co-ordinate the regulation of inflammation and metabolism because elevated levels of plasma lipids are characteristics of both obesity and inflammation. For example hyperlipidemia observed in obesity in part is responsible for the development of atherosclerosis, an inflammatory condition [73].

Research for many years have revealed that macrophages form the common link between inflammation and metabolism firstly because of the fact that adipocytes and hepatocytes of principal metabolic organs such as adipose tissue and liver respectively are in close proximity to immune cells like macrophages, lymphocytes and dendritic cells. Secondly macrophages and adipocytes share common gene expression profiles for many factors. For instance macrophages express adipocyte gene products like FABP4 and PPARγ, while adipocytes express macrophage factors like TNF-α, IL-6 and MMPs (matrix metalloproteinases) [74-76]. Finally they also share functional capabilities of each other for example, macrophages can engulf and store lipids to become atherosclerotic foam

cells similarly adipocytes can perform phagocytic and TLR induced microbicidal functions in response to infectious agents confirming that immunity and metabolic responses are co-evolved to perform cellular functions [66]. Furthermore, recent studies have documented that obesity and type 2 diabetes are characterized by macrophage infiltration into white adipose tissue and the pro-inflammatory phenotype observed in these conditions could be because of macrophages alone or in concert with adipocytes thus promoting inflammation induced insulin resistance [77]. Along with M1 macrophage infiltration there are reports which state that the polarization of macrophages residing in the adipocytes is as well affected. Because endogenously, adipose resident macrophages are identified to exhibit M2 like phenotype to perform metabolic functions like maintaining insulin sensitivity and glucose homeostasis which help in preventing diet induced obesity and type 2 diabetes. However recent evidences show that in obese conditions there is switch from M2 to M1 like phenotype overtly indicating that obesity is associated with dys-regulation in macrophage polarization [78].

1.1.6.9. Insulin resistance and effector mechanisms

In normal conditions, binding of insulin to insulin receptors of adipocytes, hepatocytes and myocytes (insulin responsive cells) results in the phosphorylation of itself and insulin receptor substrate (IRS) family thus triggering downstream signalling cascade resulting in the insulin stimulated glucose uptake. Inhibition of insulin signalling is the key event in insulin resistance. This occurs in obese when binding of TNF-α or free fatty acids to their respective receptors reduce tyrosine phosphorylation of IRS-1 and stimulate serine phosphorylation by JNK, IKK and PKC-θ kinases. Further, induction of factors such as

SOCS and iNOS proteins also contribute to cytokine mediated insulin resistance [69, 79, 80].

Growing evidences clearly indicate that obesity linked insulin resistance and type 2 diabetes are merely inflammatory diseases. Two mechanisms were proposed to explain how inflammation can trigger insulin resistance in adipocytes. Firstly *ER stress* was considered to be of central importance in triggering inflammatory responses. As an example in obesity, adipocytes undergo severe alterations in their morphology due to accumulated cholesterol and lipids. This creates a load on endoplasmic reticulum (ER stress) to reach the demands of the increased metabolism. ER stress thus leads to activation of JNK and IKK pathways which subsequently result in insulin resistance. Second mechanism to explain inflammation induced insulin resistance is *oxidative damage* where constant glucose uptake by adipocytes and endothelial cells during hyperglycemia results in excess production of ROS which further might attract inflammatory cells such as macrophages to sites of injury to exaggerate inflammation [81, 82].

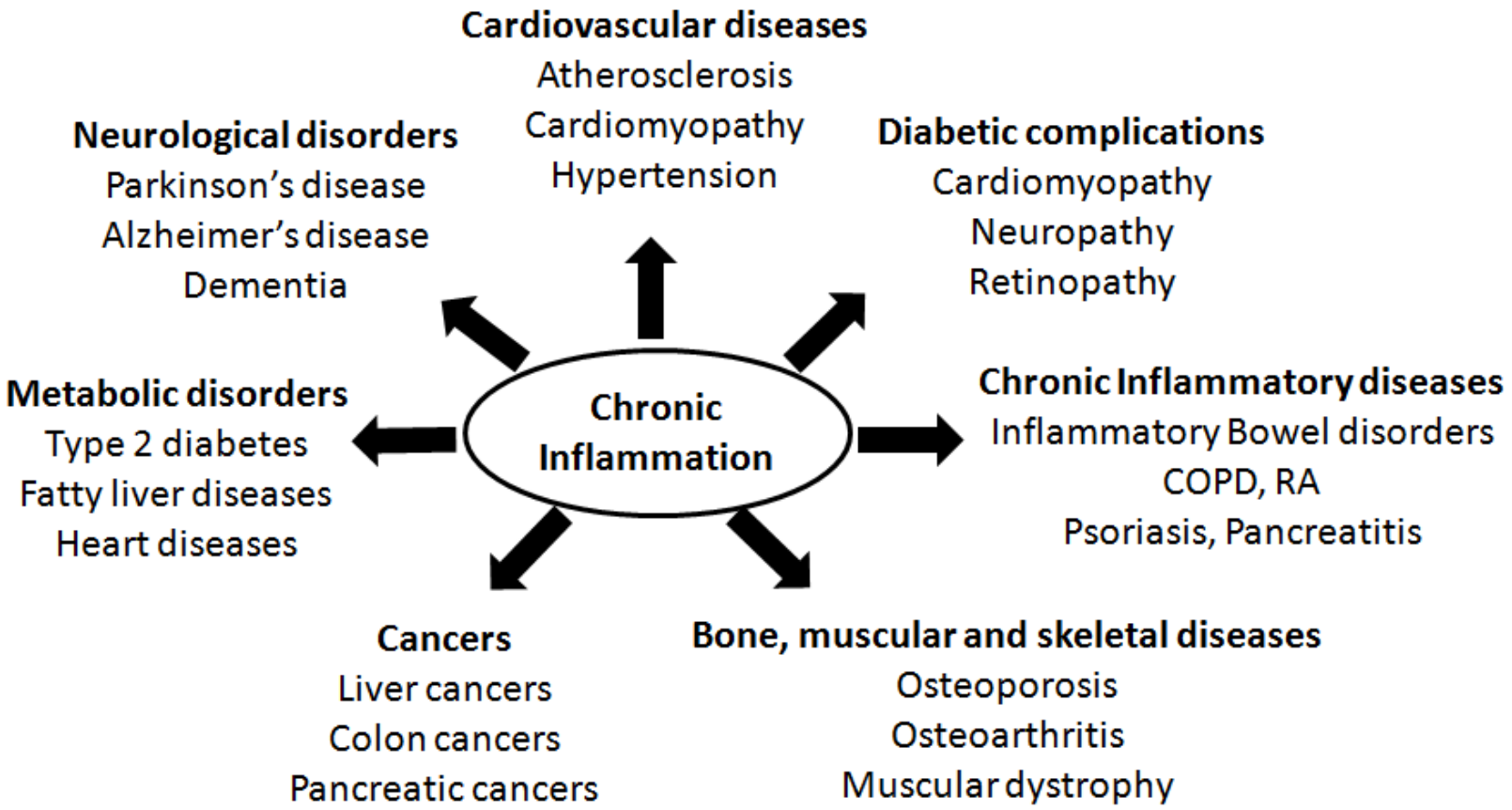


Figure 1.6. Macrophages, major culprits in inducing chronic inflammation. Uncontrolled activation of macrophage phenotypes leads to chronic inflammatory disorders such as metabolic dysfunctioning, neurological and cardiovascular diseases and several types of cancers.

1.1.7. Factors regulating macrophage polarization

Macrophage polarization is a highly dynamic process and is spatiotemporally controlled by several signalling pathways, transcriptional and post-translational regulatory networks. Several inflammatory modulators, signalling intermediates, transcriptional factors and epigenetic regulations are entailed in driving this regulatory process and more importantly STATs, SOCS, IRFs, HIFs and microRNAs play a prominent role in deciding the fate of polarized macrophages.

1.1.7.1. Role of IRFs, JAKs, STATs and other key players involved in modulating macrophage activation states

MyD88 and TRIF are the two key adaptors involved in mediating M1 responses. TLR4 stimulated by LPS or other microbial products results in activation of either of these two adaptors. MyD88 recruitment leads to activation of a series of kinases such as IRAK4, TRAF6 and IKKβ ultimately resulting in activation and translocation of NF-κB induced pro-inflammatory gene expression such as IL-12, IL-6, COX2 etc. Similarly recruitment of TRIF adaptor leads to activation of transcription factor IRF3 thus resulting in secretion of type I interferons such as IFN-α, IFN-β. The binding of these cytokines to IFNAR in turn results in activation of STAT1 transcription factor. Additionally IRF5 is also known to promote M1 responses by inducing STAT1 mediated gene expression (Figure 1.7). Overall IFN stimulated genes involving IRFs impart M1 polarization as it leads to induction of TNF, IL-12, CXCL9 etc. and promotion of Th1 and Th17 responses [20, 83, 84].

STAT mediated gene expression is regulated by the members of SOCS family. For example IL-4 in concert with IFNγ induces SOCS1 expression which in turn inhibits the

action of STAT1 thus dampening inflammatory responses. Similarly factors such as SHIPs, IRAK-M, ST2, and MyD88sh are shown to negatively regulate M1 phenotype [20, 85-87].

Canonical M2 stimuli such as IL-4, IL-13 and IL-10 results in activation of JAK/STAT pathway thus contributing to M2 phenotype. IL-4 and IL-13 binding on to IL-4Rα recruit JAK1 and JAK2 which in turn leads to STAT6 activation and translocation into the nucleus (Figure 1.7). Similarly IL-10 binding to IL-10R triggers STAT3 induced M2 responsive gene expression. In contrast to IL-4 alone, IL-4 in presence of IFNγ decreased STAT3 activation and this was found to be primarily because of up-regulated SOCS3 expression again highlighting the regulation of STAT proteins by SOCS family [20, 83, 84, 88]. Several transcription factors such as PPARγ, PPARδ, KLF-4 and c-Myc are involved in promoting M2 phenotype more importantly KLF4 and PPARγ, where myeloid specific deficiency of KLF4 and PPARγ halted M2 phenotype resulting in exaggerated inflammatory lesion in Apo E deficient and LDL receptor knockout mice respectively [89, 90]. In parallel two independent studies showed that PPARγ deficient macrophages and KLF4 knockout mice failed to protect the animals from inflammation induced insulin resistance. In addition STAT6 was shown to coordinate with both PPARγ and KLF4 in inducing M2 phenotype while inhibiting M1 by sequestering co-factors required for NF-κB activation [91, 92]. Furthermore deletion of IRF4/KLF6 transcription factors could not produce M (IL-4) macrophages indicating that IRF4/KLF6 are obligatory for inducing M2 phenotype while PPARγ and PPARδ were required for strengthening IL-4 responses [27, 93, 94].

1.1.7.2. Role of HIF-1α and HIF-2α on macrophage polarization

Hypoxia inducible factors (HIFs) are known to regulate macrophage activation states because the inflammatory and damaged sites are deprived of oxygen and nutrients and this hypoxia condition is known to regulate macrophage polarization by two isoforms of hypoxia inducible factors, HIF-1α and HIF-2α [20].

During sepsis condition, HIF-1α was found to be induced by NF-κB (Figure 1.7), however recent studies revealed that HIF-1α can promote M2 like phenotype as well [95, 96]. Similarly HIF-2 was shown to induce M2 like phenotype but contrasting results showed that it promotes higher inflammatory responses when compared to HIF-1 [97, 98]. The conflicting roles of HIFs could be probably because of the reason that hypoxia profoundly induces the expression of angiogenic and metastatic genes such as MMP7, VEGFA etc. in hypoxic inflammatory conditions like atherosclerosis, obesity and cancer where they decrease inflammation and promote tumour development. Due to co-existence of M1 and M2 population the paradoxical roles of HIF could be possibly observed. Recent evidence clearly demonstrated that HIF-1 and HIF-2 influence macrophage polarization by modulating NO synthase and arginase gene in cytokine induced, transcription dependent manner suggesting spatiotemporal regulation of macrophage polarization by HIFs [99].

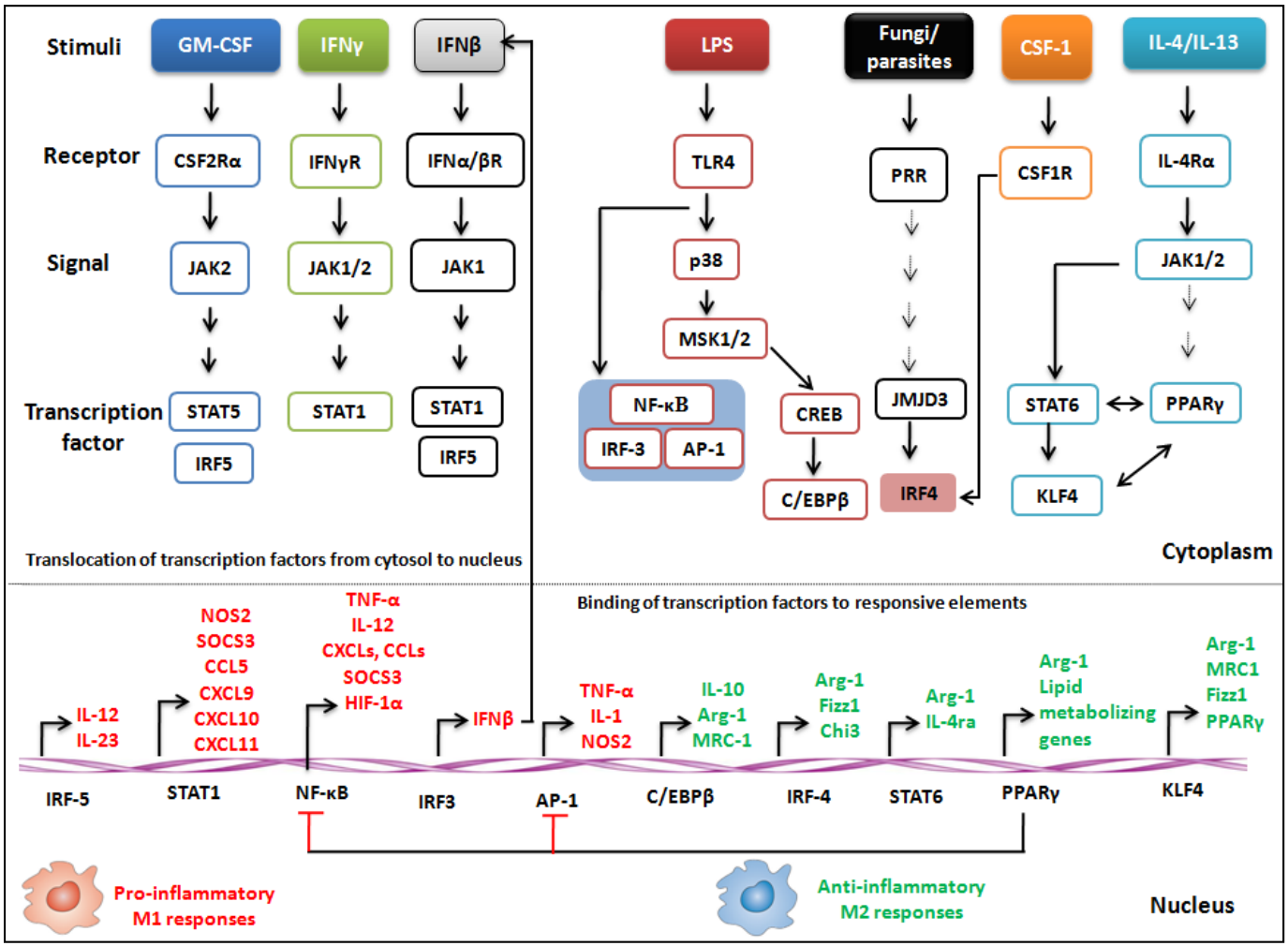


Figure 1.7. Cellular factors and signal transduction pathways involved in macrophage polarization. The stimulation at the receptor level transduces specific signals towards nucleus to mediate downstream effector functions. Transcription factors mediating pro-inflammatory responses (M1) are represented in red and anti-inflammatory in green (M2).

1.1.7.3. Effect of energy sensors TSC-mTORC, PTEN and AKT on macrophage activation states

mTORC1 (Mechanistic Target of Rapamycin- sensitive Complex), a key nutrient and metabolic sensor was shown to drive macrophage polarization and it is well reported that in obese, mTORC can re-program adipose resident M2 (maintenance of insulin sensitivity) macrophages to M1 which in turn is thought to be responsible for causing insulin resistance and type 2 diabetes [100]. Constitutive activation of mTORC was shown to induce M1 responses. Similarly several independent studies have shown that deletion of proteins required for anabolic growth such as AKT and PTEN resulted in improved IL-4 induced macrophage responses, while ablation of TSC1 an inhibitor of mTORC resulted in opposing effects additionally leading to increased pro-inflammatory responses [101-103]. These results clearly suggested that the external cues profoundly influence macrophage energy sensors which in turn affect macrophage polarization.

1.1.7.4. Impact of growth factors, enzymes and signalling molecules on macrophage polarization

In the recent times GM-CSF (granulocyte monocyte colony stimulating factor) and M-CSF (macrophage colony stimulating factor) were identified as regulatory cytokines controlling M1 pro-inflammatory and M2 anti-inflammatory responses respectively [26]. However Jaguin et al. in the year 2013 showed that in contrast to mouse macrophages, both GM-CSF and M-CSF polarize human macrophages to M1 phenotype (similar expression profiles) in presence of LPS and IFNγ thus highlighting the notion that mouse macrophages are distinct from human macrophages and grouping of macrophages needs considerable attention [104, 105]. In addition to growth factors, transcriptome profiling of

IL-4 stimulated cells revealed several enzymes and signalling modulators such as transglutaminase 2, mannose receptor, prostaglandin-endoperoxide synthase, BMP-7, FABP4, LXR-α and CISH that were shown to induce M2 phenotype. Similarly factors such as activin A, P2Y (2)R and BTK were shown to promote M1 polarization [20, 106-110].

1.1.7.5. Effect of epigenetic modulation on macrophage polarization

Emerging evidence clearly demonstrate that the macrophage polarization is controlled not only at transcriptional level but also at the epigenetic level. The epigenetic events are established in coordinating the transcriptional regulations of various genes in presence and absence of stimulations where several studies reported that during LPS induced TLR signaling, methylation and acetylation patterns on cytokine responsive elements are altered when compared to their basal levels which in turn alters macrophage phenotype. For instance HDAC3 was shown to induce strong M1 responses by deacetylating MPK1 and promoting MAPK induced inflammatory gene expression while simultaneously dampening M2 functions, similarly H3K27 demethylase Jmjd3 was shown to promote LPS induced pro-inflammatory responses because 70% of LPS inducible genes were Jmjd3 targets [111-113]. These observations clearly demonstrate that epigenetics play a crucial role in regulating macrophage polarization.

1.1.7.6. Role of microRNAs in modulating macrophage plasticity

Lately several accumulating evidence identified microRNAs as the major regulators of several cellular processes including immune responses. MicroRNAs are shown to be involved in promoting both innate and adaptive immune responses (Table 1.2, 1.3) but

their role is poorly studied in macrophage polarization [114, 115]. Since the balance between M1 and M2 macrophage responses is very sensitive and complex, there is a compelling pre-requisite to deeply understand the regulation of macrophage polarization because elucidating molecular mechanisms in macrophage dys-regulated diseases can help us in developing novel therapeutic strategies. Thus, we are interested in identifying the role of microRNAs in driving macrophage polarization because they are the *master regulators* of all the aforementioned factors.

Table 1.2. MicroRNAs involved in innate immune responses

MicroRNA	Target	Effect
miR-9	NF-κB	Negative regulator of TLR4 signalling
miR-19	TLR2	Negative regulator of TLR2 mediated inflammation
miR-21	PDCD4, IL-12	Negative regulator of TLR4 signalling
miR-27b	PPARγ	Enhanced responses towards LPS
miR-105	TLR2	Negative regulator of TLR2 mediated inflammation
miR-106a	IL-10	Decreased IL-10
miR-125b	TNF-α: IRF4	Diminished inflammation: enhanced macrophage activation
miR-145	TIRAP	Negative regulator of TLR signalling
miR-146a	TRAF6, IRAK1, IRAK2	Negative regulator of TLR signalling
miR-155	AID, MYD88, SOCS, SHIP1, TAB2, C/EBP-β	Promotes inflammation
miR-223	IKKα; Pknox1	Promotes inflammation
Let-7i, let-7e	TLR4	Negative regulator of inflammation

Table 1.3. MicroRNAs involved in adaptive immune responses

MicroRNA	Target	Effect
miR-10	Ncor2; BCL6	Increased T_{reg} effects
miR-17-92	Bim, PTEN	Increased CD4 T- cell proliferation
miR-29b	T-bet; IFNγ	Decreased Th1 responses
miR-146a	IRAK1; TRAF6; STAT1	Decreased TCR signalling; Increased T_{reg} Effects
miR-155	C-Maf	Increased Th1, Th17 effects; increased T_{reg} survival and decreased Th2 responses
miR-181a	SHP1, 2; DUSP5, 6	Increased TCR signalling
miR-182	Foxo1	Increased CD4 T cell expansion
miR-301a	PIAS3	Increased Th17 responses
miR-326	Ets	Increased Th17 responses
miR-150	C-Myb	Decreased B cell expansion
miR-155	PU.1; SHIP-1; AID	Increased antibody secretion and class-switch recombination
miR-181	-	Increased B cell expansion

1.2. MicroRNAs and their pathophysiology

MicroRNAs (miRNAs / miRs) belong to a class of small regulatory RNA molecules whose main function is to regulate gene expression at post-transcriptional level [116, 117]. They are observed to be expressed in green algae, plants, animals including viruses and about 60% of protein coding genes are known to be under the control of microRNAs [118-121]. They are chiefly involved in inducing RNA silencing effects (RNAi) via direct binding to messenger RNAs and besides this, mounting evidence indicate that miRs bind to proteins as well during translation, thus they are basically involved in controlling the stability of an mRNA and a protein of a particular gene [122-124]. As a consequence, microRNAs may control a variety of physiological functions such as DNA replication, cell cycle progression, differentiation, development, metabolism, neuronal cell fate and many other cellular processes [125-128].

1.2.1. MicroRNA discovery

MicroRNAs were first discovered in 1993 by a group led by Victor Ambros and simultaneously several other groups have identified that lin-14 mRNA involved in the early larval development of *C. elegans* was found to be repressed by a small non-coding RNA transcribed from lin-4 gene [129, 130]. Later lin-4 was discovered as the first in the class of small non-coding RNA "MicroRNA". Subsequently, let-7 RNA was similarly shown to repress lin-41 gene and was categorized under microRNAs [131]. Soon after this, several newly discovered RNAs were grouped into a major class of small non-coding RNA molecules known as microRNAs. To date about 1881 human microRNAs have been identified.

1.2.2. Structural elements and their functions

MicroRNAs are single stranded RNA molecules comprising of a short stretch of RNA oligonucleotides of about 22-26 bases in length [118, 131]. They are first transcribed as *primary double stranded microRNAs* in the nucleus which are characterized by hair pin loop flanked by a specialized 5' cap and 3' poly A tail. The flanking sequences are then trimmed during nuclear processing to form *pre-mature/ precursor microRNAs.* The product is further processed in the cytoplasm to form *mature double stranded miRNA* that is devoid of a hairpin loop [132, 133]. The double stranded RNA is subsequently processed into single stranded mature and functional microRNA, the binding of which to the 3'UTR of mRNA leads the formation of a thermodynamically favorable miRNA-mRNA hybrid structure resulting in the destabilization of messenger RNA [118, 132]. Mature microRNAs are characterized by the presence of a unique site in their sequence termed as *seed region* which confers target specificity [133]. The position from 2-8 nucleotides (5'-3' orientation) in their sequence represents the seed region and perfect complementarity of this sequence to the target (5' UTR and coding region in case of plants) results in mRNA degradation which is usually observed in plants [134]. However animal microRNAs match only 6-8 bases of their seed region against target mRNA 3' and 5' UTR and hence they generally do not result in transcript cleavage instead they induce gene silencing effects by either transcriptional or translational halt which will be discussed in the next section [135-138]. Each microRNA may repress several hundreds of mRNA targets at the same time each mRNA may be regulated by multiple microRNAs. This is purely because of the existing diversity across seed regions of miRNAs and target sequences of the messenger RNAs and this type of targeting where complementary

region of mRNA interacts with seed region of miRNA is termed as canonical targeting. Previous work by many researchers showed that most of the mRNAs are also targeted by non-canonical interactions, however contemporary reports clearly suggests that though mRNAs interact non-canonically with miRNAs, they do not mediate effective or biological repression and their existence needs further evaluation [139].

1.2.3. Cellular functions

Till date nine mechanisms of microRNA mediated gene repressions (Table 1.4) have been experimentally validated where they act at both transcriptional and translational levels. Binding of microRNA to mRNA results in conformational change in such a way that it disturbs eIF4 mediated ribosomal stability around the start codon. Similarly their interaction may lead to reduced formation of 80S subunits due to decreased assembly of 60S, 40S subunits and ribosome drop-off. Because of reduced translational initiation and elongation, translational repression occurs. Furthermore binding of microRNA to mRNAs transcriptionally can lead to destabilization or cleavage of messenger RNAs and mRNAs bound to RISC complexes subsequently may enter into P bodies where they are finally destroyed [140, 141]. Recently microRNAs are also shown to bind to the promoter regions of genes which may result in transcriptional halt [142-144].

Table 1.4. Mechanisms describing modes of microRNA action

1. 40S cap inhibition 2. 60S joining inhibition 3. Elongation inhibition 4. Ribosome drop-off (premature termination) 5. Co-translational protein degradation	**Translational inhibition mechanisms**
6. mRNA sequestration in P-bodies 7. mRNA decay (degradation/destabilization) 8. mRNA cleavage 9.Chromatin re-organization at the gene promoter resulting in transcriptional halt	**Transcriptional and post-transcriptional inhibition mechanisms**

In the very recent times Morozova *et al.* have proposed a unifying mathematical model which postulated that all the above mentioned mechanisms may potentially co-exist and work simultaneously because, the type of microRNA action in a biological system depends on several intrinsic parameters of both microRNAs and mRNA (Figure 1.8) [140].

In contrast to RNAi, *Li et al*. for the first time discovered RNAa (RNA activation) referring to RNA induced gene activation [145]. For the first time Place *et al*. have found that miR-373 induced RNAa effects by inducing promoter expression on their target genes and based on these results Huang *et al*. have shown how endogenous microRNA can exploit RNAa to promote cancer progression [142, 146].

Animal microRNAs are well known to target 3' UTR but accumulating evidence demonstrate that similar to plant miRs, mammalian miRs may also target 5' UTR and Coding DNA sequence (CDS) of mRNAs [138, 147-149]. Traditional 3'UTR targeting involves interaction with 5' ends (3U5P) of human microRNA while non-canonical 5' UTR targeting shows specificity towards 3' ends (5U3P) of microRNAs [149]. Recently Lee *et al*. reported a novel class of microRNA targets containing simultaneous 5'UTR and 3' UTR interaction sites. They suggest that many microRNAs contain interaction sites with mRNA 3' and 5' UTRs through their 5p and 3p arms respectively and this type of targeting is validated experimentally for the first time against a single mRNA [150]. Latest research suggests that besides mRNA targeting, miRNA may also bind to promoter regions on the DNA segments to either activate or inhibit the transcription [142-144].

1.2.4. Biogenesis

MicroRNAs originate from different parts of nuclear DNA and they are usually transcribed as long precursor RNA molecules which are processed into functional microRNAs [132, 133]. For instance they may be derived from exons and introns of coding or non-coding transcriptional units of a mRNA, or can arise from tRNAs (transfer RNAs), rRNAs (ribosomal RNAs) or snoRNAs (small nucleolar RNAs) and thus they can be transcribed by all the three RNA polymerases [151-155]. However till now only two microRNAs (hsa-miR-663, mmu-miR-712) were identified to be formed from ribosomal RNA and the exact mechanism of RNA polymerase I dependent microRNA generation is yet to be elucidated [156]. Accumulating evidence suggests that microRNAs may be the products of transcription, nuclease digestion or splicing reaction and these miRs can be transcribed by their own promoters or can be derived during host gene expression [156-159]. Further some miRs are co-expressed in the form of cluster (polycistronic unit) under the same promoter and thus belong to same family. For example, miR-23a~27a~24-2 are located as a cluster on human chromosome 19 sharing a common and host gene independent promoter and are transcribed as a single primary miRNA transcript [160].

The microRNA biogenesis is a finely tuned process and is evolutionarily conserved. DGCR8, (known as PASHA in invertebrates) a protein required for the recognition of primary microRNA associates with DROSHA, an RNase III enzyme to form a microprocessor complex whose function is to cleave primary miRNA about eleven nucleotides from the hairpin base to generate a pre-miRNA with 5’ phosphate and 3’ hydroxyl group [161, 162]. Certain pre-miRs bypass this microprocessor action and are

spliced out directly from introns of the host genes and such miRs are termed as *Mirtrons.* Subsequently pre-miRNAs are further processed in the cytoplasm by an endonuclease DICER (RNase III) which cuts at 5' and 3' ends resulting in a double stranded microRNA [163]. One of the thermodynamically stable strands (guide strand) is incorporated into the catalytic complex termed as RISC (RNA Inducing Silencing Complex) that aids in binding of miR to the complementary 3'UTR of mRNA leaving the other strand for degradation (passenger strand) (Figure 1.8). Sometimes both the strands remain stable and functional, thus in such cases both of them are loaded onto separate RISC complexes to target their respective mRNAs. RISC comprises of DICER and many argonaute proteins and miRNAs associated with RISC are denoted as miRISC [115, 164-166]. Argonautes preferentially choose miRs with more number of targets over those which have few or no targets [167]

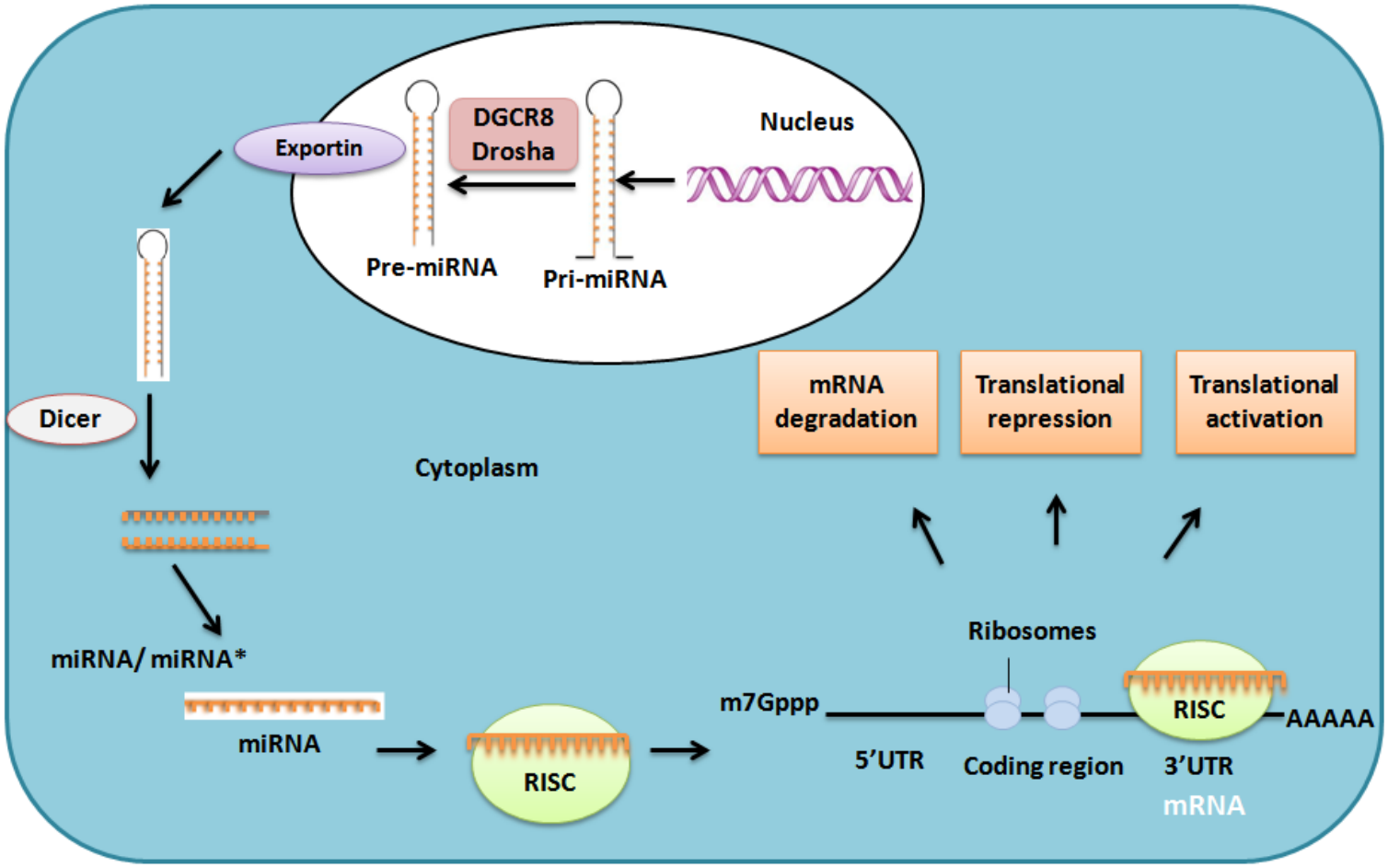


Figure 1.8. MicroRNAs regulate gene expression at post-transcriptional level. MicroRNA biogenesis begins in the nucleus and their functions are executed in the cytoplasm, where it alters transcription and translational machinery for repressing/activating protein synthesis.

1.2.5. MicroRNA nomenclature

Since microRNAs are diverse in their numbers, sequences, abundance and exhibit species specificity there is a pre-requisite to name them distinctively and hence microRNAs follow a specific pattern of nomenclature [168-170]. Firstly microRNAs follow a sequential naming pattern in the form of 'species-microRNA-number'. For example mmu-miR-342, where mmu indicates the organism *mus musculus* (mouse) and miR represents mature microRNA. "mir" is indicative of premature microRNA and the number 342 signifies the sequence of their discovery. For instance if recently published mouse miRNA is numbered as mmu-miR-342, next published miRNA under the same species is named as mmu-miR-343. Secondly if microRNAs are identical in their mature sequences but distinct in their genomic location and premature sequences, they are designated as miR-16-1 and miR-16-2. Thirdly if mature miRs slightly differ in their sequences they are represented as miR-342a and miR-342b which denotes that they are closely related microRNAs and are expressed from mir-342a and mir-342b precursors respectively. When miRs exhibit full conservation of the mature microRNA sequence or partial conservation with respect to only seed region from 2-8 position, they are said to be members of a single microRNA family which are believed to share common functions. Sometimes they are observed to exist as non-randomly arranged co-localized structures across the genes to perform a plethora of functions. For example, miR-15a and miR-16 are located as a cluster on chromosome 13 and are shown to regulate the development of chronic lymphocytic leukemia (CLL). As discussed above when both the strands of mature form are functional, depending on the abundance in their expression levels they are represented differently. For instance the strand which is predominantly expressed is

represented as miR-56 and the strand which is relatively less expressed (opposite arm of the precursor) is designated as miR-56*. When the expression levels are undetermined, they are represented as miR-142-5p (from the 5'arm) and miR-142-3p (from 3' arm) depending on their origin from two strands of premature form.

1.2.6. RNA editing

Several microRNAs are shown to undergo RNA editing (insertions, deletions, base substitutions) which is relatively a rare phenomenon [171]. The main purpose of RNA editing is to exhibit diversity across microRNAs due to their small size in order to compensate all the cellular functions. RNA editing generally includes base modification of cytidine (C) to uridine (U) and adenosine (A) to inosine (I) resulting in the alterations in the encoded protein translated from an mRNA. Similarly miRNAs usually undergo A to I alterations with the help of ADARs (Adenosine Deaminase Acting on RNAs) in primary or premature duplexes resulting in the changes in their secondary structures where I behaves as G which subsequently affects heterochromatin formation, processing, splicing and target binding [172-174].

1.2.7. MicroRNA turnover

The expression of microRNA is tightly controlled at several levels including microRNA transcription, processing and degradation in order to maintain tissue homeostasis [175]. The turnover of microRNAs depends on several factors like argonaute proteins which preferentially select thermodynamically favorable strands while degrading the unstable ones. It is well shown that exonucleases like xrn-2 in *C. elegans* and small RNA degrading nucleases (SDN) in plants degrade miRNAs in 5'-3' and 3'-5' orientation

respectively [176]. Though several nucleases exist in animals their role in regulating miRNA stability is poorly defined. Furthermore plant miRs ending with adenines on their 3' end and addition of adenines to the 3' end of mammalian miRNA-122 are shown to be effectively stable [167, 177]. Similarly addition of methyl moiety on to the 3' ends of plant miRs is known to stabilize the microRNA by blocking its uridylation [178]. In contrast uridylation is also shown to stabilize many other miRNAs [179]. Recent reports suggest that the miRNA activity could be regulated by methylation on 5' ends of pre-miRNA which is probably considered as an epigenetic modification [180, 181]. This is based on the observation of Xhemalce *et al*. that the RNA methyl transferase, BCDIN3D, directly methylates 5'monophosphate of pre-miR-145 and prevents its maturation. This methylation is consequently shown to inhibit its processing by DICER thus leaving high levels of pre-miRNA and low levels of mature miRNA. As a result these expression levels are shown to have a high impact on breast cancer development [182].

1.2.8. MicroRNA dys-regulation in diseases

Since microRNAs are known to drive major physiological processes, any excesses or deficiencies in their expression levels can lead to perilous outcomes. Further, miRs may be used as diagnostic and prognostic biomarkers. Human diseases associated with dys-regulated microRNAs will be reviewed in the following section.

1.2.8.1. Cancer

Majority of the cancer types are observed to contain dys-regulated signatures of microRNA expression. The microRNAs which are down-regulated in the disease state are considered as *tumour suppressor microRNAs* as they are shown to effectively repress

tumour inducible genes. On the other hand microRNAs that are highly over-expressed in cancer tissues are termed as *oncogenic microRNAs* as they are well described to repress the spatiotemporal expression of tumour suppressor genes thus promoting cancer development [183]. As an example, reproductive cancers (endometrial, ovarian, breast) show a mixed miRNA signature: increased expression of miRs such as miR-21, miR-155, miR-191 and miR-210 whilst decreased expression of miR-205, miR-145, miR-10b and miR-125b [184, 185] . Similarly there are hundreds of microRNAs identified till date which are potentially implicated in several types of cancers.

1.2.8.2. Neurodevelopmental disorders

High throughput screening for identifying microRNAs in non-neoplastic diseases is slowly emerging [186]. Compared to all other organs, more number of miRNAs were shown to be expressed in the brain and it is considered as a highly dynamic process: a number of miRs are expressed during early development of brain which gradually decreases in the later stages of development [187, 188]. It has recently been observed that microRNA deregulation is implicated in neurological disorders like Alzheimer's and Parkinson's disease, Schizophrenia, Down syndrome and many other related disorders [189].

1.2.8.3. Metabolic diseases

MicroRNAs are known to play a key role in controlling metabolic homeostasis especially cholesterol, lipid and glucose metabolism, the dys-regulation of which leads to critical metabolic diseases like non-alcoholic fatty liver diseases, cardiovascular diseases, insulin resistance and type 2 diabetes [190, 191]. For example miR-33a and miR-33b are

implicated in cholesterol and lipid metabolism and similarly miR-103 and miR-107 are shown to regulate insulin signaling and glucose homeostasis [192, 193]. MicroRNAs regulate glucose metabolism at multiple stages starting from insulin synthesis to glucose uptake and breakdown in respective target tissues like adipose, liver and skeletal muscle. For example miR-124a, miR-9, miR-96a, and miR-33 were shown to control insulin release while miR-15a is shown to regulate insulin biosynthesis in insulin producing pancreatic beta cells [190, 194]. Moreover microRNAs are shown to be explicitly involved in adipogenesis and skeletal muscle differentiation and any de-regulation may lead to obesity, a condition which involves altered metabolism resulting in insulin resistance [195-198]. Thus any alterations in microRNA expression levels may disrupt metabolic balance and lead to fatal outcomes.

1.2.8.4. Cardiovascular diseases

The well being of human mainly depends on the functionality of the vascular system as it supplies nutrients, oxygen and essential components to all the parts of the body. miR-21, miR-29a, miR-129, miR-210, miR-211, miR-320, miR-423, and let-7c are shown to be expressed in foetal heart during embryonic development while miR-1, miR-133, miR-16, miR-126, miR-27b, miR-30d, miR-143, miR-208 and let -7 family are known to play a pivotal role during heart development in adults, nonetheless dys-regulated levels of these miRs were reported in cardiac failure cases such as atherosclerosis, arrhythmias, hypertensions, coronary syndromes and heart strokes [199]. miR-33a and miR-33b are implicated in dyslipidemia as they are known to regulate sterol regulatory element binding proteins (SREBP) along with ATP-binding cassette (ABC) transporters that control cholesterol transport [200]. Inhibition of these miRs led to decreased

hypercholesterolemia and improved dyslipidemia, thus resulting in reducing heart failures [201].

1.2.8.5. Viral infections

The role of microRNAs in controlling viral responses in human diseases has slowly begun to come to light. Several miRs have been observed to be dys-regulated in viral diseases such as HIV, HCV and influenza virus induced infections [202, 203]. Accumulating findings suggest that viral RNAs despite being small can encode miRs on their own but these findings needs to be further verified [204]. Alternatively viruses exploit host miRNAs for their survival. For instance miR-122 expression is remarkably up-regulated in the liver of hepatitis C infected patients which is further shown to bind to the 5'UTR of HCV RNA and aid in its viral replication [205].

1.2.8.6. Inflammatory diseases

MiRNAs are shown to regulate all the physiological processes hence their dys-regulation is linked to several diseases such as autoimmune disorders, skin, nephrological diseases, skeletal muscle syndromes etc. [198, 206-208]. On the other hand all the above indicated diseases are shown to be caused under chronic inflammatory conditions. Although several reports revealed the role of microRNAs in inflammatory cells like vascular endothelial cells, granulocytes, leukocyte, monocytes and macrophages in causing inflammation induced diseases, the functional role of microRNAs in macrophage polarization and associated diseases is poorly studied and hence we have undertaken the objective of studying the microRNA signature in polarized macrophages and their regulatory role in inflammation induced insulin resistance (Figure 1.9) [209-211].

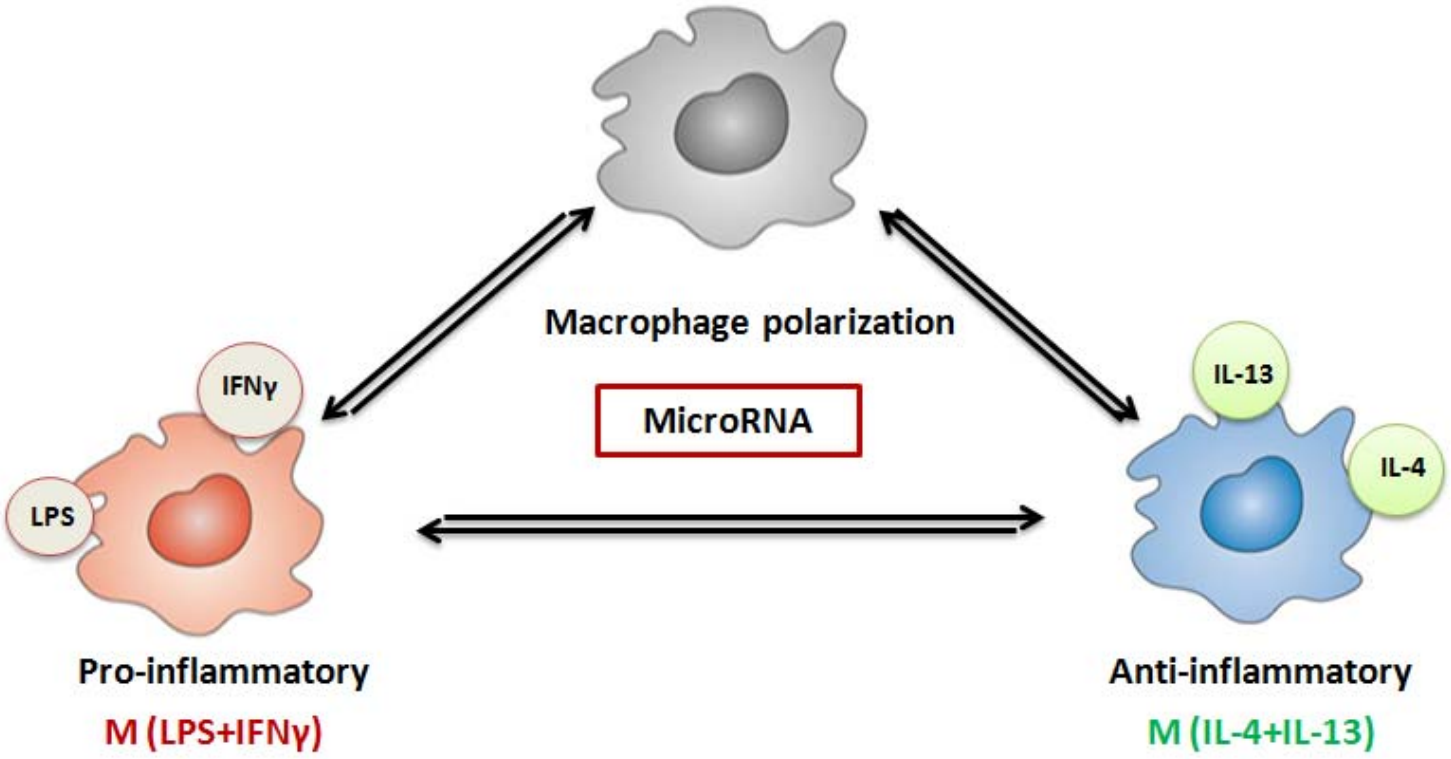


Figure 1.9. MicroRNAs regulate macrophage polarization. MicroRNAs, tiny regulators of cells control diverse functions such as inflammatory responses and macrophage polarization

1.2.9. MicroRNA based therapy

MicroRNA therapy can be achieved using two approaches: miRNA mimics and antagonists. Mimics are used to restore the loss of functions in a cell which are suppressed in a diseased state. This approach is termed as miRNA replacement therapy and is currently in use. MRX34 is the first identified double stranded miRNA mimic developed for mimicking the effects of miR-34 in vivo. It is shown to inhibit oncogenic pathways and stimulate anti-tumour responses and thus it serves as a potent tumour suppressing miRNA. It has currently passed phase I clinical trials and is about to enter into phase II after achieving additional data read-outs in multiple tumour cells [212]. Similarly antagonists include miRNA inhibitors which suppress the function of endogenous microRNA. These inhibitors irreversibly bind to the miRNAs thereby preventing their actions. Miravirsen is the first miRNA inhibitor developed against miR-122 to treat chronic hepatitis C. It is a locked nucleic acid based anti-sense oligonucleotide and is presently being tested in phase II clinical trials [213, 214]. Further miRna THERAPEUTICS have announced Let-7 as another microRNA candidate for miRNA replacement therapy, which was successful in its preclinical trials and is about to enter into phase I trials to treat non-small cell lung cancers (NSCLC) [215]. Besides the above molecules, antagonists for miR-208/499 and miR-195 have been developed which are presently being tested under preclinical trials to treat chronic heart failures and post myocardial infarction remodelling respectively [216, 217]. Administration of these chemically modified miRs can restore normal functions of a cell thus helping in treating a diseased condition. Similarly several microRNAs are being tested at the preclinical stage and their success mainly depends on the safety and efficacy. There are a variety of

delivery strategies to ensure improved biodistribution, enhanced permeability and retention (EPR), targeted delivery and reduced toxicity and all these features may be feasible using nanotechnology [218, 219]. Nanoparticles being tiny in size can be conveniently modified with chemical moieties and stabilizing agents to mediate safer and long term effects inside the cells. For instance, nanoparticles may be coated with cholesterol and PEG to facilitate uptake of miRs by the cells thereby enhancing their circulating half life. Further, the stability of RNA molecules may be improved by making appropriate base modifications with chemical moieties (methyl, chloro, fluoro groups). Furthermore, they can be ligated with antibodies or ligands against specific endothelial receptors to mediate improved bioavailability and targeted delivery [220]. MRX34 is currently known to be encapsulated in liposomal nanoparticle formulation called SMARTICLES that contains mixture of lipids with amphoteric properties which can be easily accessed by the cells due to their alterations across physiological pH [221].

1.3. Role of microRNA-712 in inflammation and associated diseases

The microRNA profiling data obtained from microarray analysis in macrophages stimulated with LPS and IFNγ revealed a functionally novel mouse specific microRNA known as miR-712 which is a poorly characterized microRNA. In fact, when we have initiated this study in 2011, not even a single report was available in the literature. Based on nucleotide BLAST analysis we have identified that miR-712 precursor is probably transcribed from ITS2 (Internal transcribed spacer) region of 45S pre-ribosomal RNA (Figure 1.10). Supporting our speculation, Son *et al.* in the year 2013 have shown that miR-712, a mechanosensitive miRNA, has an unconventional origin, generated from 45S pre-ribosomal RNA (Rn45s) in an exonuclease XRN-1 dependent manner. They showed

that knockdown of XRN-1 up regulates miR-712 expression in disturbed flow subjected endothelial cells. miR-712 is termed as an atypical microRNA because unlike conventional miRNAs it is generated in DGCR8/DROSHA independent but exonuclease and DICER dependent fashion. In addition to this Son et al identified another human specific microRNA-663 which also originates in similar fashion but it is derived from ITS1 region of Rn45s gene [156].

1.3.1. miR-712 in atherosclerosis

Son et al. have reported that miR-712 is a flow sensitive microRNA and is significantly up-regulated by disturbed flow in mouse endothelial cells, vascular smooth muscle cells and blood. In the same study, it was demonstrated that miR-712 targets TIMP3, a key regulator of inflammation, thereby activating MMP 2/9 and ADAM 10/17/TS4 which are mainly responsible for triggering pro-atherogenic responses. They have also identified miR-205 as a human functional homolog of miR-712: both miR-205 and miR-712 share common seed sequence and about 50 percent common mRNA targets (of 5384 miRs modulated during d-flow, 1904 miRs were found to be common) [156, 222].

1.3.2. miR-712 biogenesis

In order to better understand miR-712 biogenesis, we need to first unravel the complexity involved in ribosomal RNA assembly as the miRNA is located on 45s preRNA (Figure 1.10). The ribosomal subunits 18S, 5.8S and 28S are transcribed as a single unit separated by internal transcribed spacers (ITS1, ITS2). ITS regions are removed in all the organisms in a controlled manner without which there will be termination and or degradation of rRNA and the mechanisms involved in this process are poorly defined.

Now for the first time in the year 2015 Lisa *et al*. have helped us in comprehending how ITS2 processing is coordinated during the maturation of 5.8S and 28S rRNA. During ribosomal assembly, ITS2 processing is initiated and coordinated by Las1-Grc3-Rat1-Rai1 pathway. Las1 endonuclease cleaves at C2 site in ITS2 region forming a cyclic phosphodiester bond between 3' of 7S RNA (5.8S precursor) and a 5' hydroxyl group at 26S preRNA (28S precursor). Subsequently Grc3 adds phosphate to 5'OH which is now the substrate of Rat-1p endonuclease and its co-factor Rai1 [223]. Rat-1p is a nucleolar enzyme involved in the degradation of monophosphates in 5'-3' direction and thus aids in proficient ribosomal maturation. Rat-1p and Xrn-1p are similar in their functions (functional homologs for 5'-3' exonuclease activity) except that Rat-1 is predominantly located in the nucleus while Xrn-1 is present in the cytoplasm. Furthermore Johnson *et al.* and other groups have showed that in yeast cells, cytoplasmic Xrn-1 is functionally interchangeable with a nuclear enzyme Rat-1p in its absence, albeit with less efficiency, when mislocalized to other compartments [224, 225]. However no reports give clear information under what conditions these enzymes are interchangeable in vivo.

To date Rat-1p is shown to be prominently involved in 28S ribosomal RNA maturation and there is no direct evidence to indicate that Xrn-1 performs nuclear functions naturally, however the role of Xrn-1 in ITS-2 processing and 28S ribosomal maturation is evident only from mutational and deletion studies [226]. Whether Xrn-1 plays a direct or an indirect role in miR-712 generation is yet to be determined. Thus there is a necessity to understand the exact role of Xrn-1 in ribosomal processing in order to appreciate the biogenesis of miR-712. On the other hand in atherosclerosis or in any diseased condition, tremendous damage occurs to the cells which in turn affect ribosomal processing and

protein synthesis [227, 228]. Thus miR-712 biogenesis is a cause or a consequence (miR-712 could be later responsible for disease progression by feed forward mechanism) of atherosclerosis is still undefined. Their biogenesis can be clearly envisaged if the expression and degradation patterns of miR-712 and rRNA subunits can be monitored in a variety of disease conditions.

1.3.3. Anti-miR-712 nanoparticles

miR-712 being a mouse specific microRNA, although cannot be directly used for human applications, can still primarily be used as model to study various diseases associated with it. However in diseased conditions where its functional homolog miR-205 expression is compromised, replacement with miR-712 may have beneficial effects. Or under certain circumstances, miR-712 can exert potential functional effects compared to its homologs. Hence if we can identify such conditions, we may apply them for human benefits. Since anti-miR-712 nanoparticles have been designed to prevent atherosclerotic lesions in mouse, similar strategy can be employed in designing miR-712 mimic or antagonist to treat diseases in a tissue or a cell targeted manner [229, 230].

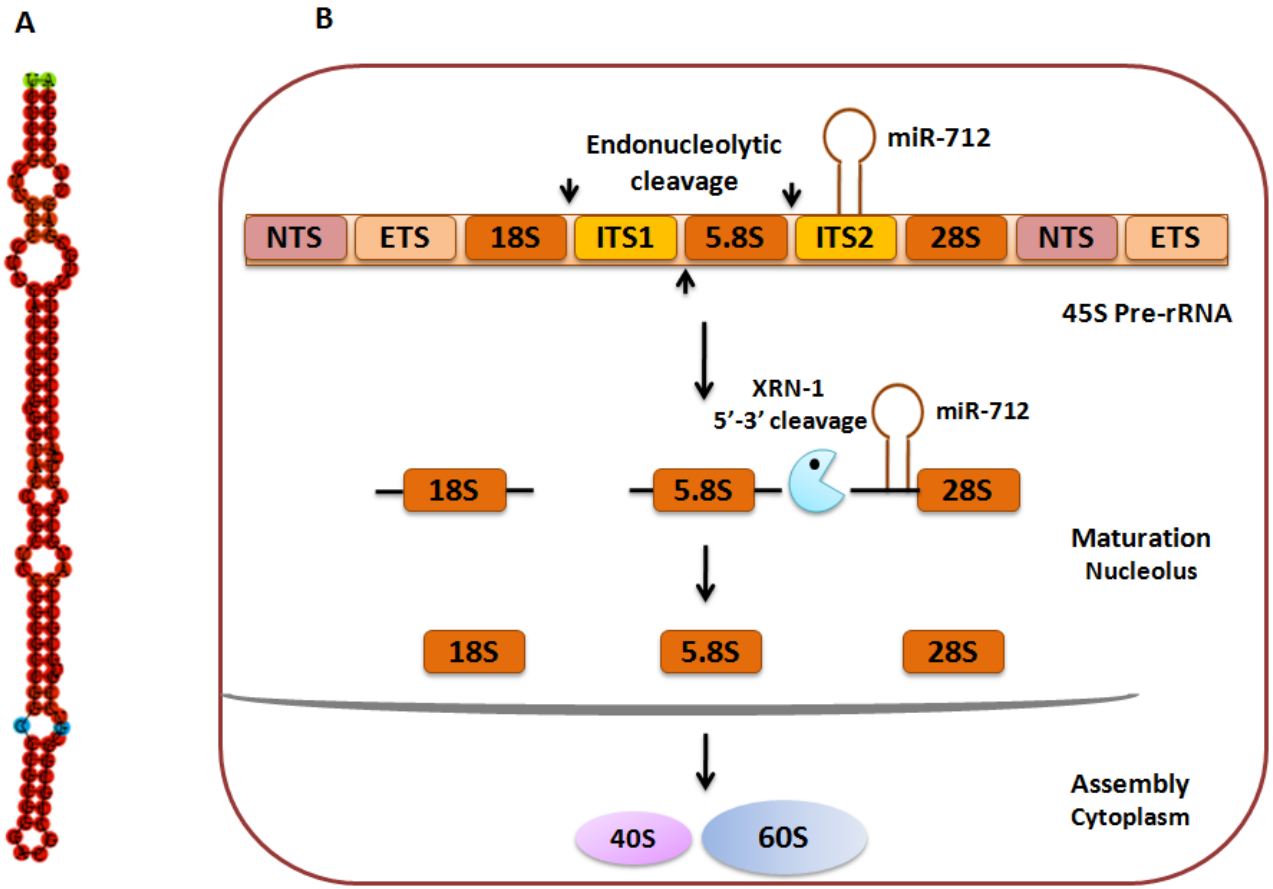


Figure 1.10. Structure and biogenesis of miR-712. (A) **Secondary structure of pre-mir-712.** mir-712 structure drawn based on minimum free energy scores (MFE) by RNAfold. (B) **miR-712 originates from ITS2 region of 45s pre-ribosomal RNA.** Biogenesis of miR-712 requires endo- and exo-nucleolytic cleavages in the ITS2 regions of 45s pre-ribosomal RNA.

1.4. Role of microRNA-16 in inflammation and associated diseases

The microRNA profiling data obtained from our microarray analysis in macrophages stimulated with LPS and IFNγ showed moderately decreased miR-16 expression levels. MiR-16 is known to be predominantly involved in regulating B cell associated cancers [231]. Although it is ubiquitously expressed in all the cells, the precise role of miR-16 in regulating other pathophysiological processes is poorly studied especially in inflammation associated diseases which is gaining ground in the recent times [232]. Although there are several independent studies describing the role of miR-16 in inflammation, obesity and type 2 diabetes, its role in inflammation induced insulin resistance is poorly studied [233-235]. Hence we have undertaken the objective to define the roles of miR-16 in inflammation induced insulin resistance and we observed that ectopic expression of miR-16 enhanced insulin stimulated glucose uptake in skeletal myoblasts by up-regulating GLUT4 and MEF2A which are key players involved in insulin stimulated glucose uptake. Collectively, our data highlight the functional role of miR-16 in ameliorating inflammation induced insulin resistance [236].

1.4.1. miR-16 genomic annotation

miR-16 belongs to miR-15 family and both miRs are evolutionarily clustered to function as tumour suppressor genes. The clustered miRs are transcribed as homologs from two different regions of human genome. miR-15a/16-1 is transcribed from chromosome 13q14 locus and this cluster encodes four miRs namely miR-15a, miR-15a*, miR-16-1, miR-16-1*. Similarly miR-15b/16-2 cluster from chromosome 3q26 locus generates miR15b, miR-15b*, miR-16-2 and miR-16-2*. The host gene for miR-15a/16 is intronic region of DLEU2 (Deleted in Leukemia) gene, while miR-15b/16-2 is hosted in the

intron of SMC4 (Structural Maintenance of Chromosomal proteins) gene (Figure 1.11). DLEU2 gene generates eight non-coding transcripts and is considered as a potent tumour suppressor gene which is often observed to be deleted in chronic lymphocytic leukemia (CLL), myeloma, mantle cell lymphoma and prostrate cancers while there are four coding transcripts of SMC4 gene which are known to regulate chromosomal rearrangements and mitotic events and this locus is implicated in diseases such as diabetes, pulmonary and cardiac problems nevertheless recent findings also portrays its implications in several types of cancers [237].

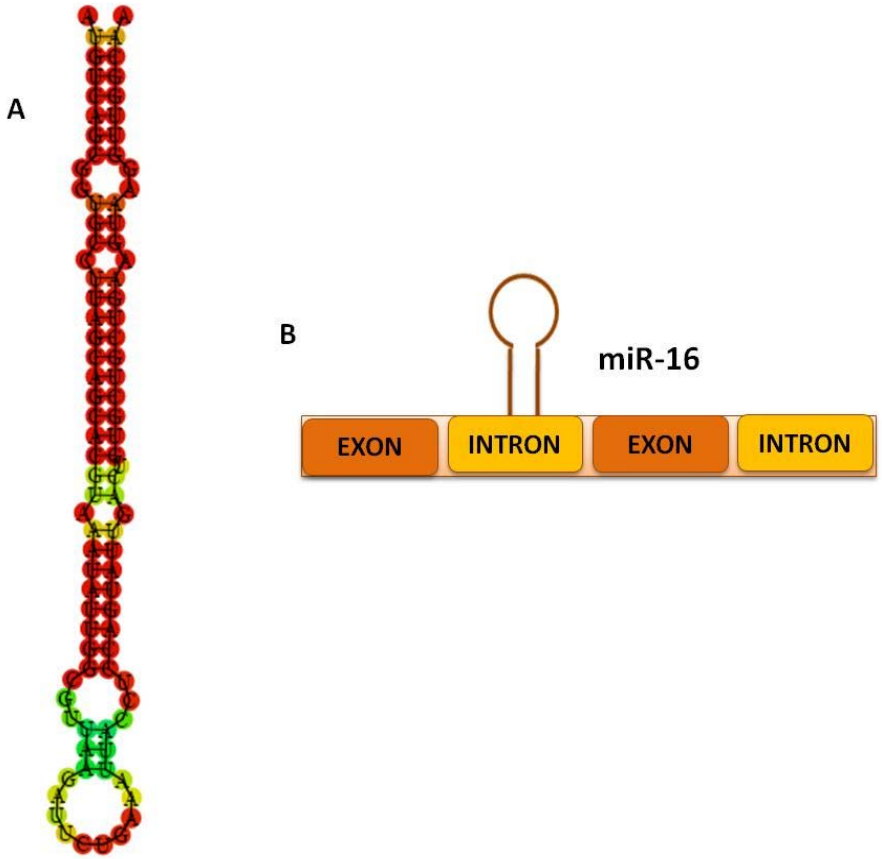


Figure 1.11. Structure and biogenesis of miR-16. (A) Secondary structure of pre-mir-16. mir-16 secondary structure drawn based on minimum free energy scores (MFE) by RNAfold.

(B) miR-16-1/2 originates from DLEU2/SMC4 genes. miR-16-1/2 genes are located on the chromosome 14/3 within the introns of DLEU2/SMC4 genes respectively.

1.4.2. MiR-16 a potent tumour suppressor

Research for several years has revealed that miR-15a/16-1 is a tumour suppressor gene which is frequently deleted or down regulated in CLL, pituitary adenomas and prostate carcinomas [232, 238, 239]. miR-15a/16-1 cluster on chromosome 13q14 is highly expressed in B cells compared to miR-15b/16-2 indicating that the former is chiefly involved in maintaining B cell homeostasis. Although miR-15a/16-1 and miR-15b/16-2 are similar in their sequences, they are shown to exhibit distinct roles in the development of tumourigenesis [240, 241]. The functional roles of miR-15a/16-1 are well illuminated in the current scenario; however because of the controversial roles of miR-15b/16-2, their implications in cancers are still being elucidated.

The transcriptome and proteome analysis revealed that these miRs, targeted genes which were involved in regulating cell proliferation and anti-apoptotic functions. The transcriptome profile showed that miR-15a/16-1 targeted 20% of mRNAs containing AU rich elements in their 3'UTRs indicating that they execute ARE (AU rich elements) mediated regulation while proteome changes displayed Wt1 (Wilms tumour), Mcl1(Myeloid cell leukaemia), cyclin-D1 and Wnt-3a, proteins majorly involved in cell proliferation as their potential targets [232]. miR-15a/16-1 was mainly shown to suppress tumour formation by inhibiting the expression of Bcl2 (B-cell lymphoma 2), a critical oncogene implicated in a variety of malignancies [232, 242]. This clearly suggests that miR-15a/16-1 cluster is involved in regulating apoptosis and thus suppress tumourigenesis.

CLL is the most common type of leukemia occurring worldwide and represents one-third of all leukaemia cases. It is a heterogeneous disease occurring in two forms: *Indolent* and

aggressive [243]. Indolent is a form where tumours are slow growing and does not require immediate treatment soon after the diagnosis. This type of cancer requires waiting for the symptoms to develop and typically have good prognosis with high survival rate. On the other hand aggressive type of cancer is a rapidly developing tumour which seeks urgent attention. Intensive chemotherapy in addition to radiotherapy may be required to treat such cancers. Such types of cancers usually have very poor prognosis with low survival rates. CLL is a disease which is often associated with in the families and very recently it has been observed that the germ-line mutations in miR-16-1 was responsible for hereditary cancers and patients affected with CLL are prone to develop secondary malignancies because of such mutations across different genomic location [241].

Bioinformatics and experimental evidence suggested p53 binding sites upstream to both miR-15a/16-1 and miR-15b/16-2 cluster indicating that they are transcriptionally regulated by tumour protein 53 (TP53) and increased levels of miR-15/16 in turn negatively regulated p53. Thus these experiments clearly demonstrated that miR-15a/16-1 and TP53 are engaged in feedback circulatory loop in a normal healthy cell: p53 transactivates miR-15a/16-1 expression and increased expression of microRNAs in turn halts p53 expression. However in indolent CLL, this circuit fails to function normally because of the interplay between tumour suppressor miRNAs; miR-15a/16-1 and miR-34a (chromosome 11). Deletion of 13q14 (Indolent CLL) results in decreased targeting of Bcl2 because of which apoptosis will be majorly inhibited, and secondly the active p53 which is not targeted by miR15a/16 in turn transactivates miR-34a. Increased miR-34a further targets ZAP70, and low levels of ZAP70 (Indolent CLL) indicates high survival rate (good prognosis) in CLL patients. On the other hand, aggressive CLL which is often

because of 11q23 (miR-34a) deletion, showed reduced targeting of ZAP70 thus indicating very low survival rate (poor prognosis). Several such studies suggest that the progression of CLL depends on the cross talks among different microRNAs such as miR-181b, miR-29, miR-155, miR-17, and miR-92 [244, 245]. Thus the interactions among these microRNAs across various chromosomes determine the outcome of the disease.

Mounting evidence indicate that apart from chromosomal deletions at 13q14 locus, allelic selection, mutations, defective primary miRNA processing and epigenetic modifications at the microRNA locus also play a role in the development of CLL [244]. Veronese *et al.* in the year 2015 have showed that miR-15a/16-1 expression occurs in monoallelic fashion where one of the alleles of miR-15a/16-1 is transcribed by RNA polymerase II along with DLEU2 gene while the second allele transcription involves RNA polymerase III activity independent of host gene expression. In 13q14 deleted patients, RNA polymerase III driven miR-15a/16-1 expression was observed which was correlated with high ZAP70 levels (predicting poor prognosis), on the contrary ZAP70 expression was least in cases where miR-15a/16-1 was transcribed via RNA polymerase II. This indicates that allele specific transcriptional regulation exists for miR-15a/16-1 expression which can help in distinguishing indolent and aggressive types in CLL [246]. Further a single nucleotide polymorphism (SNP) from C to T at +7 nucleotide in 3' direction of pri-mir-16 was observed in few patients which was later identified to be responsible for decreased expression of miR-16. This SNP was found to be a germ-line mutation and was responsible for deletion of the respective allele of miR-15a/16-1 cluster. This was further supported by the observations that such mutations were not observed in somatic cells and secondly one of the CLL affected patient's mother and sister died of CLL and breast

cancer respectively [232]. It is believed that mutations in 40 bases before and after pre-miRNA sequence can influence the transcription efficiency. Recently A to G mutation was observed 100 bases upstream miR-15 stem-loop in two CLL patients [246]. Although these mutations did not affect the expression of pri-miR-15a/16-1, the mature forms of miR-15a were more radically down regulated compared to miR-16-1 indicating that point mutations may lead to defective microRNA maturation. Higher levels of miR-16 compared to miR-15 were believed to be because of the miR-16 expression levels compensated by alternative genomic location and consequently these SNPs lead to reduced DROSHA processing activity [247].

In other types of CLL despite no genomic mutations were observed, miR-15a/16-1 expression were found to be significantly down-regulated albeit with high pri-miR levels. Although mRNA expression levels of DROSHA, DGCR8 other miRNA processing complex were unaffected, DROSHA processing activity was found to be reduced only with respect to miR-15a/16-1 cluster as other miRs used in the study such as miR-155 processing were unaltered. Thus DROSHA activity was not globally defective and this shortcoming was specific towards miR-15 cluster. In the same study they observed that, ADARB1 (Adenosine Deaminase RNA specific B1) was shown to inhibit pri-mir processing by RNA editing [247]. ADARs are known to modify specific adenosine residues on pri-miRNA into inosine which makes them resistant against DROSHA cleavage. They further showed that A to G mutation resulting in defective processing of miR-15a/16-1 was abrogated, when RNA binding domain and nuclear localization signal domain of ADARB1 was deleted indicating that miR-15a/16-1 adenosines are the

potential targets of ADARB1 thus resulting in poor activity of DROSHA consequently leading to decreased microRNA expression levels.

Apart from above mentioned factors, epigenetic modifications are also considered to be important regulators of microRNA expression and their functions. Histone acetylation and methylation are known to control RNA polymerase II mediated miR-15/16 transcription and conversely HDACs (Histone Deacetylase) are known to inhibit miR-15/16 promoter activity. Compelling evidence indicate that HDAC 1, 2 and 3 are aberrantly over-expressed in CLL and are shown to silence miR-15/16 expression [181]. Further inhibition of HDAC 1, 2 and 3 was shown to restore acetylation and accumulation of H3K4Me2 at miR-15/16 promoter resulting in increased expression of both miRNA and host gene DLEU2 (but not SMC4) suggesting that HDACs potentially target tumour suppressors [181]. On the other hand hypermethylation on DLEU2 promoter was shown to down-regulate dleu2 expression in pediatric AML (Amyloid Leukemia) which in turn hampered miR-15a/16-1 expression indicating that host gene expression alone can alter microRNA expression again clearly suggesting that tumour suppressors act in a coordinated fashion [248].

In addition recent findings suggested that DLEU7, a candidate tumour suppressor on chromosome 13q14 gene was found to be located telomeric to miR-15a/16-1 and both the microRNA cluster and DLEU7 were observed to cooperatively act as tumour suppressors. DLEU7 was shown to inhibit NF-κB and NFAT which were well known to promote cell survival and proliferation. DLEU7 inhibition was shown to activate NF-κB and NFAT via TNF signaling while miR-16 inactivation caused constitutive expression of bcl2 and mcl1 thus contributing to CLL [241].

1.4.3. miR-16 induces cell cycle arrest

Besides controlling cell proliferation and survival, miR-16 is shown to regulate cell cycle events. miR-16 is known to promote cell cycle arrest at G1/S transition phase by targeting cyclins D1, D3 and their respective kinases CDK6 and cyclin E1. Thus miR-16 targeting these genes results in preventing phosphorylation and triggering activation of Rb (Retinoblastoma) which can then readily bind and block E2F, a transcription factor responsible for driving cells from G1 to S phase. This was further supported by the observation that inhibition of miR-15/16 enhanced E2F induced G1/S phase transition. Additionally it was shown that miR-15a/b, miR-16-1/2 and their host gene expression required E2F1 activity indicating that a feedback loop exists for restricting miRNA expression and controlling cell cycle events similar to p53 regulation [249].

1.4.4. miR-16 regulates chronic inflammatory diseases

Recent findings highlight the importance of miR-16 in playing a key role in modulating inflammatory diseases. Though several studies reported the expression of miR-16 in immune cells, for the first time the role of miR-16 was identified to be associated with inflammation by Li *et al.* in 2010 where they demonstrated that during monocyte-macrophage differentiation miR-16 was down-regulated with concomitant increase in IKKα expression levels. They have further illustrated that during differentiation, in order to prevent macrophage hyperactivation miR-16 was found to inhibit non-canonical NF-κB transactivation by directly targeting IKKα and thereby modulating TRAF, NIK and P52 expression. Thus they corroborate that miR-16 differentially regulates inflammatory responses in naive and stimulated macrophages in order to control macrophage hyperactivation which can progressively damage the tissue [250]. Further Shin *et al.* in

the year 2011 have shown that miR-16 expression is dependent on NF-κB binding on its promoter sites [251]. Thus the above two studies signify that miR-16 and NF-κB expressions are regulated by each other in a feedback fashion. Multiple findings suggested that in LPS treated monocytes or macrophages, miR-16 was able to down-regulate TLR4, IRAK-1, TNF-α, IL-6 etc. expression indicating that miR-16 negatively regulates inflammatory responses [252]. Conversely Zhou *et al.* have shown that LPS triggers miR-16 expression in MAPK dependent and NF-κB independent manner in U937 cells and positively aids in inflammation by transactivating IL-8, IL-6 and IL-1α [253]. Additionally miR-16 expression levels were observed to be remarkably up regulated in inflammatory diseases; Crohn's disease and ulcerative colitis and similar trend was also observed in rheumatoid arthritis [254-256]. However in patients with early rheumatoid arthritis, low levels of miR-16 was observed in their sera indicating that they can serve as diagnostic biomarkers which change over the period and monitoring of which might be helpful in predicting the disease outcome [257]. Correspondingly independent reports have shown that miR-16 expression levels are rigorously up-regulated in both adult and neonatal sepsis respectively [258, 259]. Similarly several reports demonstrated differential expression of miR-16 under various conditions such as LPS, LTA etc. in various cell types but their functional roles have not been clearly elucidated till date [260, 261]. Besides the above mentioned observations, miR-16 expression levels were found to be decreased in retinal endothelial cells (RECs) cultured in hyperglycemic conditions and ectopic expression of miR-16 decreased TNF-α and SOCS3 which consequently increased IGFBP3 (Insulin like growth factor binding protein) clearly suggesting that miR-16 can prevent insulin resistance during

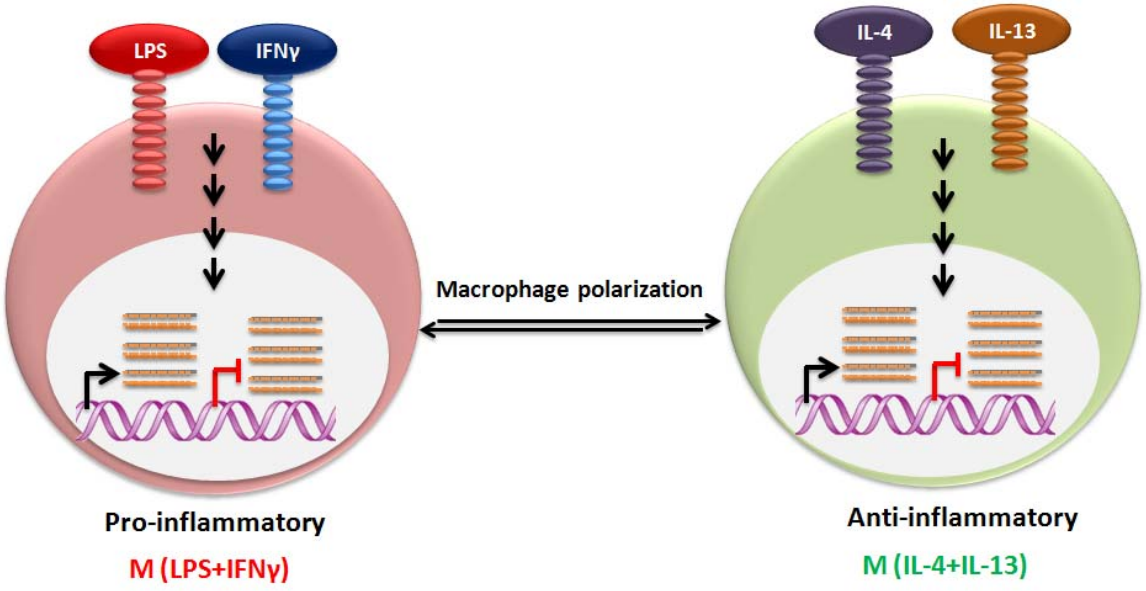
hyperglycemic conditions [262]. Likewise Lee *et al.* in the year 2015 have demonstrated that miR-16 levels were down-regulated in insulin resistant rat models and over-expression of miR-16 resulted in controlled protein accretion in skeletal muscle thus establishing its role in muscle development [263].

1.4.5. Neurodegenerative disease

miR-16 expression levels were found to be decreased in primary neurons derived from Alzheimer's disease rats (AD). Over-expression of miR-16 signified that it can prevent apoptosis and improve cell viability by targeting 3'UTR of AAP (Amyloid precursor protein) which is generally deposited in the diseased brains thus contributing to AD [264]. On the contrary miR-16 is known to promote Parkinson's disease by targeting Hsp70 which is responsible for proper folding of α-synuclein, accumulation of which leads to progression of Parkinson's disease. Though the role of Hsp70 in α-synuclein aggregation is poorly understood, its regulation by miR-16 is revealed for the first time in this paper [265].

Chapter 2

MicroRNAs are differentially modulated in M (LPS+IFNγ) and M (IL-4+IL-13) polarized macrophages



MicroRNAs are differentially modulated in M (LPS+IFNγ) and M (IL-4+IL-13) macrophages. Macrophages when polarized during LPS+IFNγ or IL-4+IL-13 treatments, display characteristic microRNA signature where certain microRNAs are up-regulated while some other microRNAs are down-regulated. The differentially modulated microRNAs in turn regulate macrophage inflammatory responses.

Significance of work:

Understanding the role of microRNAs in the regulation of macrophage plasticity will likely permit us to decode the molecular basis of macrophage polarization. This may help to devise novel therapeutic strategies to treat diseases such as cancers, metabolic disorders etc. which are associated with the dys-regulated macrophage polarization. The overall objective of the proposed study is to gain insights into the molecular mechanisms that govern macrophage plasticity.

Towards the identification of microRNA signature of LPS+IFNγ and IL-4+IL-13 activated macrophages, we first optimized the conditions for stimulating RAW 264.7 cells (a frequently used macrophage cell line) to study macrophage plasticity.

2.1. LPS+IFNγ and IL-4 are potent inflammatory insults

Stimulation of macrophage with 1 μg/ml of LPS and 100 ng/ml of IFNγ resulted in enhanced expression of several M (LPS+IFNγ) markers: iNOS (an enzyme involved in catalyzing nitric oxide (NO) production from arginase) (Figure 2.1A), cytokines (IRF-1, IL-6 and TNF-α) (Figure 2.1B) and ROS generation (Figure 2.1C) in LPS+IFNγ exposed macrophages suggesting that the conditions were optimal for the polarization of macrophages by LPS and IFNγ towards M (LPS+IFNγ) phenotype. Moreover, IL-4 treated macrophages displayed enhanced expression of arginase (ARG-1), mannose receptor C type lectin (MRC-1), YM-1 and IL-10 expression that are the characteristic of repair capable anti-inflammatory macrophages (Figure 2.1D).

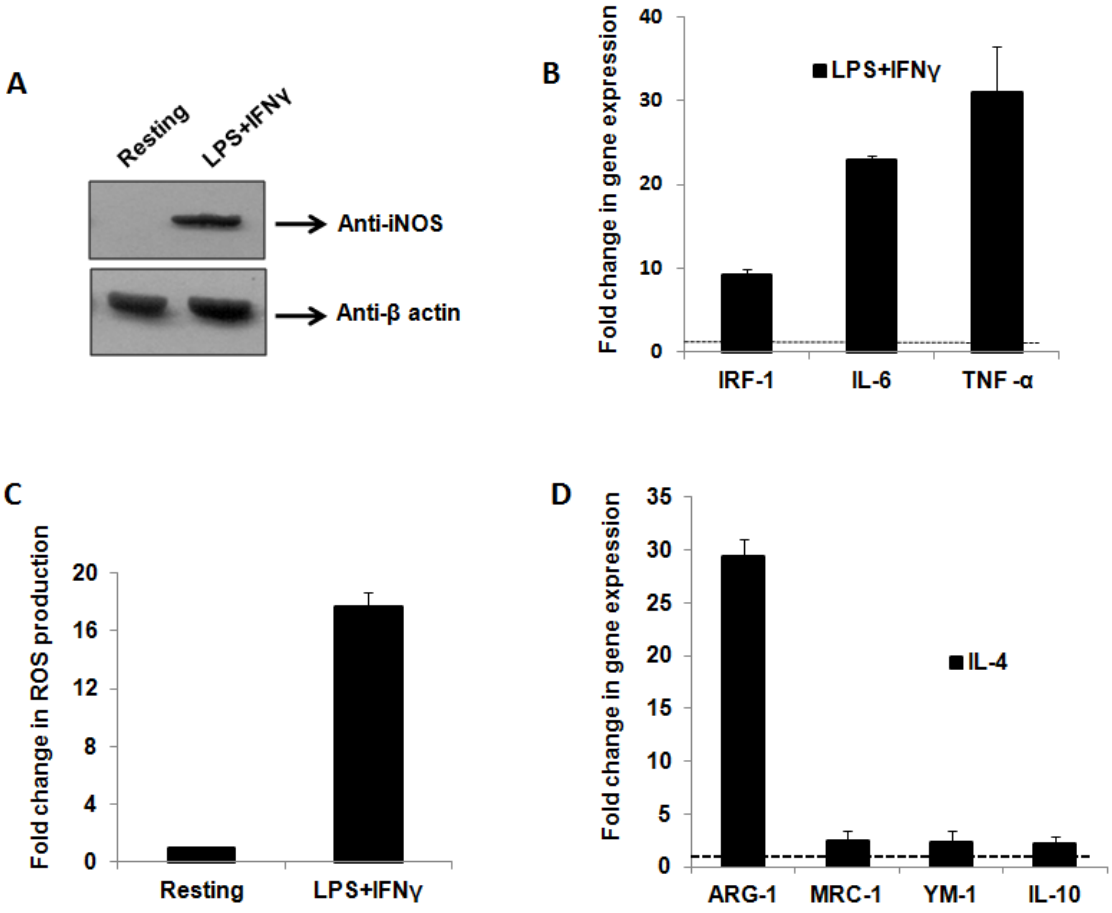


Figure 2.1. LPS and IFNγ induce iNOS expression, pro-inflammatory cytokine secretion, reactive oxygen species production while IL-4 enhances anti-inflammatory gene expression. RAW 264.7 cells were left un-stimulated or stimulated with LPS+IFNγ for 10 h and (A) cell lysates were analyzed by immunoblotting with iNOS antibody and β-actin antibody as the loading control. (B) Cells were lysed in TRIzol and cDNA was used for determining gene expression. β-actin was used as the control gene for normalization. Data shown are mean ± SD. (C) Post 24 h of treatment with LPS+IFNγ, amount of ROS in the cell lysates were measured by fluorometry using DCF-DA. Data shown are mean ± SD. RAW 264.7 cells were left un-stimulated or stimulated with IL-4+IL-13 for 10 h and cells were lysed in TRIzol and cDNA was used for determining gene expression. β-actin was used as the control gene for normalization.

2.2. MicroRNAs are differentially modulated in polarized macrophages

To study the differential regulation of microRNAs in polarized macrophages, RAW 264.7 cells were exposed to LPS+IFNγ or IL-4+IL-13 for 24 h or left untreated and the microRNAs were analyzed using Affymetrix chip array 2. The results are presented in Figure 2.2. The experiment was performed in duplicates and the expression pattern was similar in both the replicates under each treatment and the data shown is an average of both the replicate sets. In all the graphs, 0 indicates baseline value (control), miRs with values >0 are considered as over-expressed and <0 are considered as down-regulated. Wherever applicable, over-expressed, down-regulated and normally expressed miRs are represented in red, blue and yellow colors, respectively. miRs with fold change greater than 1.5 are considered to be differentially regulated.

The data presented in Figure 2.2A-2.2C show that several microRNA were differentially expressed in polarized macrophages. We observed that many miRNAs were differentially regulated by LPS and IFNγ treatment compared to IL-4 and IL-13 treatment. Since numbers of microRNAs are too high, the quality control of data is performed by using principal component analysis (PCA) which reduces the variance in microRNA population by removing number of unreliable samples from the replicates across all the three treatments (Figure 2.2C). Correspondingly hierarchical clustering (HCL) analysis was performed to identify microRNAs that were similar across given treatments. The cluster entities grouped microRNAs into single unit because of similar type of expression pattern. This method employs agglomerative approach (bottom up) where microRNAs with similar expression profiles are joined together to form a group which subsequently form clusters and several pairs of clusters in turn are merged to form a dendrogram which

is an indicative of relative microRNAs (Figure 2.2D). Further, differentially regulated miRNAs are plotted using volcano plots which plots fold change versus statistical significance. In these graphs, miRs whose fold change and p values are ≥ 1.5 and ≤ 0.05, respectively, are considered to be significant (Figure 2.2E-2.2G).

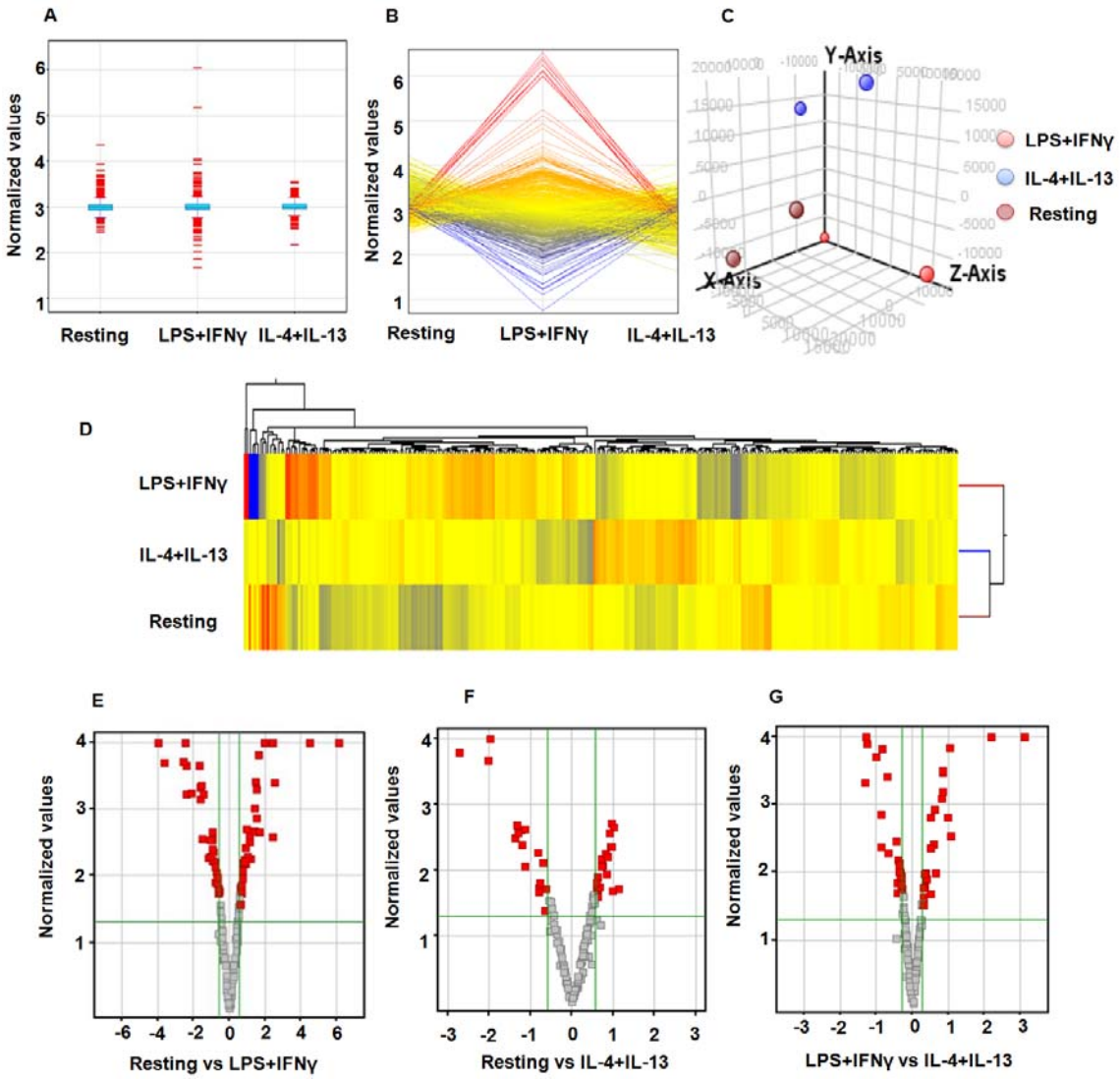


Figure 2.2. MicroRNAs are differentially modulated in polarized macrophages. The differential expression pattern of microRNAs in polarized macrophages is identified using following graphical analysis. (A) Box- and-Whisker plot, (B) Profile plot, (C) PCA analysis, (D) HCL plot and (E-G) Volcano plot

2.3. Total number of microRNAs modulated in M (LPS+IFNγ) and M (IL-4+IL-13) macrophages

Our microarray profiling data identified 1407 microRNAs across all the three sample groups and about 221 microRNAs were found to be differentially expressed during LPS+IFNγ stimulation, of which 107 miRs were up-regulated and 114 miRs were found to be down-regulated. Further, 155 miRNAs were observed to exhibit alterations in their expression levels upon IL-4+IL-13 treatment of which 76 miRNAs were found to be up-regulated and 79 miRNAs were down-regulated (Figure 2.3A). Next, we have identified 83 microRNAs which exhibited similar trend in their expression patterns in both the groups of activated macrophages. Among them, 32 miRNAs were commonly observed to be up-regulated and 51 miRNAs were commonly down-regulated (Figure 2.3B, 2.3C). Additionally we observed that only two microRNAs; miR-22* and miR-125b* displayed contrasting expression levels under both the polarizing conditions (Table 2.1).

Consistent with the literature, we have observed several microRNAs were modulated under inflammatory conditions in our profiling study (Table 2.2). Top 25 microRNAs which exhibited alterations in their expression patterns under both the conditions are listed in the following tables (Table 2.3-2.6).

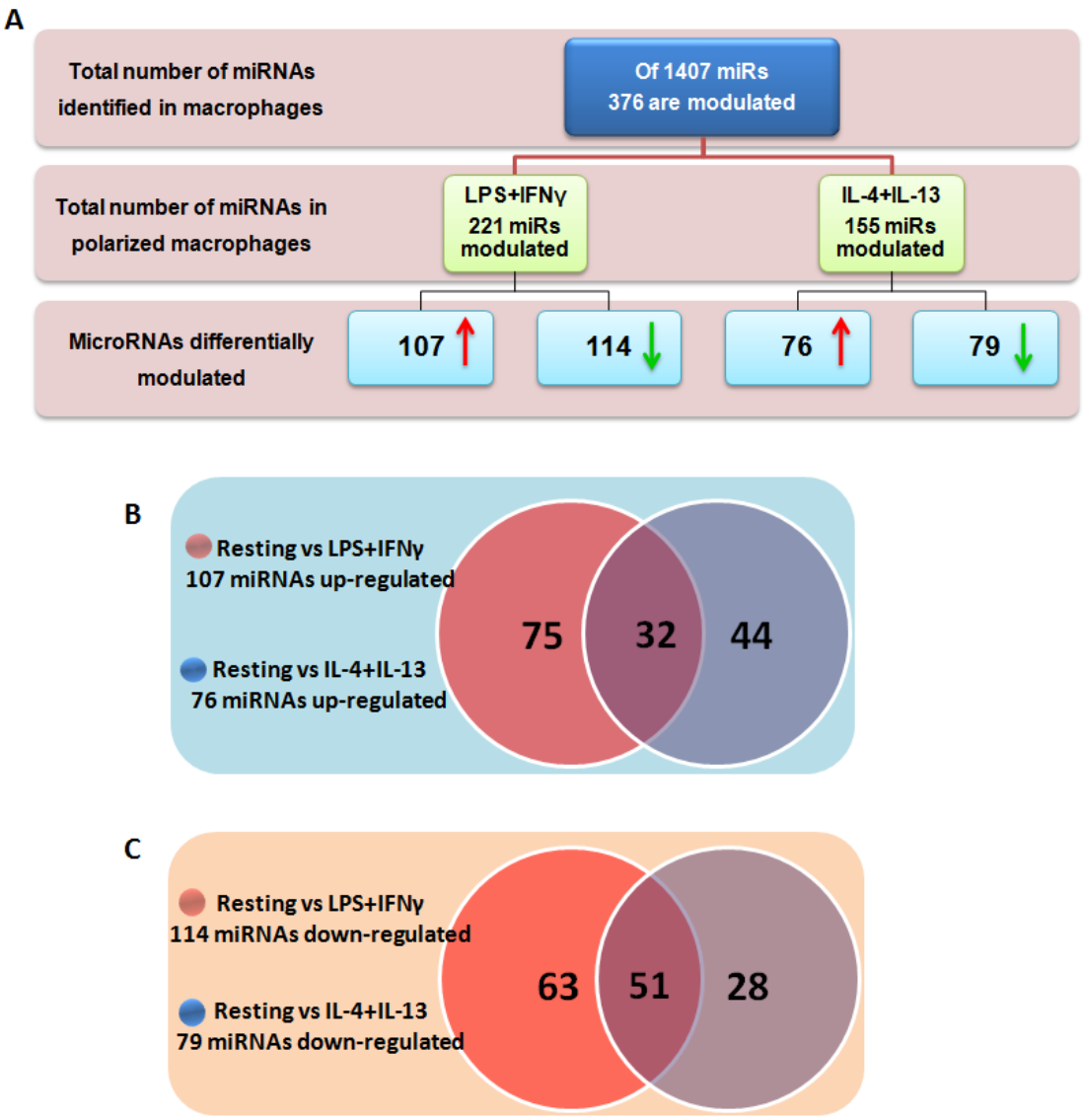


Figure 2.3. Schematic of total number of microRNAs differentially regulated in polarized macrophages.

(A) Overview of total microRNA signature in LPS+IFNγ and IL-4+IL-13 polarized macrophages.

(B) Venn diagram representing total number of up-regulated and commonly regulated microRNAs in LPS+IFNγ and IL-4+IL-13 polarized macrophages.

(C) Venn diagram representing total number of down-regulated and commonly regulated microRNAs in LPS+IFNγ and IL-4+IL-13 polarized macrophages

Table 2.1. MicroRNAs displaying opposite trend in their expression pattern under LPS+IFNγ and IL-4+IL-13 treatment

S.No	MicroRNA	LPS+IFNγ	IL-4+IL-13
1	mmu-miR-22-star	2.0	-1.7
2	mmu-miR-125b-star	-1.5	2.2

Table 2.2. MicroRNAs modulated in this study are differentially regulated under various inflammatory insults

S.No	MicroRNAs altered	Polarizing condition	Trend	Cell type	Reference
1	miR-155	M (LPS+IFNγ)	Increase	Human MDMs	Graff *et al.* 2012 [266]
2	miR-147	M (LPS)	Increase	Mouse peritoneal macrophages	Liu G *et. al* 2009 [267]
3	miR-149	M (LPS)/ M (BCG)	Decrease	RAW 264.7	Xu G *et. al* 2014 [268]
4	miR-146a	M (LPS)	Increase	RAW 264.7	Schulte *et al.* 2011 [269]
5	miR-146b	M (LPS)	Increase	THP-1	Taganov D. *et. al* 2006 [270]
6	miR-125a-5p	M (LPS)	Increase	PMA differentiated THP-1	Graff *et al.* 2012 [266]
7	miR-125a-3p	M (LPS+IFNγ)	Increase	Human MDMs	Graff *et al.* 2012 [266]
8	miR-204	M (LPS+IFNγ)	Increase	Mouse BMDMs	Zhang *et al.* 2013 [271]
9	miR-27a-star	M (IgG+LPS)	Increase	Human MDMs	Graff *et al.* 2012 [266]
10	miR-181a	M (LPS+IFNγ)	Increase	Mouse BMDMs	Zhang *et al.* 2013 [271]

Table 2.3. Top 25 microRNAs that are up-regulated in LPS+IFNγ polarized macrophages. hp indicates precursor microRNA.

S.No	MicroRNA	Fold increase during LPS+IFNγ treatment
1	mmu-miR-155	68.8
2	hp_mmu-mir-155	22.7
3	mmu-miR-679	5.8
4	mmu-miR-147	5.3
5	mmu-miR-713	5.3
6	mmu-miR-21-star	3.8
7	mmu-miR-700	3.6
8	hp_mmu-mir-692-2_x	3.1
9	mmu-miR-1186b	3.1
10	mmu-miR-149	3.0
11	hp_mmu-mir-692-1_s	3.0
12	mmu-miR-1937a	2.9
13	mmu-miR-669h-3p	2.9
14	mmu-miR-3473	2.9
15	mmu-miR-3470a	2.8
16	mmu-miR-210	2.8
17	mmu-miR-1937b	2.8
18	hp_mmu-mir-2135-5_x	2.7
19	mmu-miR-146a	2.7
20	mmu-miR-146b	2.6
21	mmu-miR-125a-5p	2.6
22	mmu-miR-1274a	2.5
23	mmu-miR-1937c	2.3
24	mmu-miR-207	2.2
25	mmu-miR-291b-5p	2.2

Table 2.4. Top 25 microRNAs that are up-regulated in IL-4+IL-13 polarized macrophages

S.No	MicroRNA	Fold increase during IL-4+IL-13 treatment
1	mmu-miR-467b-star	2.45
2	mmu-miR-1964	2.24
3	mmu-miR-125b-star	2.18
4	mmu-miR-188-5p	2.08
5	hp_mmu-mir-872	2.07
6	mmu-miR-532-3p	2.03
7	mmu-miR-491	2.01
8	hp_mmu-mir-692-2_x	2.00
9	mmu-miR-501-5p	1.96
10	mmu-miR-330-star	1.96
11	mmu-miR-15a-star	1.93
12	mmu-miR-324-3p	1.91
13	mmu-miR-1981	1.90
14	mmu-miR-1274a	1.89
15	hp_mmu-mir-2135-5_x	1.86
16	hp_mmu-mir-758_x	1.86
17	mmu-miR-1939	1.85
18	mmu-miR-1937c	1.85
19	mmu-miR-2133	1.84
20	hp_mmu-mir-469	1.83
21	mmu-miR-378-star	1.82
22	hp_mmu-mir-485	1.80
23	mmu-miR-1937b	1.80
24	mmu-miR-194	1.80
25	hp_mmu-mir-692-2_s	1.80

Table 2.5. Top 25 microRNAs that are down-regulated in LPS+IFNγ polarized macrophages

S.No	MicroRNA	Fold decrease during LPS+IFNγ treatment
1	mmu-miR-27a-star	16.13
2	mmu-miR-2136	12.57
3	mmu-miR-712	6.08
4	mmu-miR-374	5.73
5	mmu-miR-30c-2-star	5.49
6	mmu-miR-675-5p	5.33
7	mmu-miR-27b-star	5.31
8	mmu-miR-19a	4.53
9	mmu-miR-615-5p	4.46
10	mmu-miR-1902	4.35
11	mmu-miR-290-5p	4.32
12	mmu-miR-135a-star	3.95
13	mmu-miR-30e-star	3.89
14	mmu-miR-30a-star	3.78
15	mmu-miR-26b	3.70
16	mmu-miR-451	3.34
17	mmu-miR-877-star	3.20
18	mmu-miR-20a-star	3.12
19	mmu-miR-322-star	3.09
20	mmu-miR-200b-star	3.08
21	mmu-miR-130a	3.03
22	mmu-miR-695	3.02
23	mmu-miR-1945	2.99
24	mmu-miR-93-star	2.82
25	mmu-miR-2132	2.79

Table 2.6. Top 25 microRNAs that are down-regulated in IL-4+IL-13 polarized macrophages

S.No	MicroRNA	Fold decrease during IL-4+IL-13 treatment
1	mmu-miR-2136	6.62
2	mmu-miR-26b	5.08
3	mmu-miR-130a	4.93
4	mmu-miR-675-5p	4.09
5	mmu-miR-695	3.99
6	mmu-miR-29b	3.93
7	mmu-miR-451	3.60
8	mmu-miR-374	3.34
9	mmu-miR-322	2.87
10	mmu-miR-223	2.81
11	mmu-miR-29c	2.59
12	mmu-miR-27a-star	2.57
13	mmu-miR-19a	2.52
14	mmu-miR-678	2.52
15	mmu-miR-669j	2.51
16	mmu-miR-872-star	2.47
17	mmu-miR-301b	2.45
18	mmu-miR-32	2.36
19	mmu-miR-200b-star	2.36
20	mmu-miR-186	2.33
21	mmu-miR-301a	2.30
22	mmu-miR-21	2.29
23	mmu-miR-1945	2.19
24	mmu-miR-1957	2.18
25	mmu-miR-322-star	2.14

2.4. Validation of microarray profiling results using miR-155 as reference microRNA by qPCR analysis

We have validated microarray data by performing qPCR analysis primarily using miR-155 as the reference miRNA as it is a well studied microRNA in macrophage inflammatory responses (Figure 2.4A). Consistent with the literature and profiling studies, we noted that miR-155 expression was robustly up-regulated in M (LPS+IFNγ) macrophages (Figure 2.4B). We have additionally validated its expression levels in primary bone marrow derived macrophages (BMDMs) where we observed that miR-155 levels were significantly up-regulated upon LPS+IFNγ treatment in primary macrophages as observed in RAW 264.7 cell line (Figure 2.4C). However we did not observe any alterations in miR-155 expression upon IL-4+IL-13 treatment (Figure 2.4D, 2.4E). The gene expression for all the miRNAs were normalized using RNU6 control gene. Further, analysis of the PCR products on agarose gel displayed miR-155 (~70 bp) and U6 (~97bp) amplicons at their respective molecular weights (Figure 2.4F) suggesting that miRNAs and control gene U6 are specifically amplified.

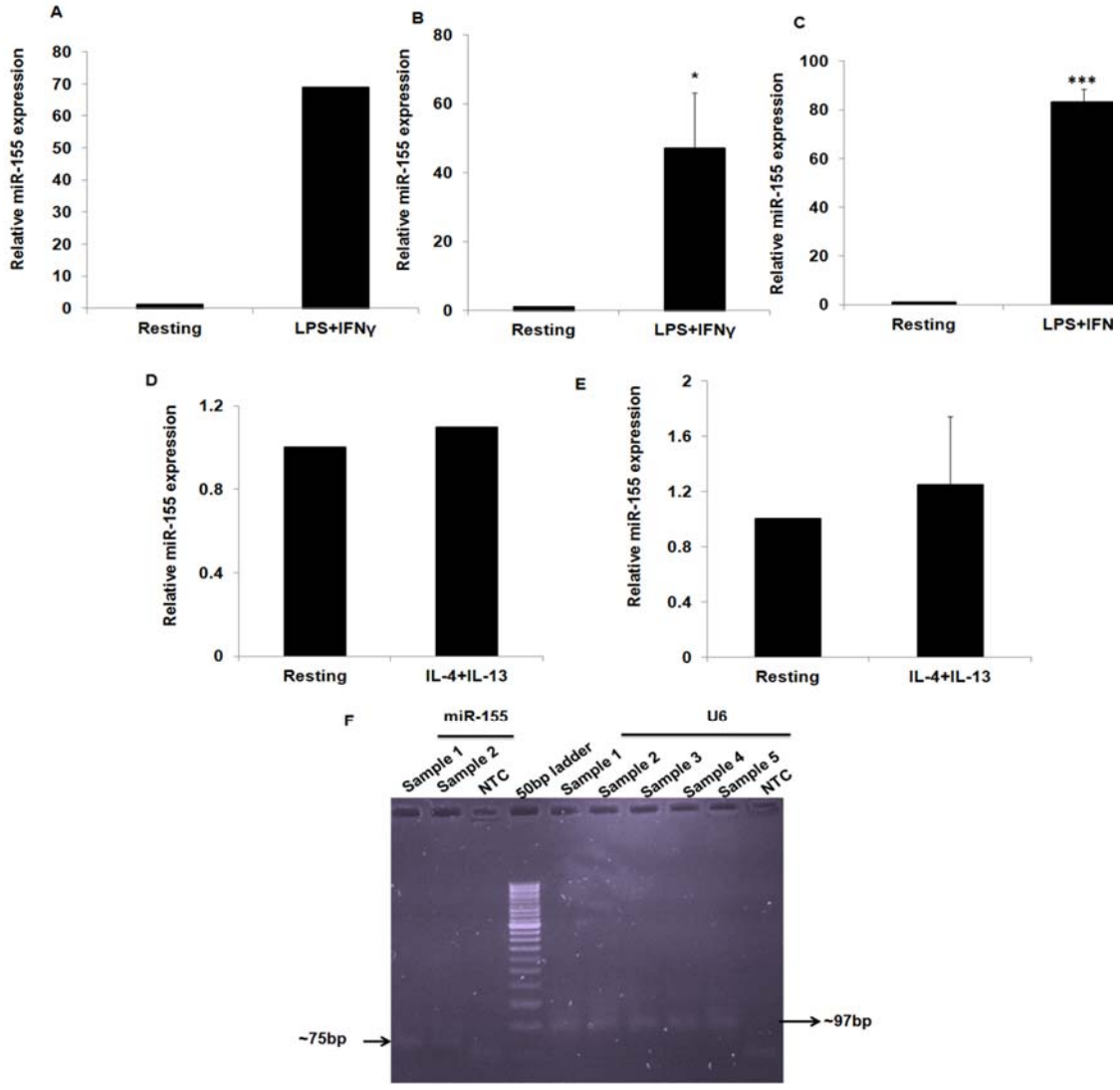


Figure 2.4. miRNA-155 expression is up-regulated in M (LPS+IFNγ) polarized macrophages, while no significant change was observed in M (IL-4+IL-13) polarized RAW 264.7 cells. (A) RAW 264.7 macrophage cells were stimulated with LPS+IFNγ and (D) IL-4+IL-13 for 24 h and stimulated RAW 264.7 samples were outsourced for Affymetrix chip based microRNA analysis. (B) Macrophages and (C) BMDMs after treatment with LPS+IFNγ and (E) macrophages treated with IL-4+IL-13 stimuli for indicated time points were lysed in TRIzol and cDNA was used for determining miRNA expression and data was normalized to control U6. Data shown are a representative of three independent experiments (Mean ± SEM). (F) Post qPCR, amplified products were run on agarose gel.

Having validated the profiling results, we primarily focused on functionally novel microRNAs. We identified around 101 and 51 functionally novel miRNAs in LPS+IFNγ and IL-4+IL-13 polarized macrophages, respectively. Among them 62 miRs were found to be up-regulated and 39 were down-regulated in LPS+IFNγ polarized macrophages (Table 2.7) and similarly in IL-4+IL-13 polarized macrophages 32 miRs were up-regulated and 19 were down-regulated (Table 2.8). When we have initiated this work not even a single report was available on the functional roles of these novel miRs. Amongst them we observed that 21 novel microRNAs were commonly up-regulated in both the treatments and 16 miRs were commonly down-regulated (Table 2.8). Of all the miRs identified during our study, we have selected miR-712 and miR-16 for further analysis (Table 2.9).

Table 2.7. MicroRNAs identified to be differentially regulated in LPS+IFNγ polarized macrophages for the first time in this study (functionally novel). Italicized microRNAs represent down-regulated miRs in LPS+IFNγ polarized macrophages

S.No	Novel microRNAs in LPS+IFNγ polarized macrophages	S.No	Novel microRNAs in LPS+IFNγ polarized macrophages	S.No	Novel microRNAs in LPS+IFNγ polarized macrophages	S.No	Novel microRNAs in LPS+IFNγ polarized macrophages
1	mmu-miR-679	26	mmu-miR-1964	51	mmu-miR-2146	76	*mmu-miR-2183*
2	mmu-miR-713	27	mmu-miR-669f	52	mmu-miR-672	77	*mmu-miR-1967*
3	mmu-miR-700	28	hp_mmu-mir-3470b_x	53	hp_mmu-mir-1944	78	*mmu-miR-25*
4	mmu-miR-1186b	29	mmu-miR-690	54	hp_mmu-mir-1937b-5_s	79	*mmu-miR-546*
5	hp_mmu-mir-692-1_s	30	hp_mmu-mir-466h_x	55	hp_mmu-mir-1195	80	*mmu-miR-667*
6	mmu-miR-1937a	31	hp_mmu-mir-466f-3_x	56	hp_mmu-mir-653	81	*mmu-miR-450b-5p*
7	mmu-miR-3473	32	hp_mmu-mir-1957	57	hp_mmu-mir-669d_x	82	*mmu-miR-1930*

8	mmu-miR-3470a	33	hp_mmu-mir-485	58	mmu-miR-330-star	83	*mmu-miR-1306_s*
9	mmu-miR-1937b	34	mmu-miR-466h	59	hp_mmu-mir-1934	84	*hp_mmu-mir-693_x*
10	mmu-miR-669h-3p	35	mmu-miR-1907	60	hp_mmu-mir-3473	85	*mmu-miR-760*
11	hp_mmu-mir-2135-5_x	36	mmu-miR-466f-5p	61	hp_mmu-mir-1937b-3	86	*mmu-miR-327*
12	mmu-miR-1274a	37	mmu-miR-466f	62	mmu-miR-676	87	*mmu-miR-1893*
13	mmu-miR-1937c	38	hp_mmu-mir-700	63	*mmu-miR-2137*	88	*mmu-miR-669j*
14	mmu-miR-207	39	mmu-miR-1943	64	*mmu-miR-1957*	89	*mmu-miR-678*
15	mmu-miR-291b-5p	40	hp_mmu-mir-874	65	*mmu-miR-669i*	90	*hp_mmu-mir-1941*
16	mmu-miR-720	41	mmu-miR-1894-5p	66	*mmu-miR-718*	91	*mmu-miR-877*
17	mmu-miR-875-3p	42	mmu-miR-574-3p	67	*mmu-miR-668*	92	*mmu-miR-1968*
18	hp_mmu-mir-692-2_s	43	mmu-miR-2135	68	*mmu-miR-1199*	93	*mmu-miR-872-star*
19	mmu-miR-3470b	44	mmu-miR-3472	69	*mmu-miR-1956*	94	*mmu-miR-2132*

20	hp_mmu-mir-466f-1_x	45	hp_mmu-mir-1196	70	*hp_mmu-mir-540*	95	*mmu-miR-1945*
21	hp_mmu-mir-466k_x	46	hp_mmu-mir-466j_x	71	*hp_mmu-mir-680-3*	96	*mmu-miR-695*
22	hp_mmu-mir-692-1_x	47	hp_mmu-mir-1953	72	*hp_mmu-mir-669l*	97	*mmu-miR-877-star*
23	hp_mmu-mir-1946a	48	mmu-miR-466i	73	*hp_mmu-mir-695*	98	*mmu-miR-1902*
24	hp_mmu-mir-466f-2_x	49	mmu-miR-712-star	74	*mmu-miR-3474*	99	*mmu-miR-615-5p*
25	mmu-miR-466j	50	hp_mmu-mir-873	75	*hp_mmu-mir-219-1*	100	*mmu-miR-712*
						101	*mmu-miR-2136*

Table 2.8. MicroRNAs identified to be differentially modulated in IL-4+IL-13 polarized macrophages for the first time in this study (functionally novel). Italicized microRNAs represent down-regulated miRs in IL-4+IL-13 polarized macrophages and shaded miRs represent commonly up-regulated or down-regulated miRs with respect to LPS+IFNγ microRNA signature.

S.No	Novel microRNAs in IL-4+IL-13 polarized macrophages	S.No	Novel microRNAs in IL-4+IL-13 polarized macrophages
1	mmu-miR-1964	27	hp_mmu-mir-1904
2	hp_mmu-mir-692-2_x	28	hp_mmu-mir-1946a
3	mmu-miR-330-star	29	mmu-miR-2135
4	mmu-miR-1981	30	mmu-miR-568
5	mmu-miR-1274a	31	mmu-miR-466f-5p
6	hp_mmu-mir-2135-5_x	32	hp_mmu-mir-692-1_s
7	mmu-miR-1939	33	*mmu-miR-1960*
8	mmu-miR-1937c	34	*hp_mmu-mir-219-1*
9	mmu-miR-2133	35	*mmu-miR-2132*
10	hp_mmu-mir-485	36	*mmu-miR-877*
11	mmu-miR-1937b	37	*mmu-miR-668*
12	hp_mmu-mir-692-2_s	38	*mmu-miR-1306*
13	mmu-miR-3472	39	*mmu-miR-615-5p*
14	mmu-miR-712-star	40	*mmu-miR-450b-5p*
15	hp_mmu-mir-1956	41	*mmu-miR-1897-5p*
16	mmu-miR-1937a	42	*mmu-miR-1962*
17	mmu-miR-574-3p	43	*hp_mmu-mir-1941*
18	hp_mmu-mir-1944	44	*mmu-miR-1902*
19	hp_mmu-mir-692-1_x	45	*mmu-miR-1957*
20	hp_mmu-mir-3470b_x	46	*mmu-miR-1945*
21	mmu-miR-720	47	*mmu-miR-872-star*
22	mmu-miR-1894-5p	48	*mmu-miR-669j*
23	mmu-miR-1198	49	*mmu-miR-678*
24	hp_mmu-mir-1963	50	*mmu-miR-695*
25	mmu-miR-1934	51	*mmu-miR-2136*
26	mmu-miR-1955		

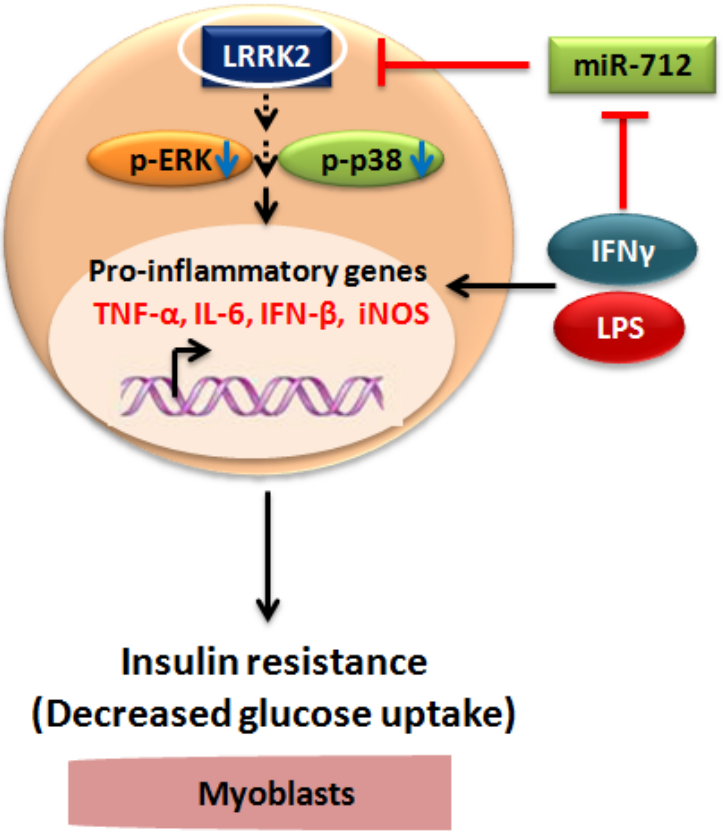
Table 2.9. miR-16 and miR-712 are selected for further analysis using miR-155 as a reference microRNA

S.No	MicroRNA	Modulation in LPS+IFNγ (fold)	Modulation in IL-4+IL-13 (fold)	Remarks	Mature microRNA sequence
1	miR-155	68.8	-1.1	Multifunctional miRNA: cancers, inflammation, immunity, hematopoietic (Faraoni et al. 2009) [272]	TTAATGCTAATTGTGA TAGGGGT
2	miR-712	-6.08	-1.04	Functional role is not studied	CTCCTTCACCCGGGCG GTACC
3	miR-16	-1.58	-1.32	Well studied in cancers. Role in sepsis, diabetes, arthritis but mechanism is poorly defined [232, 256, 259, 273]	TAGCAGCACGTAAATA TTGGCA

2.5. Summary

Overall we identified 1407 microRNAs from our profiling results, of which 376 (~26%) were observed to be differentially regulated during macrophage polarization. Among them, total of 152 microRNAs were found to be novel and we have selected miR-712 and miR-16 for further analysis after validating profiling results using miR-155 as a control microRNA using qPCR analysis (Table 2.9).

Chapter 3: MicroRNA-712 attenuates M (LPS+IFNγ) mediated inflammation induced insulin resistance in skeletal myoblasts



MicroRNA-712 suppresses macrophage pro-inflammatory responses and restores myoblast insulin sensitivity during chronic inflammation. miR-712 expression is decreased in macrophages during inflammatory conditions, while ectopic expression of miR-712 targets LRRK2 mRNA and reduces phosphorylation of ERK1/2 and p38 in macrophages and this results in attenuation of inflammation induced insulin resistance in skeletal myoblasts suggesting its pivotal role in regulating inflammatory responses and insulin dependent glucose metabolism.

MicroRNA-712 being functionally novel and significantly modulated during inflammatory LPS+IFNγ stimulation, there was a necessity to study its precise role during macrophage activation. Although it was very recently found to have a role in inducing atherosclerosis, we were interested in identifying its functions in macrophage polarization and associated diseases such as insulin resistance. Therefore in order to delineate its role in macrophage mediated inflammation, we performed gain-of-function experiments in macrophages and observed that unlike its causative role in atherosclerosis in endothelial cells, miR-712 expression was able to dampen macrophage pro-inflammatory responses thus restoring insulin sensitivity in skeletal myoblasts.

3.1. miR-712 is ubiquitously expressed in mice tissues

miR-712 is a novel mouse specific microRNA and its tissue distribution is not known, thus we determined miR-712 expression pattern in various tissues of mice by qPCR analysis (Figure 3.1A). Specific miRNA-712 amplicons were detected in all the tissues tested. These results suggest that miR-712 is probably ubiquitously expressed (Figure 3.1B).

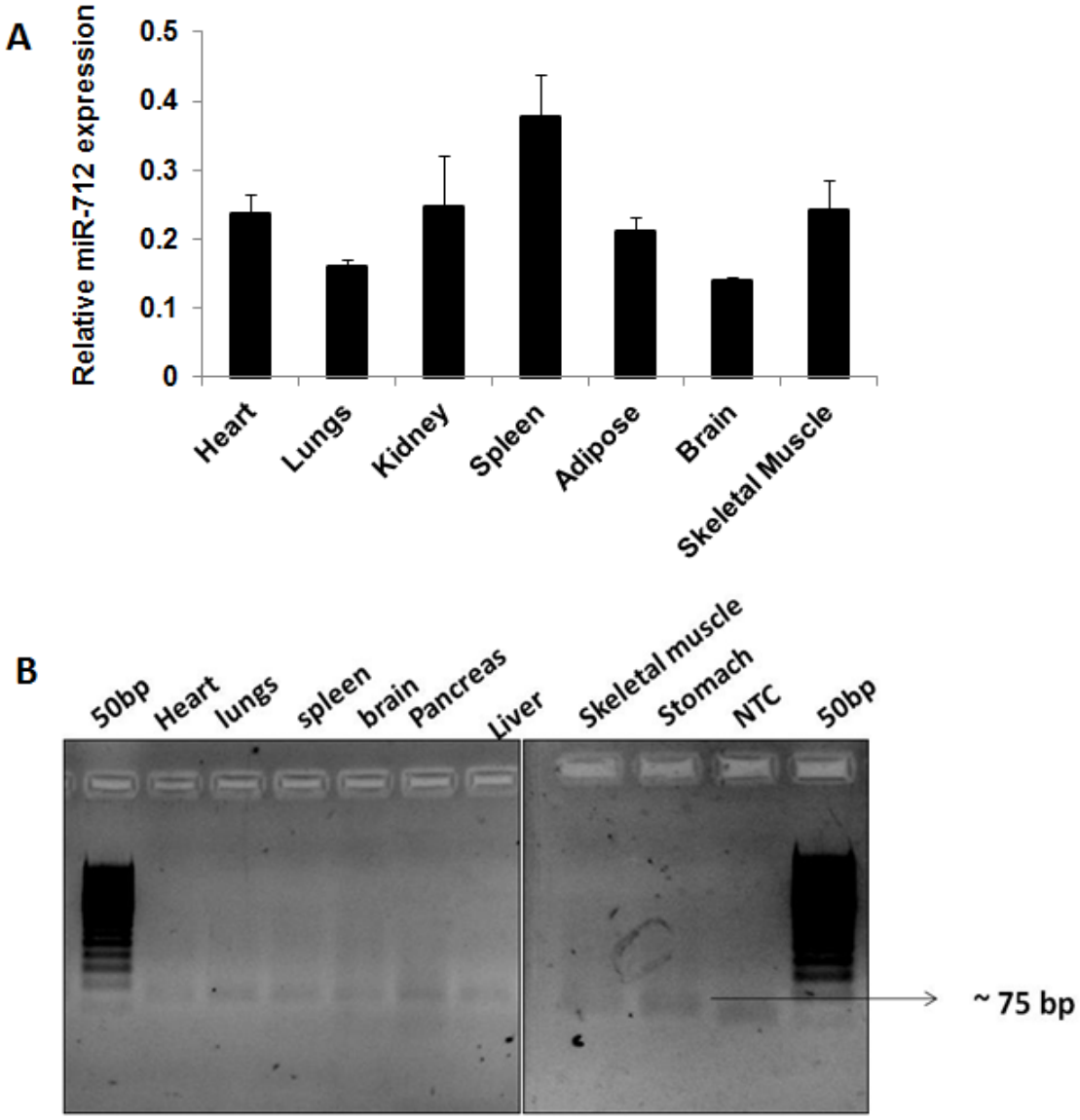


Figure 3.1. miRNA-712 is ubiquitously expressed in mice tissues. (A) RNA was isolated from snap frozen tissues of C57/BL6 mice using trizol, cDNA was used for determining miRNA expression and data was normalized to control U6. Data shown are a representative of two mice (Mean ± SD). (B) Post qPCR run, samples were analyzed on agarose gel.

3.2. Functional analysis of miR-712 based on computational approach

The functionality of miR-712 is poorly understood, thus we carried out the pathway analysis of miR-712 using miRSystem tools to know the potential functional roles of miR-712. Our results suggested that miR-712 may have a major role in regulating inflammation as most of the genes being targeted by this microRNA are linked to macrophage driven inflammatory and infectious pathways while few other targets are known to participate in metabolism (Table 3.1). When we have analyzed metabolic functions associated with macrophages, we observed that majority of the pathways affected by miR-712 were associated with macrophage functions. Similar results were observed using DIANA software, another computational tool.

3.3. miR-712 predicted to regulate macrophage associated inflammation, infections and metabolism

Pathway analysis revealed that miR-712 may putatively target genes which are majorly involved in both innate and adaptive immune responses. For instance, we observed that miR-712 may target multiple mRNAs whose protein products were reported to be involved in TLR signalling, phagocytosis, Fc receptor mediated functions, chemokines and cytokine pathways and B cell & T cell receptor signalling pathways (Table 3.1).

Further we noted that miR-712 may also influence pathways involved in parasitic infections such as malaria, amoebiasis, Chagas disease and toxoplasmosis. In addition we have also observed that it can putatively target genes regulating viral diseases such Hepatitis C and PRIONs (Table 3.1).

Furthermore *in silico* analysis revealed that miR-712 may repress genes involved in inflammatory diseases such as Cardiovascular, Alzheimer's, Parkinson's disease and

amyotrophic lateral sclerosis. These observations clearly suggested that miR-712 may have a crucial role in regulating immune responses, infections and inflammation (Table 3.1).

Additionally we have observed that miR-712 may target genes involved in insulin signalling and lipid metabolism suggesting that miR-712 may show a critical role in controlling metabolism and related diseases (Table 3.1).

Table 3.1. miR-712 is predicted to regulate macrophage associated inflammation, infections and metabolism. * The scores were obtained by dividing the expression levels of desired microRNA to all the miRNAs in a cell along with significance values [274].

S.No	Targeted pathway (KEGG)	Score*	Targets
1	TGF-beta signalling pathway	2.8	E2F5,GDF6, RBL2, SMAD5,SP1
2	Hepatitis C	1.9	CLDN11,GSK3B, PIAS2, SOS1, TRAF3
3	Insulin signalling pathway	1.9	GSK2B,PPP1R3A,RHO Q,SORBS1,SOS1
4	MAPK signalling pathway	1.8	DUSP7,FGF5,MAP2K4, PP3R1,SOS1,STK3,TAO K1
5	Bacterial invasion of epithelial cells	1.5	CLTC,DNM3,ITGA5
6	Arrhythmogenic right ventricular Cardiomyopathy (arvc)	1.5	GJA1,ITGA5,LMNA
7	B cell receptor signalling pathway	1.4	GSK3B,PPP3R1,SOS1
8	Prion diseases	1.3	LAMC1,NCAM1
9	Dilated cardiomyopathy	1.3	GNAS,ITGA5,LMNA
10	T cell receptor signalling pathway	1.1	GSK3B,PPP3R1,SOS1
11	Amoebiasis	1.1	COL4A1,GNAS,LAMC1
12	Inositol phosphate metabolism	1.0	INPP4A,INPPL1
13	Cell adhesion molecules_(cams)	0.9	CADM1,CLDN11,NCA M1
14	VEGF signalling pathway	0.8	VEGFA,PPP3R1
15	Phosphatidylinositol signalling system	0.8	INPP4A,INPPL1
16	PPAR signalling pathway	0.8	ACSL1,SORBS1
17	Fc epsilon RI signalling pathway	0.8	MAP2K4, SOS1
18	Huntington's disease	0.7	CLTC1, CREB1, SP1

19	Hypertrophic cardiomyopathy (hcm)	0.8	ITGA,LMNA
20	Alzheimer's disease	0.8	GSK3B,LRP1,PPP3R1
21	Fc gamma receptor mediated phagocytosis	0.8	DNM3,MARCKS
22	Toll-like receptor signalling pathway	0.7	MAP2K4,TRAF3
23	Chagas disease	0.7	MAP3K4,GNAS
24	Cytokine-cytokine receptor interaction	0.6	GDF6,VEGFA
25	Ether lipid metabolism	0.6	AGPS
26	Natural killer cell mediated cytotoxicity	0.6	PPP3R1,SOS1
27	JAK-STAT signalling pathway	0.6	PIAS2,SOS1
28	Chemokine signalling pathway	0.6	GSK3B,SOS1
29	Sphingolipid metabolism	0.5	SGMS1
30	Phagosome	0.5	ITGA5
31	Fatty acid metabolism	0.5	ACSL1
32	mTOR signalling pathway	0.5	VEGFA
33	Malaria	0.5	LRP1
34	Amyotrophic lateral sclerosis (als)	0.5	PP3R1
35	Parkinson's disease	0.5	LRRK2
36	Toxoplasmosis	0.5	LAMC1
37	Adipocytokine signalling pathway	0.5	ACSL1
38	Rig-I-like receptor signalling pathway	0.5	TRAF3
39	Leukocyte transendothelial migration	0.4	CLDN11
40	Antigen processing and presentation	0.4	CREB1
41	Arachidonic acid metabolism	0.4	GPX3

3.4. miR-712 is associated with different types of cancers

In addition to inflammation and metabolic disorders, bioinformatic analysis revealed that miR-712 may target genes that are linked to cell cycle, apoptosis and cancer. Since polarized macrophages were found to have a prominent role in tumour formation and cancer progression, we strongly anticipate that miR-712 has a regulatory role in controlling macrophage induced tumour development (Table 3.2). Top 20 putative pathways targeted by miR-712 are graphically represented in Figure 3.2.

Based on these observations we anticipate that miR-712 has a strong role in driving macrophage mediated inflammatory responses and we have initiated our studies by validating its expression levels in LPS+IFNγ polarized macrophages.

Table 3.2. miR-712 may target pathways involved in cell cycle and cancers. * The scores were obtained by dividing the expression levels of desired microRNA to all the miRNAs in a cell along with significance values.

S.No	Targeted pathway (KEGG)	Score*
1	Pathways in cancer	2.3
2	Small cell lung cancer	2.0
3	ErbB signalling pathway	2.0
4	Cell cycle	1.5
5	Prostate cancer	1.3
6	Endometrial cancer	1.1
7	Acute myeloid leukemia	1.0
8	Renal cell carcinoma	0.9
9	VEGF signalling pathway	0.8
10	Wnt signalling pathway	0.6
11	Bladder cancer	0.5
12	Notch signalling pathway	0.5
13	Hedgehog signalling pathway	0.5
14	Non-small cell lung cancer	0.5
15	Basal cell carcinoma	0.5
16	Colorectal cancer	0.5
17	Glioma	0.5
18	Pancreatic cancer	0.4
19	Melanoma	0.4
20	Chronic myeloid leukemia	0.4
21	Apoptosis	0.4

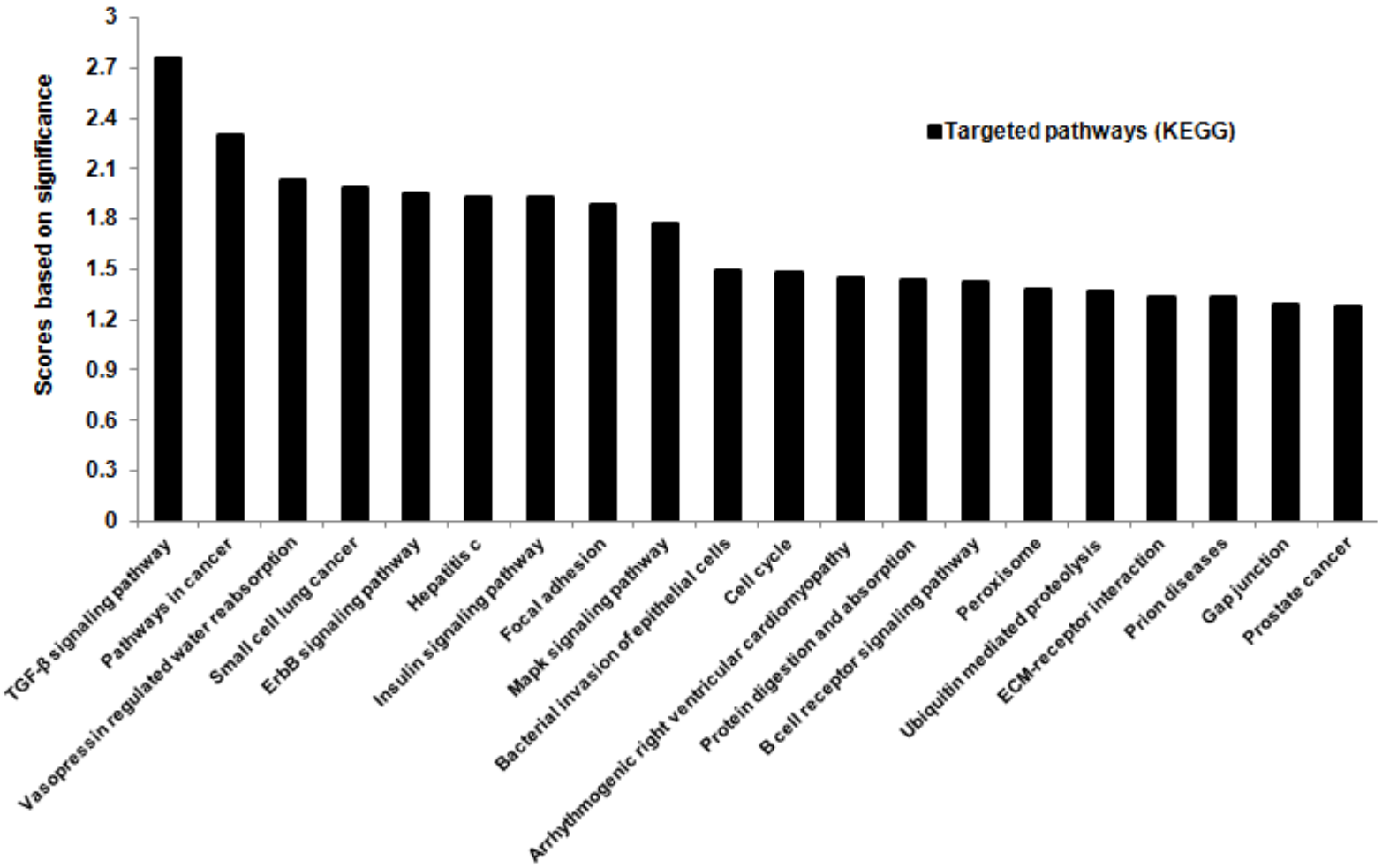


Figure 3.2. Top 20 putative pathways predicted to be targeted by miR-712. The microRNA-712 targeting pathways were predicted using miRSystem analysis tools. The scores were obtained by dividing the expression levels of desired microRNA to all the miRNAs (microRNA enrichment) in a cell along with significance values [274].

3.5. miR-712 is differentially regulated in LPS+IFNγ polarized macrophages

We validated microarray profiling results (Figure 3.3A, 3.3C) by initially studying the kinetics of miR-712 expression in LPS+IFNγ treated macrophages by qPCR analysis. Stimulation with LPS+IFNγ did not change the expression of miR-712 at 6 h and 12 h (Figure 3.3B). However, we observed that miR-712 levels were decreased from 24 h onwards which was consistent with the profiling data and this reduction was persistent till 48 h (Figure 3.3B). Similar to profiling results we did not find any significant changes in IL-4+IL-13 polarized macrophages or macrophages treated with IL-4 alone for 8 and 24 h (3.3D, 3.3E).

3.6. miR-712 expression is down-regulated in primary BMDMs

We have additionally validated miR-712 expression analysis in primary bone marrow derived macrophages (BMDMs) where we observed miR-712 levels were more robustly down-regulated (33 fold) in BMDMs compared to macrophage cell line (Figure 3.4).

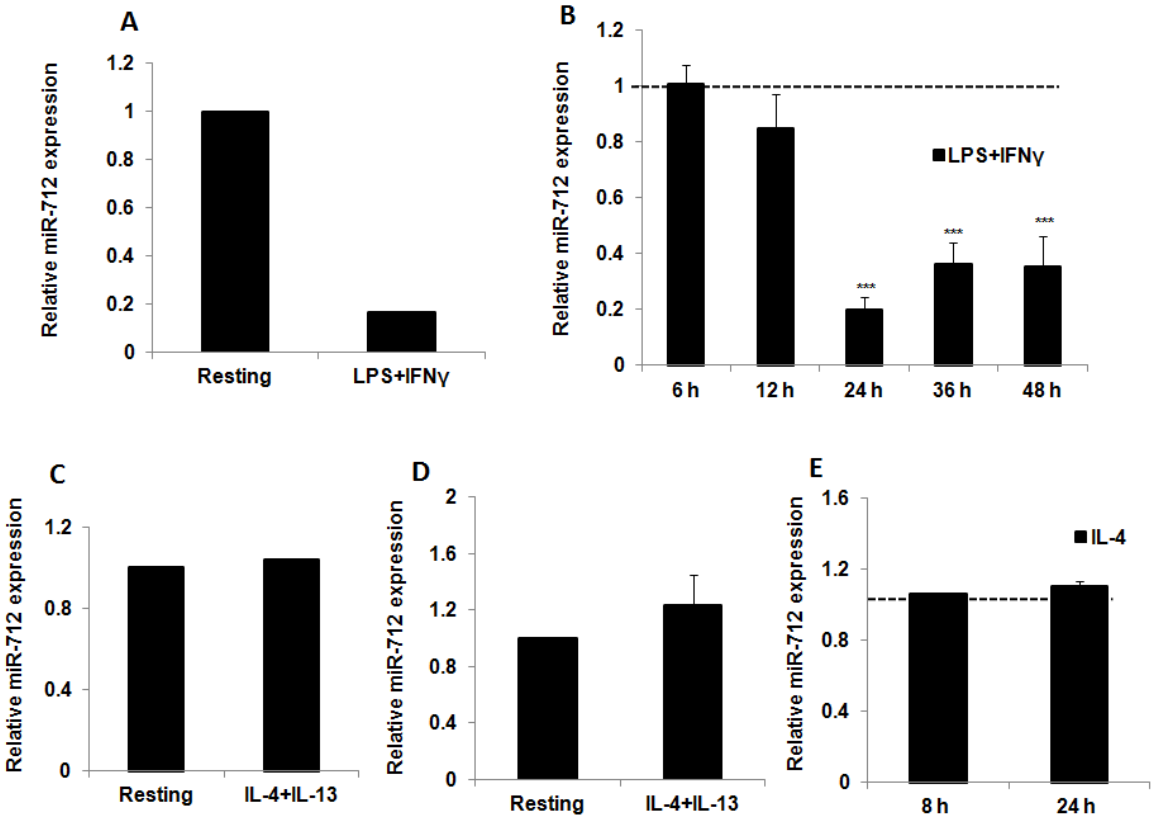


Figure 3.3. miRNA-712 expression is down-regulated in M (LPS+IFNγ) polarized macrophages, while no significant change was observed in M (IL-4+IL-13) polarized RAW 264.7 cells. (A, C) RAW 264.7 macrophage cells were stimulated with LPS+IFNγ or IL-4+IL-13 for 24 h and treated samples were outsourced for Affymetrix chip based microRNA analysis. RAW 264.7 cells after treatment with (B) LPS+IFNγ, (D) IL-4+IL-13 and (E) IL-4 stimuli for indicated time points were lysed in TRIzol and cDNA was used for determining miRNA expression and data was normalized to control U6. Dashed line indicates resting or un-stimulated cells. Data shown are a representative of three independent experiments (Mean ± SEM).

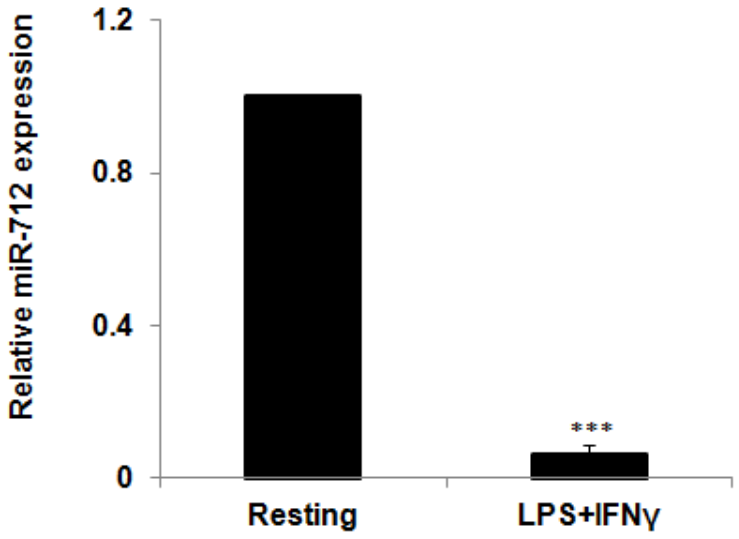


Figure 3.4. miR-712 expression is down-regulated in M (LPS+IFNγ) polarized primary bone marrow derived macrophages. Primary bone marrow macrophages were derived from bone marrow cells by culturing in the presence of M-CSF (20ng/ml) for 7 days and BMDMs (bone marrow derived macrophages) were stimulated with LPS+IFNγ for 24 h, total RNA was isolated and miR-712 were analyzed by qPCR. Data shown are a representative of two independent experiments (Mean ± SD).

3.7. miR-712 expression is down-regulated in palmitate activated and *Mycobacterium bovis* infected RAW 264.7 cells.

We next wished to study whether the reduction of miR-712 levels is specific to LPS+IFNγ stimulation or it is a common effect observed in pro-inflammatory macrophages. For this, we measured the expression of miR-712 in macrophages infected with *Mycobacterium bovis* and palmitate treated macrophages. M. bovis infection induces activation of macrophages to produce several pro-inflammatory mediators. Similarly, in obese individuals, circulating levels of free fatty acids (FFAs) are increased. FFAs are implicated in exerting insulin resistance through multiple mechanisms. Palmitate polarizes macrophages towards a pro-inflammatory phenotype which in turn hampers insulin signaling in neighbouring adipose cells or skeletal muscle via paracrine effects. Hence we were interested in determining the effect of M. bovis infection and palmitate on miR-712 expression in RAW 264.7 cells. Consistent with the data of LPS and IFNγ stimulated macrophages we observed that miR-712 levels were also reduced in M. bovis infected and palmitate treated RAW 264.7 cells (Figure 3.5A, 3.5B). This suggests that miR-712 is responsive to several pro-inflammatory stimuli and may likely emerge as a key player in the regulation of macrophage inflammatory responses.

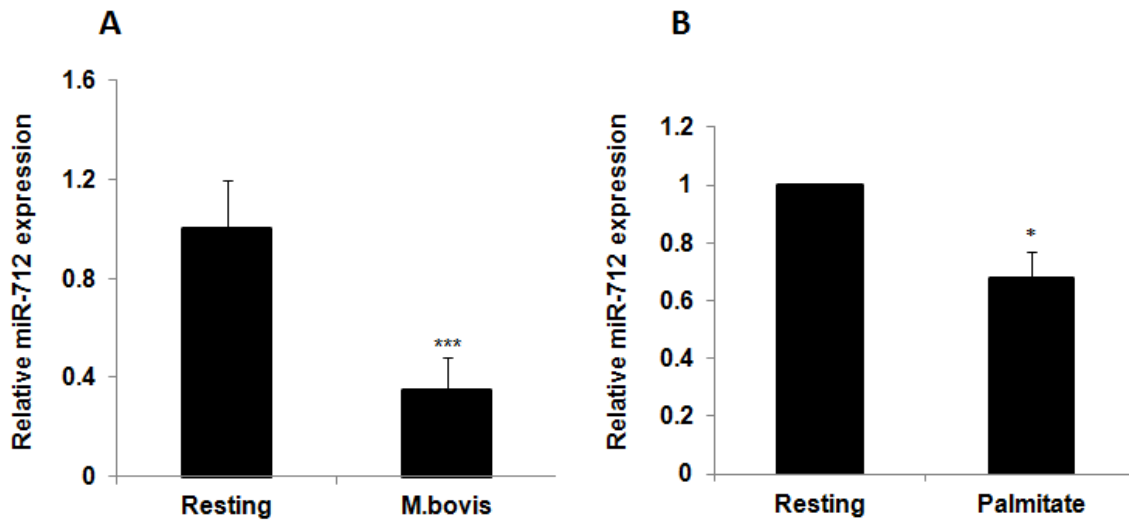


Figure 3.5. miRNA-712 expression is down-regulated in *Mycobacterium bovis* infected and palmitate treated RAW 264.7 cells. (A) RAW 264.7 cells after infecting with *M.bovis* for 3 h were washed twice and incubated in penicillin and streptomycin containing medium for another 24 h. Then cells were lysed in TRIzol and cDNA was used for determining miRNA expression and data was normalized to control U6. (B)) RAW 264.7 macrophage cells were stimulated with 500µM of palmitate for 24 h and treated samples were lysed in TRIzol and cDNA was used for determining miRNA expression and data was normalized to control U6. Data shown are a representative of three independent experiments (Mean ± SEM).

3.8. Forced expression of miR-712 dampens LPS+IFNγ-induced macrophage inflammatory responses

To understand the functional role of miR-712 during different activation states, RAW 264.7 macrophage cells were nucleofected with control mimic or miR-712 mimic and post 16 h of transfection, cells were either un-stimulated (Resting, R) or stimulated with LPS or IFNγ or both for 10 h and cellular levels of iNOS protein were determined by western blot analysis and the levels of inflammatory cytokines in the cell free supernatants was measured using ELISA. We observed that over-expression of miR-712 decreased LPS and/or IFNγ induced expression of iNOS, a potent LPS+IFNγ induced macrophage marker (Figure 3.6A, 3.6B). Further, forced expression of miR-712 mimic significantly decreased the production of LPS+IFNγ induced TNF-α, IL-6 and IFN-β but did not affect the secretion of interferon gamma induced protein (IP-10) (Figure 3.6C-3.6F). Further, we evaluated the effect of miR-712 on reactive oxygen species and phagocytosis and we observed that miR-712 does not influence ROS production and phagocytosis (Figure 3.6G, 3.6H). In parallel, we confirmed the over-expression of miR-712 by qPCR (Figure 3.6I).

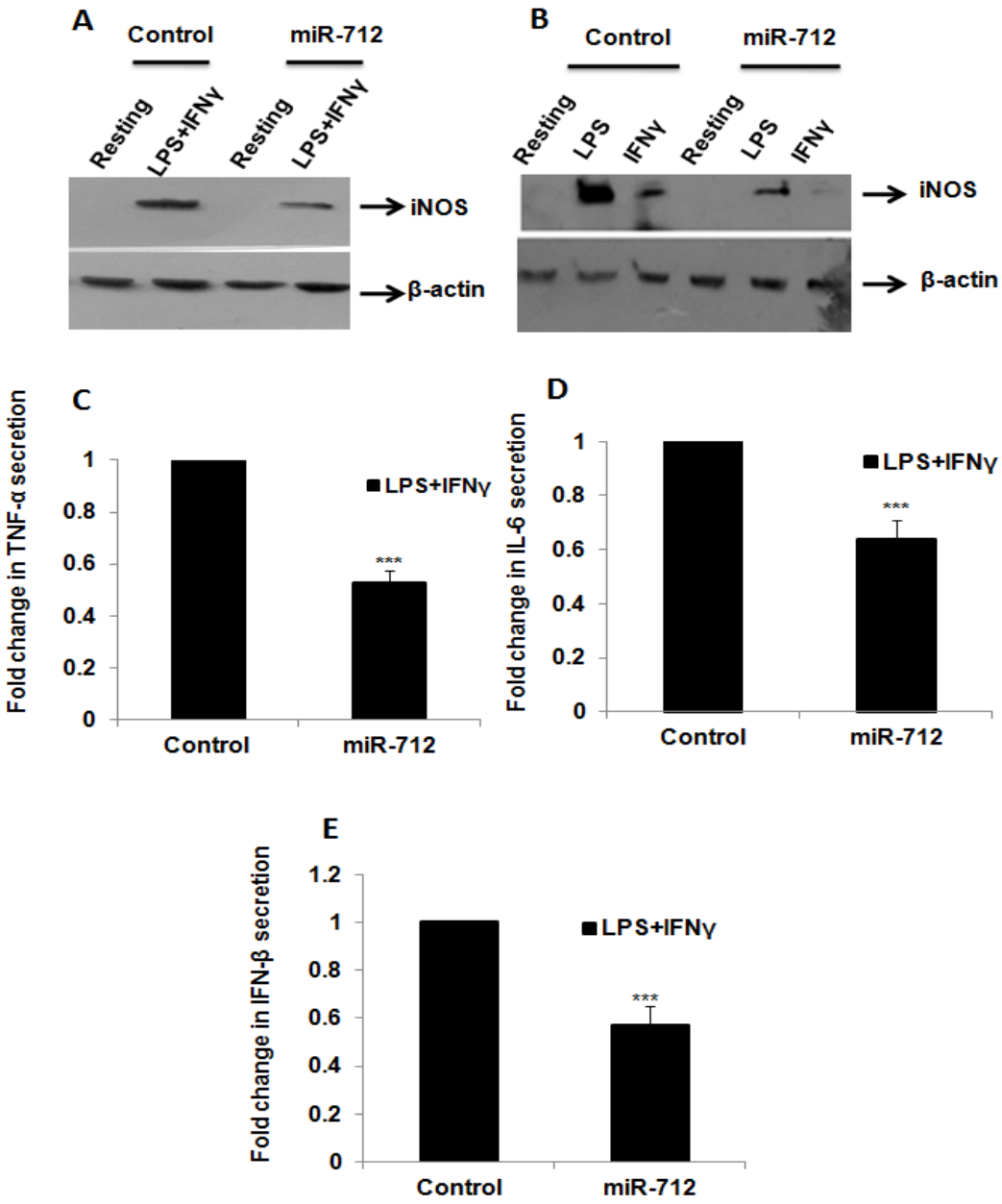


Figure 3.6. miRNA-712 dampens LPS+IFNγ induced iNOS, TNF-α, IL-6 and IFN-β mediated pro-inflammatory responses. RAW 264.7 cells transfected with control mimic or miR-712 mimics were stimulated with LPS+/- IFNγ for 10 h. (A-B) Probed for iNOS protein which was normalized to total actin. Data shown are a representative of three independent experiments. (C-E) Amount of TNF-α, IL-6 and IFN-β in the cell supernatants were measured by ELISA. Data shown are a representative of three independent experiments (Mean ± SEM).

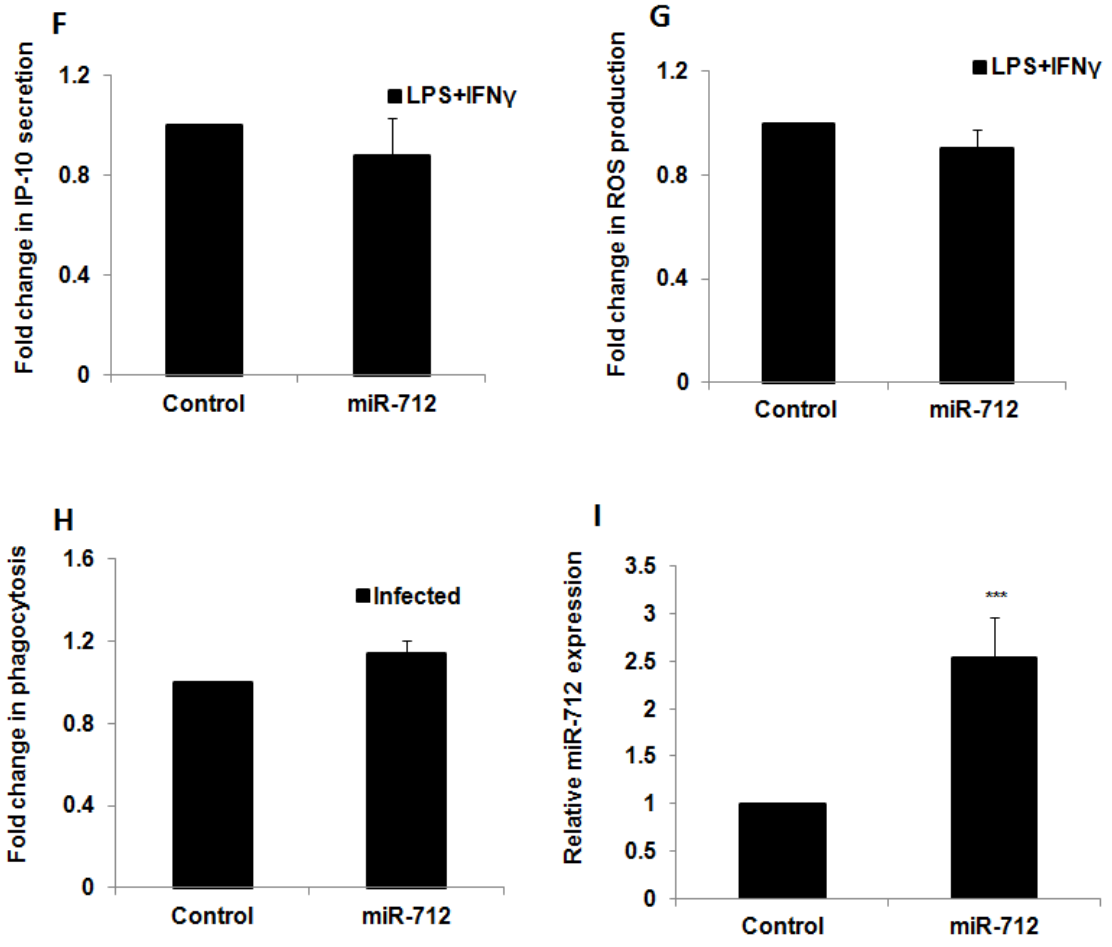


Figure 3.6. miRNA-712 dampens LPS+IFNγ induced iNOS, TNF-α, IL-6 and IFN-β mediated pro-inflammatory responses. RAW 264.7 cells transfected with control mimic or miR-712 mimics were stimulated with LPS+/- IFNγ for 10 h. F) Amount of IP-10 in the cell supernatants were measured by ELISA. Data shown are a representative of three independent experiments (Mean ± SEM). (G) Post 24 h of treatment with LPS+IFNγ, amount of ROS in the cell lysates were measured by fluorometry using DCF-DA. Data shown are a representative of three independent experiments (Mean ± SEM). (H) Post transfection, cells were infected with fluorescent bacteria or left un-infected for 3 h and phagocytosis of fluorescent bacteria by RAW 264.7 cells was determined by the measurement of fluorescence in the cell lysates by fluorometry. (I) In parallel, we validated the over-expression of mature miR-16 by qPCR. Data shown are a representative of three independent experiments (Mean ± SEM).

3.9. Forced expression of miR-712 does not influence IL-4 induced anti-inflammatory responses

To understand its role in M (IL-4) macrophages, RAW 264.7 cells were nucleofected with control mimic or miR-712 mimic. Approximately 16 h post-transfection, cells were either un-stimulated (Resting, R) or stimulated with IL-4 for 24 h and cellular levels of arginase-1 (ARG-1), IL-10 (interleukin-10) and YM-1 (chitinase) expression were determined by qPCR analysis. No significant changes in IL-4 induced markers were observed with miR-712 over-expression suggesting that its role is likely restricted to M (LPS+IFNγ) (Figure 3.7).

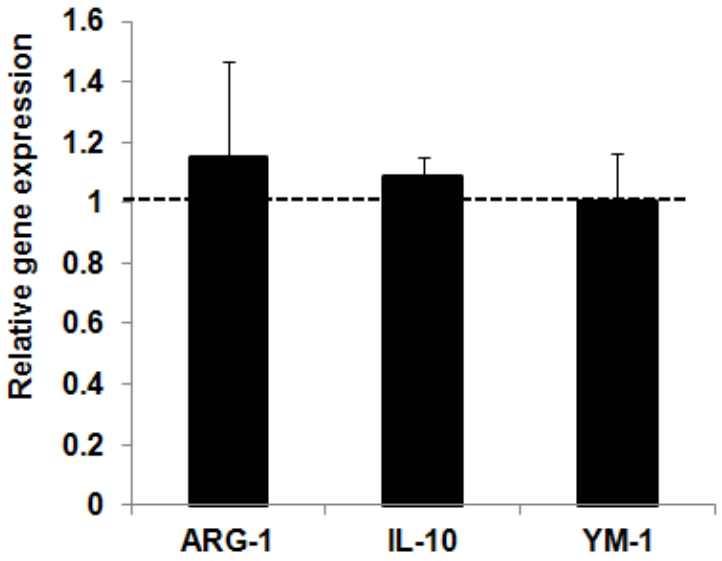


Figure 3.7. miRNA-712 does not impact IL-4 induced arginase, mannose receptor C, IL-10 and chitinase expression. RAW 264.7 cells transfected with control mimic or miR-712 mimics were stimulated with IL-4 for 24 h or left un-stimulated (dashed line) and cells were lysed in TRIzol and cDNA was used for determining gene expression. Actin was used as control gene for normalization. The data shown above are a representative of two independent experiments (Mean ± SD).

3.10. miR-712 targets LRRK2 3'UTR

In order to investigate the mechanistic basis underlying miR-712 anti-inflammatory effects, we performed computational analysis to identify the putative targets of microRNA-712. Based on *in silico* analysis, several targets of miR-712 were identified and top 20 predicted targets are listed in table 3.4. In the below mentioned table top most targets are ranked based on TargetScan analysis and the principles used by various prediction softwares are listed in table 3.3. Among several targets identified, LRRK2 was shortlisted for further analysis based on the following criteria: 1) LRRK2 is predicted by most of the search engines with a good score (Figure 3.8A-3.8C). 2) LRRK2 was shown to mediate microglial pro-inflammatory responses and its inhibition was reported to reduce iNOS expression and TNF-α secretion [275]. 3) LRRK2 expression was reported to be increased in Crohn's inflammation and it was shown to enhance NF-κB dependent gene transcription [276]. Also very recently miR-205 was identified as a human homolog of miR-712 and miR-205 was shown to target LRRK2 in Parkinson's disease (Figure 3.8D) [156]. Thus, it possible that miR-712 may inhibit LRRK2 and thereby reduce pro-inflammatory responses of LPS+IFNγ exposed macrophages.

Table 3.3. Principles of microRNA target prediction algorithms

Prediction algorithm	Parameters considered for final scores	Cut-off scores
TargetScan	seed match, 3' complementarity, local AU content, position and site type contribution	target with lowest context+score is the most favorable
MiRDB	SVM method (uses principles from four tools- MiRanda, PITA, TargetScan, miRSVR	Prediction score >80 is likely to be real
microRNA.org	seed-site pairing, site context, free-energy, and conservation	≤ 0.1
DIANA microT-CDS	free energy binding and complementarity	0.3-1 (1 being the best score) 0.6 is the cut-off score for mouse species

Table 3.4. Top 20 mRNA targets for miR-712 predicted by multiple search engines

S.No	Target gene	TargetScan	miRDB	microRNA.org	DIANA Tools
1	Chn1	-0.86	100	-0.9	0.99
2	Tm9sf2	-0.71	100	-3.3	0.95
3	Pdk1	-0.57	63	not predicted	0.86
4	Phb	-0.53	88	-1.5	0.98
5	Fam184b	-0.48	96	-1.48	0.81
6	Lrp1	-0.46	95	-1.62	not predicted
7	Nsf	-0.46	93	-1.25	0.94
8	Btbd3	-0.45	90	-1.48	0.96
9	BC030336	-0.45	97	-2.95	0.96
10	Ctps2	-0.45	97	-1.09	0.86
11	Rngtt	-0.45	100	-2.2	0.92
12	Mll1	-0.44	not predicted	-0.73	not predicted
13	Srsf10	-0.44	60	not predicted	not predicted
14	AI480653	-0.44	not predicted	-1.17	not predicted
15	Lysmd3	-0.44	89	-1.57	0.91
16	Frk	-0.43	73	-1.31	0.93
17	Gxylt1	-0.43	75	-0.21	0.9
18	Gm14137	-0.41	64	-0.91	0.85
19	Etnk1	-0.41	96	-0.01	0.87
20	Hnrnph3	-0.4	95	-1.89	not predicted

A

Target	Target Scan	Miranda	MiRDB	PITA	DIANA
Leucine rich Repeat Kinase 2 (LRRK2)	-0.35	-1.27	51	-5.85	0.86

B

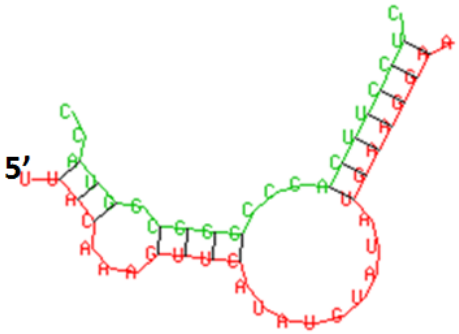
Position 185-192 of LRRK2 3'UTR

5'.... AAGUUCAUAUGUAUAUGAAGGAA....

mmu-miR-712 (seed match 8mer)

3' CCAUGGCGGGCCCACUUCCUC

C



Minimum free energy: -17.5 kcal/mol

D

hsa-miR-205 5' - UCCUUCAUUCCACCGGAGUCUG - 3'

mmu-miR-712 5' - CUCCUUCACCCGGGCGGUACC - 3'

Seed sequence similarity between miR-205 and miR-712

Figure 3.8. Bioinformatics analysis show that miRNA-712 targets LRRK2 3' UTR. (A) LRRK2 is predicted to be targeted by miR-712 by multiple search engines. (B) Seed complementarity between LRRK2 3'UTR seed sequence and miR-712 seed region (TargetScan). (C) The binding and stability between LRRK2 mRNA (red) and miR-712 (green) sequence is shown above as hybrid structure (RNAhybrid software). (D) Seed match (highlighted in yellow) between human miR-205 and mouse miR-712.

3.11. Experimental validation of LRRK2 as the target of miR-712

To validate if LRRK2 is an authentic target of miR-712, we over-expressed the miR-712 and investigated its effect on LRRK2 expression by qPCR, western blot and luciferase reporter assay. We found that miR-712 ectopic expression significantly decreased LRRK2 mRNA and protein levels (Figure 3.9A, 3.9B). Further, we noticed that over-expression of miR-712 significantly inhibited the activity of reporter clone (LRRK2 3'UTR cloned downstream of luciferase gene) (Figure 3.9C).

3.12. miR-712 down-regulates pro-inflammatory responses by reducing phosphorylation of p38 and ERK 1/2 levels.

LRRK2 is reported to activate ERK1/2 and p38 signaling in different cell types. Having observed that miR-712 reduced the expression of LRRK2, we then examined the effect of miR-712 on LPS+IFNγ induced signaling. LPS+IFNγ induced the phosphorylation of p38 and ERK1/2 and forced expression of miR-712 attenuated the magnitude of the phosphorylation of both p38 and ERK1/2 (Figure 3.10A, 3.10B).

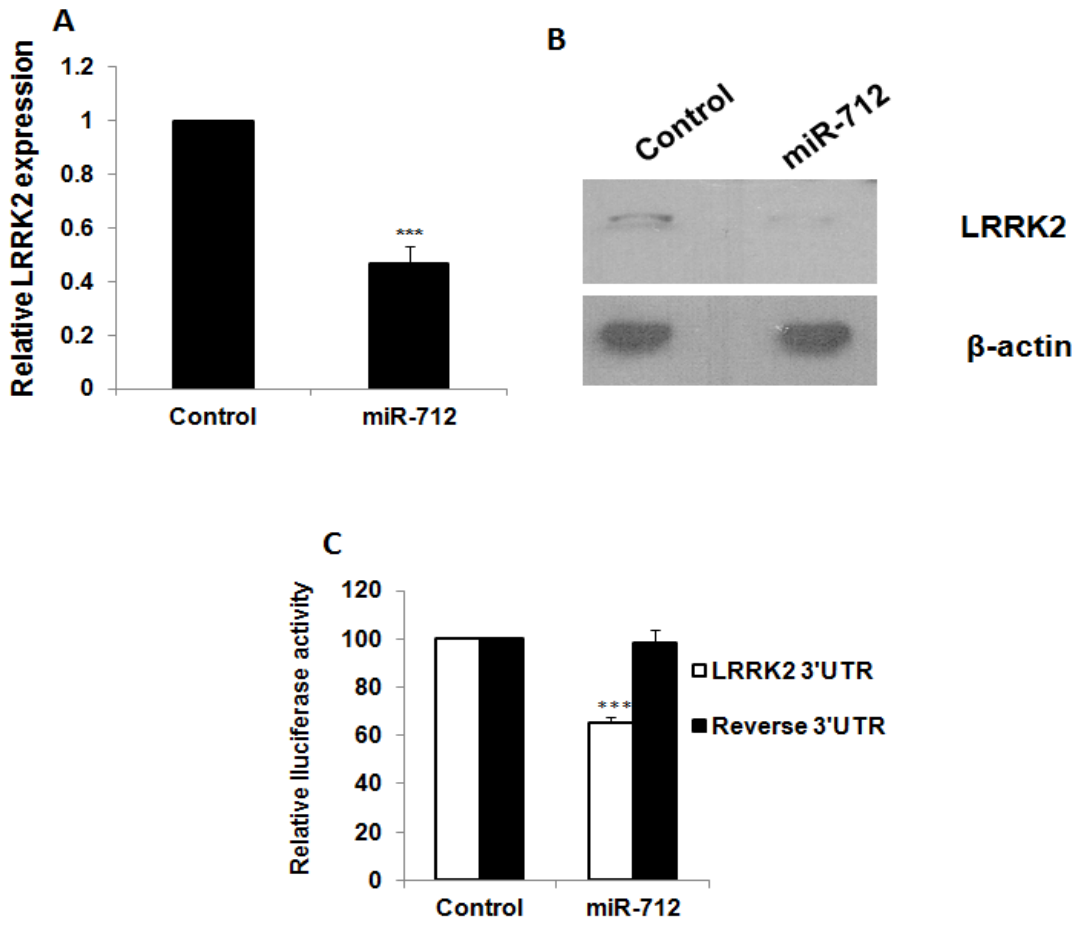


Figure 3.9. Experimental evidence show that miRNA-712 targets LRRK2 3' UTR. (A) RAW 264.7 macrophage cells were nucleofected with control mimic or miR-712 mimic. Approximately 10 h post-transfection, cells were harvested for miRNA expression analysis using qPCR. Data shown are a representative of three independent experiments (Mean ± SEM). (B) Approximately 24 h post-transfection, cells were harvested for detecting LRRK2 protein levels of by western blot analysis. Data shown are a representative of three independent experiments (Mean ± SEM). (C) HEK 293T cells were co-transfected with control mimic or miR-712 mimic along with PGL3 promoter luciferase vector or LRRK2 3'UTR clone. Post 24 h of transfection, cells were lysed and luciferase readings were measured and were normalized using renilla values. Data shown are a representative of three independent experiments (Mean ± SEM).

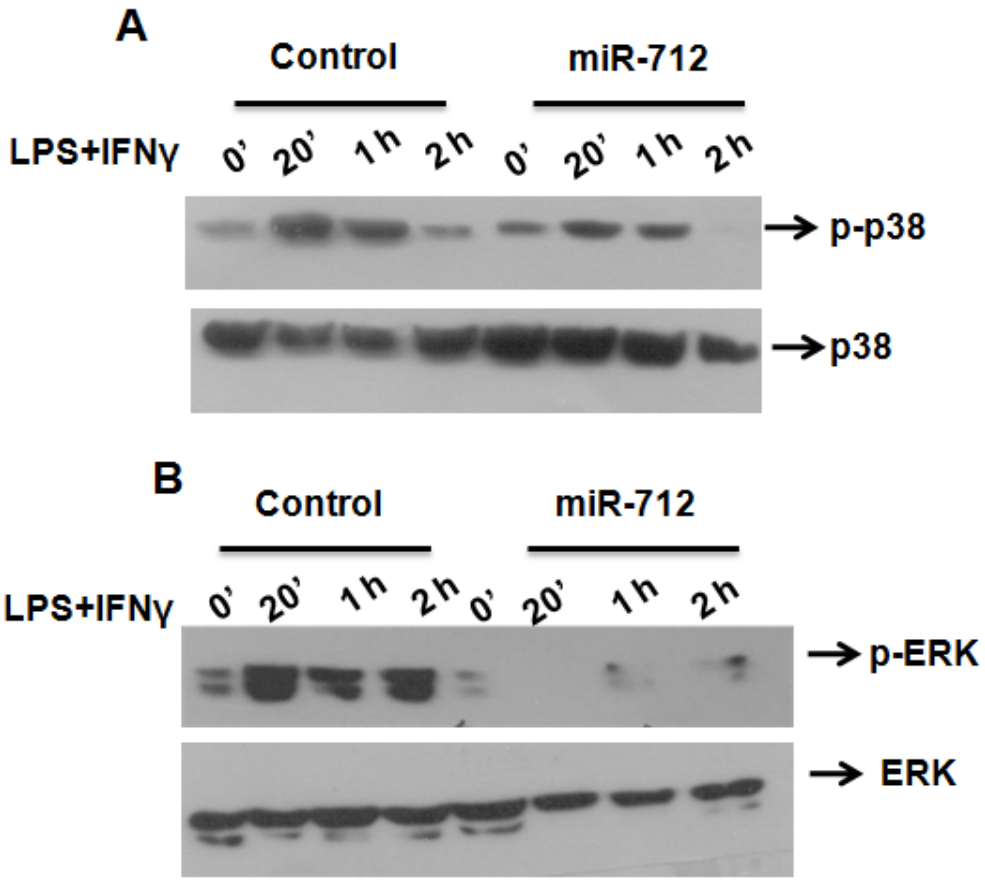


Figure 3.10. miR-712 reduces phosphorylation of p38 and ERK1/2 in M (LPS+IFNγ) polarized macrophages. RAW 264.7 cells transfected with control mimic or miR-712 mimic were stimulated with LPS+IFNγ for indicated time points and was probed for phosphorylation of p38 and ERK levels along with their respective total proteins. Data shown are a representative of three independent experiments.

3.13. Forced expression of miR-712 impairs macrophage mediated insulin resistance in skeletal myoblasts

Pro-inflammatory cytokines are shown to promote insulin resistance in insulin dependent glucose metabolizing cells. To probe the functional impact of miR-712 on the paracrine effects of M (LPS+IFNγ) macrophages on insulin stimulated glucose uptake by skeletal myoblasts, RAW 264.7 macrophage cells were nucleofected with control mimic or miR-712 mimic. Approximately 16 h post-transfection, cells were either un-stimulated (Control) or stimulated with LPS and IFNγ for 10 h and the harvested conditioned cell supernatants were added to L6 rat skeletal myoblasts for 20 h. Later cells were serum starved for 6 h followed by glucose starvation for 30 minutes and then were stimulated with insulin for 5 minutes or 10 minutes and glucose uptake was determined using 2-NBDG assay. We observed that incubation of skeletal myoblasts with LPS+IFNγ stimulated control transfectants caused a significant reduction in both un-stimulated and insulin stimulated glucose uptake. However consistent with anti-inflammatory nature of miR-712, over-expression of miR-712 in RAW 264.7 cells abrogated this paracrine inhibitory effect of M (LPS+IFNγ) cytokines on skeletal myoblast glucose uptake (Figure 3.11).

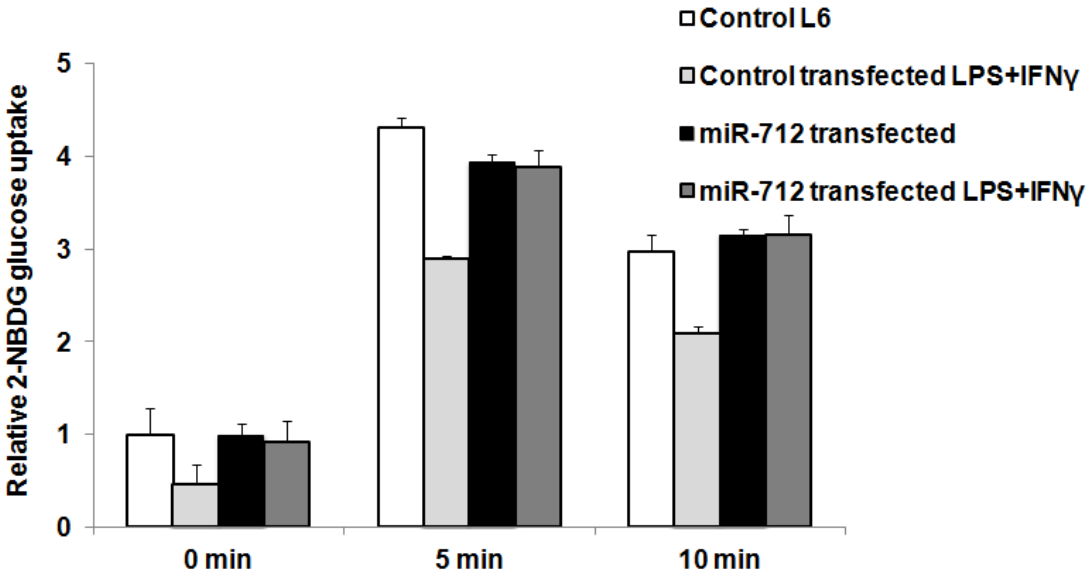


Figure 3.11. miRNA-712 suppresses inflammation induced insulin resistance in L6 myoblasts. Mean basal and insulin stimulated glucose uptake was measured using 2-NBDG by fluorometry in L6 myoblasts after 18 h of incubation with supernatants obtained from treated RAW 264.7 cells as indicated in the methods. Values are normalized with the corresponding protein content and expressed relative to basal control cells which was set as 1. Data (mean ± SD) shown are a representative of three independent experiments.

3.14. miR-712 expression is augmented in skeletal myoblasts challenged with different stressors

Next we were interested in identifying the direct role of miR-712 on skeletal myoblasts, for which we have initially determined the expression levels of miR-712 in palmitate, tunicamycin and thapsigargin treated differentiated myoblasts as all the three are well known to trigger inflammatory responses. During this treatment we have observed that unlike in macrophages, miR-712 expression levels were significantly induced in the above mentioned treatments suggesting potential regulatory function in the skeletal muscle as well (Figure 3.12A-3.12C). However the direct role of miR-712 on insulin signaling needs further evaluation.

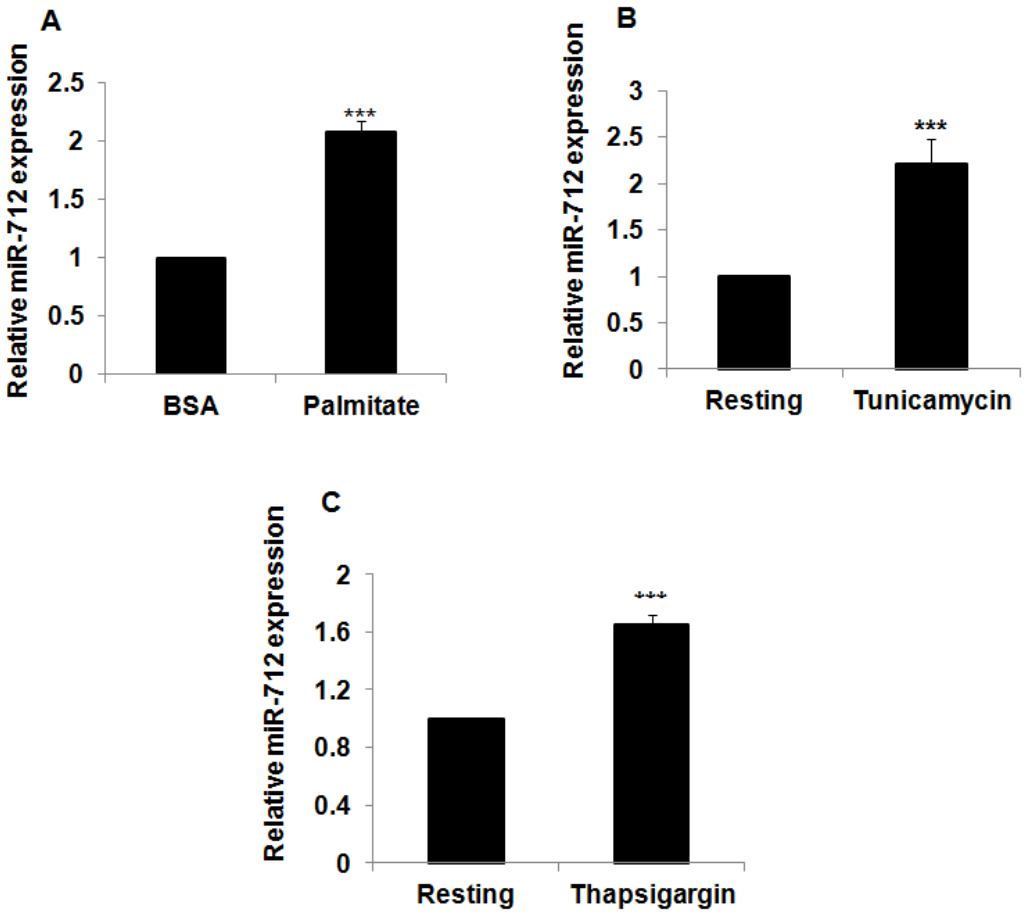
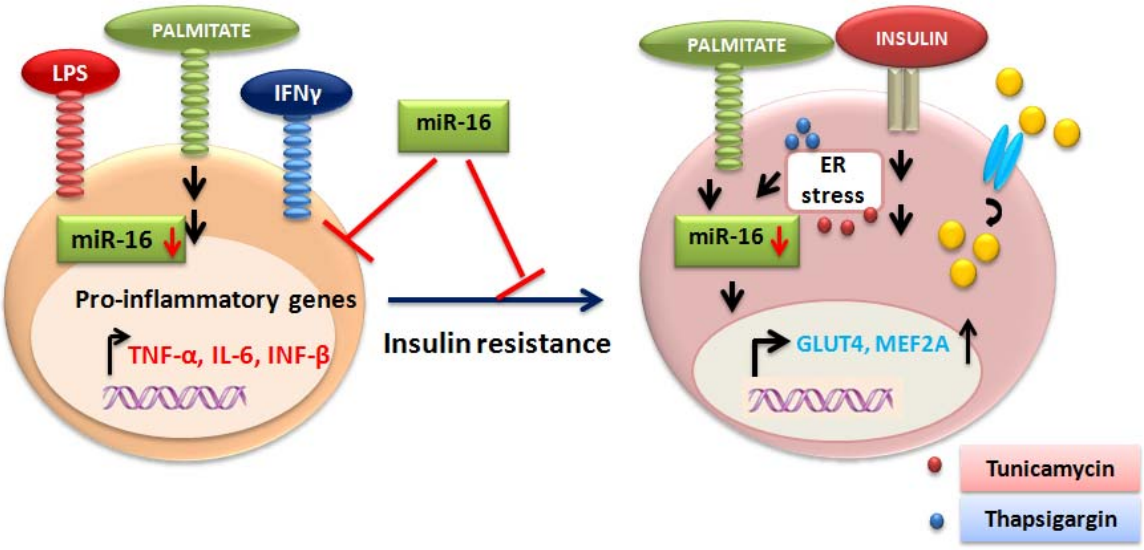


Figure 3.12. miR-712 expression is up-regulated in skeletal myoblasts upon palmitate, tunicamycin and thapsigargin treatment. C2C12 cells treated with (A) 500µM of palmitate, (B) 5µg/mL of tunicamycin and (C) 300nM of thapsigargin for 24 h were lysed in TRIzol and cDNA was prepared. cDNA was used for determining miRNA expression and data was normalized to control U6. Data shown are a representative of three independent experiments (Mean ± SEM).

3.15. Summary

Our results show that miR-712 expression is down-regulated in inflammatory conditions in macrophages. We have showed that the forced expression of miR-712 suppresses macrophage mediated pro-inflammatory responses and their downstream inhibitory effects on insulin signaling pathway. Further we have identified LRRK2 as the target of miR-712. We have showed that mechanistically miR-712 reduces phosphorylation of p38 and ERK1/2 which are required for pro-inflammatory gene expression. However its role in skeletal muscle under inflammatory condition needs further attention in order to understand its importance in insulin signaling pathway. Collectively our data highlight the important role of miR-712 in ameliorating inflammation induced insulin resistance.

Chapter 4: MicroRNA-16 attenuates M (LPS+IFNγ) mediated inflammation induced insulin resistance in skeletal myoblasts



MicroRNA-16 suppresses macrophage pro-inflammatory responses and improves myoblast insulin sensitivity. The expression of miR-16 is reduced in macrophages exposed to LPS and IFNγ and myoblasts treated with palmitate, tunicamycin and thapsigargin in skeletal myoblasts. Forced expression of miR-16 in macrophages attenuates inflammatory responses of macrophage and their paracrine inhibitory effects on insulin sensitivity in skeletal myoblasts. On the other hand forced expression of miR-16 in myoblasts improves insulin sensitivity by up-regulating MEF2A and GLUT4 expression indicative of the important role of miR-16 in inflammation induced insulin resistance.

The role of miR-16 is well defined in cancers; however its role in macrophage polarization is less studied. Although multiple studies have showed that miR-16 is expressed in immune cells and its de-regulation is linked to chronic diseases such as Crohn's disease and rheumatoid arthritis, the precise role of miR-16 in macrophage triggered inflammation and specifically in inflammation induced insulin resistance is poorly studied. Thus, in order to unmask its role in inflammatory diseases we sought to understand the role of miR-16 in macrophage polarization and its effect on insulin sensitivity. Our results showed that miR-16 expression is down-regulated in inflamed macrophages and myoblasts and over-expression of miR-16 lead to decreased macrophage mediated inflammation and improved myoblast insulin sensitivity both directly and indirectly.

4.1. miR-16 expression is down-regulated in LPS+IFNγ activated macrophages

In order to identify the role of miR-16, we have first validated the microarray data by performing qPCR analysis. Consistent with the profiling studies (Figure 4.1A), we have observed that miR-16 expression was down-regulated in M (LPS+IFNγ) macrophages (Figure 4.1B). In microarray profiling, we found that miR-16 levels were reduced by ~37% in IL-4+IL-13 treated macrophages (Figure 4.1C). However we did not observe any significant reduction of miR-16 in macrophages treated with IL-4 alone for 8 h and 24 h (Figure 4.1E) or in combination with IL-13 (Figure 4.1D) for 24 h.

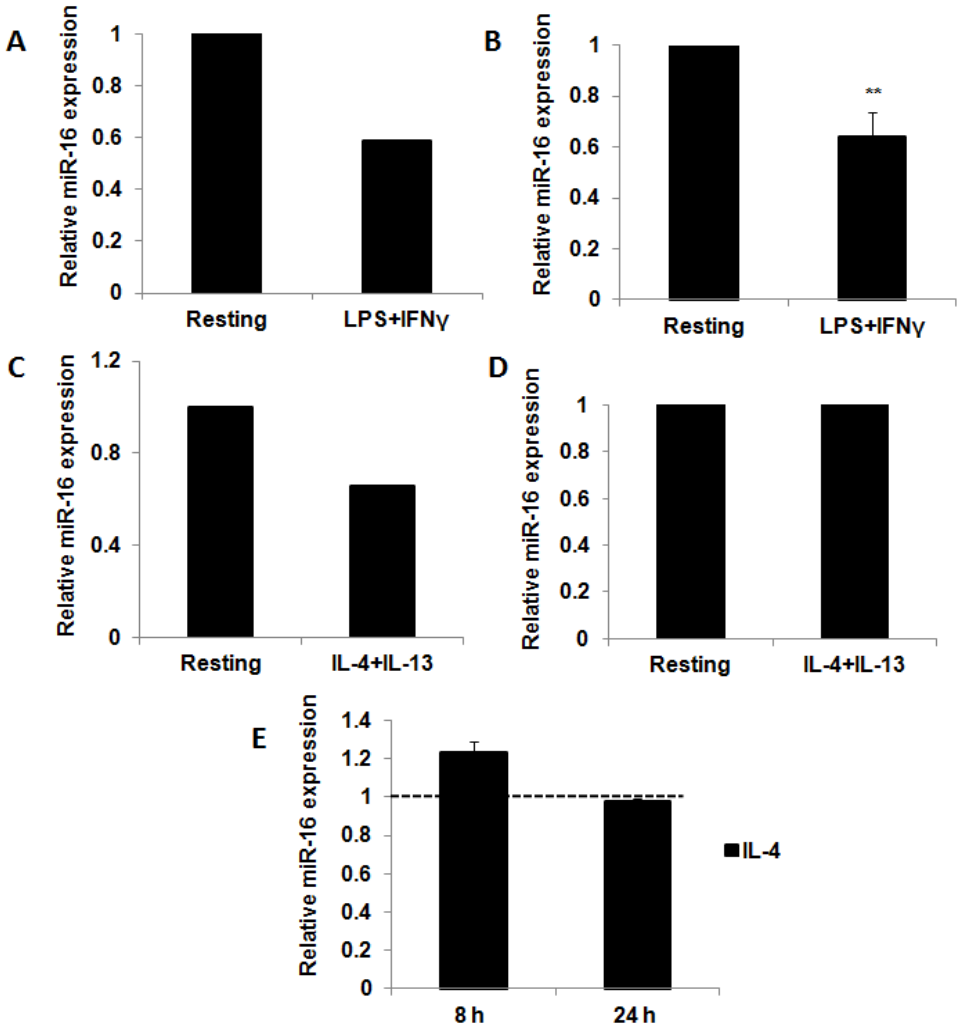


Figure 4.1. miRNA-16 expression is down-regulated in M (LPS+IFNγ) polarized macrophages, while no significant change was observed in M (IL-4 ± IL-13) polarized RAW 264.7 cells. (A, C) RAW 264.7 macrophage cells were stimulated with LPS+IFNγ or IL-4+IL-13 for 24 h and treated samples were analyzed by Affymetrix chip based microRNA analysis. (B, D, E) RAW 264.7 cells after treatment with LPS+IFNγ and IL-4 alone or in combination with IL-13 for indicated time points were lysed in TRIzol and cDNA was used for determining miRNA expression and data was normalized to control U6. Dashed line indicated resting or un-stimulated cells. Data shown are a representative of three independent experiments (Mean ± SEM).

4.2. miR-16 expression is down-regulated in palmitate activated RAW 264.7 cells

In obese individuals, circulating levels of free fatty acids (FFAs) are increased. FFAs are implicated in exerting insulin resistance through multiple mechanisms. Palmitate polarizes macrophages towards a pro-inflammatory phenotype which in turn hampers insulin signaling in neighbouring adipose cells or skeletal muscle via exerting its paracrine effects. Hence we were interested in determining the effect of palmitate on miR-16 expression in RAW 264.7 cells. Consistent with the data of LPS and IFNγ stimulated macrophages, miR-16 levels were observed to be reduced in palmitate treated RAW 264.7 cells (Figure 4.2).

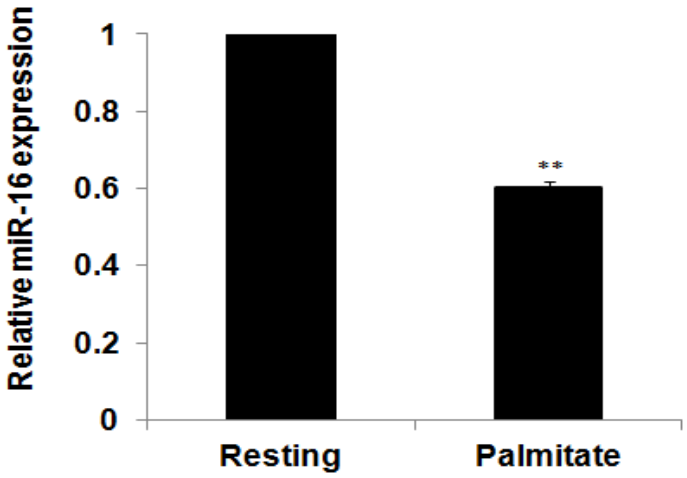


Figure 4.2. miRNA-16 expression is down-regulated in palmitate treated RAW 264.7 cells. RAW 264.7 cells after treatment with 500μM palmitate for 24 h were lysed in TRIzol and cDNA was used for determining miRNA expression and data was normalized to control U6. Data shown are a representative of three independent experiments (Mean ± SEM).

4.3. Forced expression of miR-16 dampens LPS+IFNγ induced macrophage inflammatory responses

Having observed that miR-16 levels were reduced in M (LPS+IFNγ) and palmitate exposed macrophages, we next sought to investigate the functional role of miR-16 in the regulation of macrophage polarization. For this, RAW 264.7 macrophage cells were nucleofected with control mimic or miR-16 mimic and approximately 16 h post-transfection cells were either un-stimulated (Resting, R) or stimulated with LPS+IFNγ for 10 h. The levels of inflammatory cytokines in the cell free supernatants were measured using ELISA and cellular levels of iNOS protein were determined by western blot analysis. We observed that the over-expression of miR-16 inhibited LPS+IFNγ induced TNF-α, IL-6 and IFN-β secretion (Figure 4.3A-4.3C). However miR-16 did not affect the secretion of interferon gamma induced protein (IP-10) (Figure 4.3D) and the expression of iNOS (Figure 4.3E). Further, we evaluated the effect of miR-16 on reactive oxygen species (ROS) and phagocytosis and found that that miR-16 does not influence ROS production and phagocytosis (Figure 4.3F, 4.3G). In parallel, we confirmed the over-expression of miR-16 by qPCR analysis (Figure 4.3H).

4.4. miR-16 selectively regulates IL-4 induced macrophage gene expression

To understand its role in M (IL-4) macrophages, RAW 264.7 cells were nucleofected with control mimic or miR-16 mimic. Approximately 16 h post-transfection, cells were either un-stimulated (Resting, R) or stimulated with IL-4 for 24 h and cellular levels of arginase-1 (ARG-1), mannose receptor C (MRC-1), IL-10 (interleukin-10) and YM-1 (chitinase) expression were determined using qPCR analysis. We observed that miR-16 increased ARG-1 expression by ~2 fold whereas it did not affect other markers such as

MRC-1, IL-10 and YM-1 (Figure 4.4).

4.5. Forced expression of miR-16 impairs macrophage mediated insulin resistance in skeletal myoblasts

Pro-inflammatory cytokines such as TNF-α, IL-6 and IFN-γ were shown to promote insulin resistance in insulin dependent tissues. Thus, to probe the functional impact of miR-16 on the paracrine effects of M (LPS+IFNγ) macrophages on insulin stimulated glucose uptake by skeletal myoblasts, RAW 264.7 macrophage cells were nucleofected with control mimic or miR-16 mimic. Approximately 16 h post-transfection, cells were either un-stimulated (Control) or stimulated with LPS and IFNγ for 10 h and supernatants were added to L6 rat skeletal myoblasts for 20 h. Later cells were serum starved for 6 h followed by glucose starvation for 30 minutes and then were stimulated with insulin for 5 minutes or 10 minutes and glucose uptake was determined using 2-NBDG assay. We observed that incubation of skeletal myoblasts with LPS+IFNγ stimulated control transfectants caused a significant reduction in both un-stimulated and insulin stimulated glucose uptake. However consistent with anti-inflammatory nature of miR-16, over-expression of miR-16 in RAW 264.7 cells abrogated this paracrine inhibitory effect of M (LPS+IFNγ) cytokines on skeletal myoblast glucose uptake (Figure 4.5).

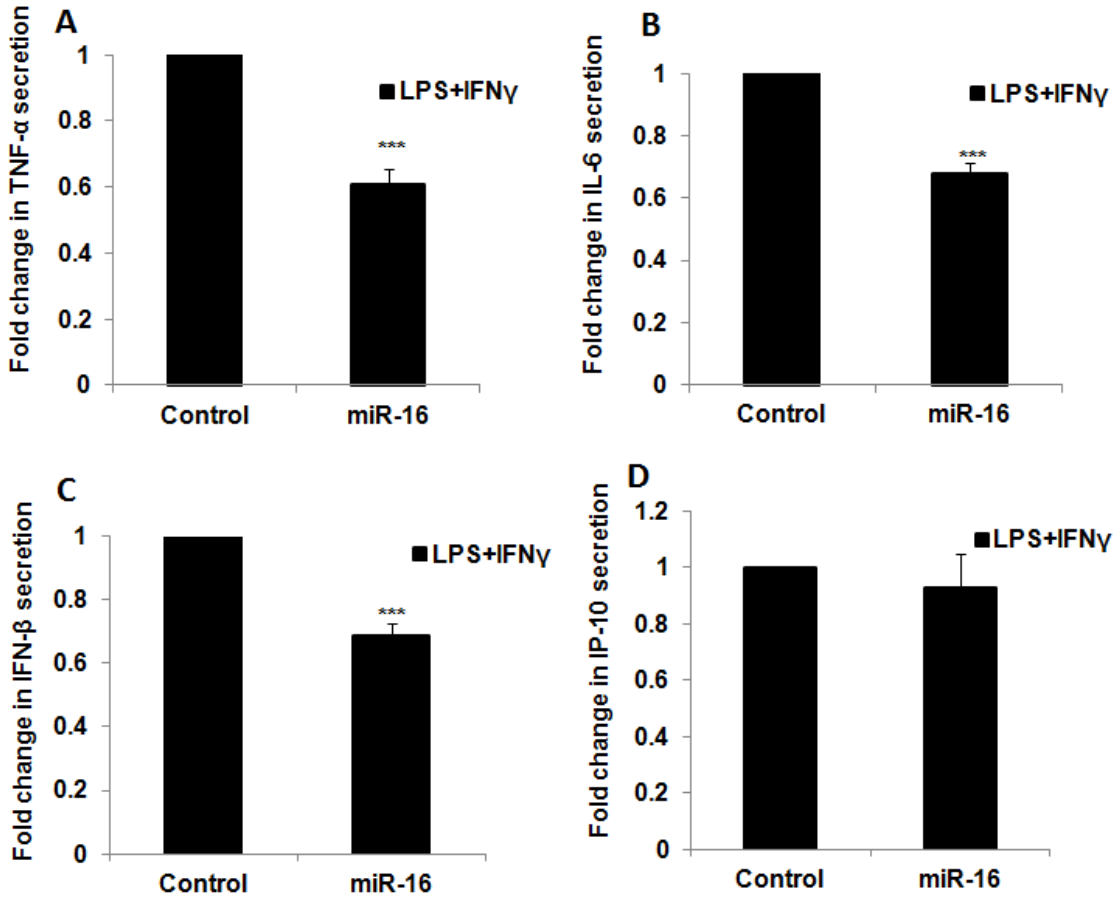


Figure 4.3. miRNA-16 dampens LPS+IFNγ induced TNF-α, IL-6 and IFN-β in LPS+IFNγ exposed macrophages. RAW 264.7 cells transfected with control mimic or miR-16 mimics were stimulated with LPS+IFNγ for 10 h. (A-D) Amount of TNF-α, IL-6, IFN-β and IP-10 in the cell supernatants were measured by ELISA. Data shown are a representative of three independent experiments (Mean ± SEM).

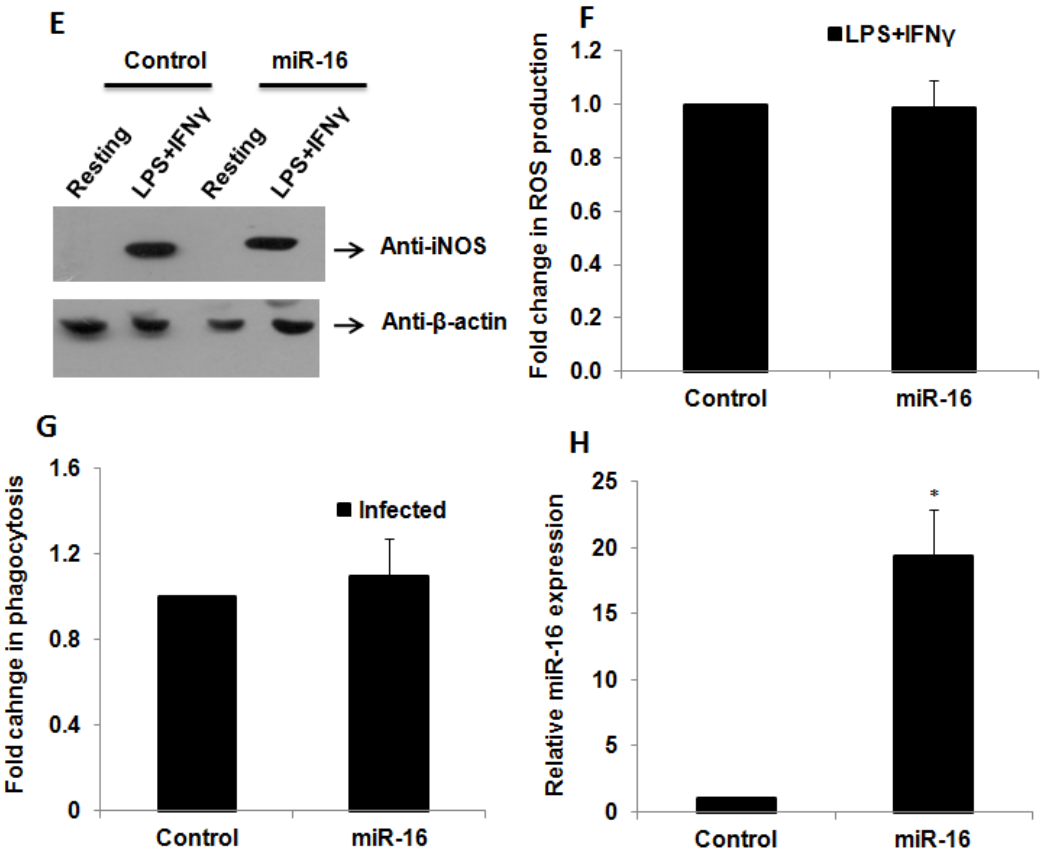


Figure 4.3. miRNA-16 dampens LPS+IFNγ induced TNF-α, IL-6 and IFN-β in LPS+IFNγ exposed macrophages. RAW 264.7 cells transfected with control mimic or miR-16 mimics were stimulated with LPS+IFNγ for 10 h. (E) Cell lysates were analyzed by immunoblotting with iNOS antibody and probed with β-actin antibody as the loading control. (F) Post 24 h of treatment with LPS+IFNγ, amount of ROS in the cell lysates were measured by fluorometry using DCF-DA. Data shown are a representative of three independent experiments (Mean ± SEM). (G) Post transfection, cells were infected with fluorescent bacteria or left un-infected for 3 h and phagocytosis of fluorescent bacteria by RAW 264.7 cells was determined by the measurement of fluorescence in the cell lysates by fluorometry. (H) In parallel, the over-expression of mature miR-16 was determined by qPCR. Data shown are a representative of three independent experiments (Mean ± SEM).

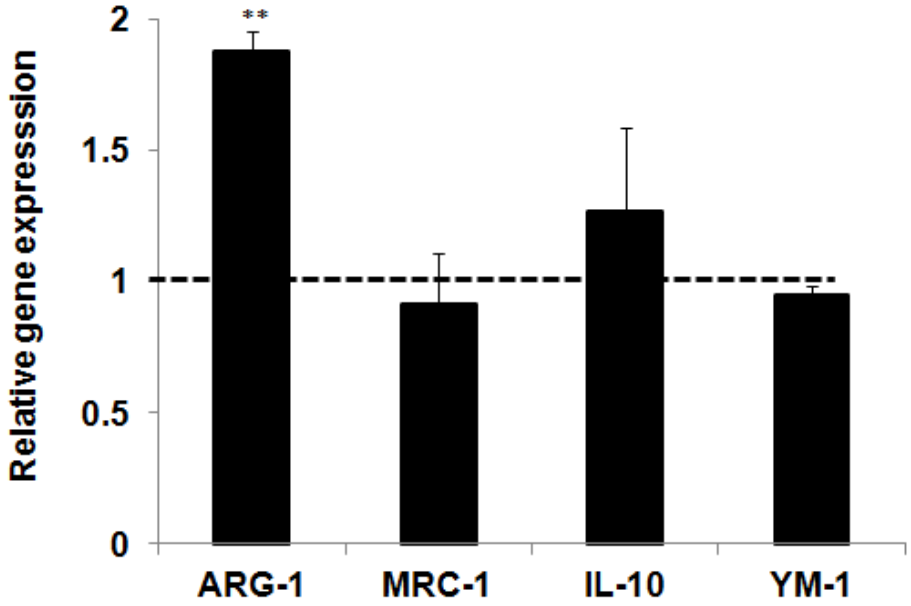


Figure 4.4. miRNA-16 promotes IL-4 induced arginase expression. RAW 264.7 cells transfected with control mimic or miR-16 mimics were stimulated with IL-4 for 24 h or left un-stimulated (dashed line) and cells were lysed in TRIzol and cDNA was used for determining gene expression. β-actin was used as the control gene for normalization. The data shown above are a representative of three independent experiments (Mean ± SEM).

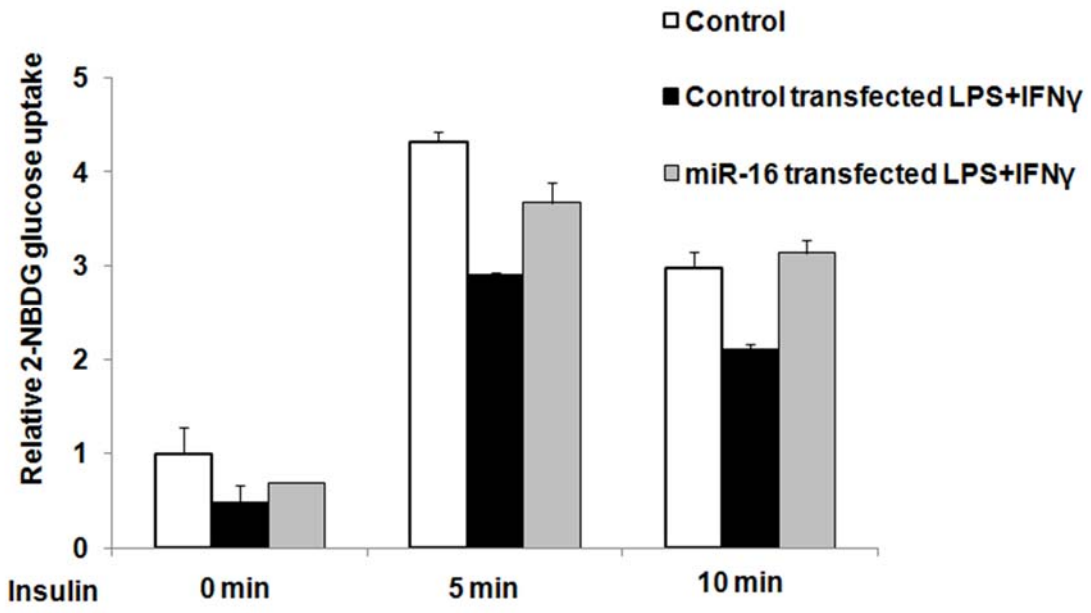


Figure 4.5. miRNA-16 suppresses inflammation induced insulin resistance in L6 myoblasts. Mean basal and insulin stimulated glucose uptake was measured using 2-NBDG by fluorometry in L6 myoblasts after 18 h of incubation with supernatants obtained from treated RAW 264.7 cells as indicated in the methods. Values are normalized with the corresponding protein content and expressed relative to basal control cells which was set as 1. Data (mean ± SD) shown are a representative of three independent experiments.

4.6. miR-16 expression is down-regulated in high sucrose diet fed (HSD) rat tissues

miRNA expression profiles from several studies showed that miR-16 is ubiquitously expressed at modest levels in all animal tissues. Since, the expression pattern of miR-16 was consistently down-regulated in inflammation; we aimed to determine the expression profiles of miR-16 in inflammation induced insulin resistance conditions. For this, we used high sucrose diet (HSD) fed rats as the model system and assessed miR-16 expression in insulin responsive organs and as observed in macrophages, miR-16 is significantly down-regulated in metabolically active rat tissues such as soleus muscle (Figure 4.6A), adipose (Figure 4.6B), and liver (Figure 4.6C).

4.7. miR-16 expression is down-regulated in skeletal myoblasts exposed to stress

Next we sought to identify the direct role of miR-16 if any in skeletal myoblasts, for which we have initially determined the expression levels of miR-16 in palmitate, tunicamycin and thapsigargin (endoplasmic reticulum (ER) stress inducers), treated differentiated myoblasts. All the three are well known to trigger inflammatory responses and ER stress which in turn induce insulin resistance. During this treatment we have observed that similar to the trend observed in macrophages, miR-16 expression levels were significantly down-regulated in palmitate treated (Figure 4.7A), and ER stress induced C2C12 myoblasts (Figure 4.7B-4.7C), suggesting its potential regulatory function in the skeletal muscle as well.

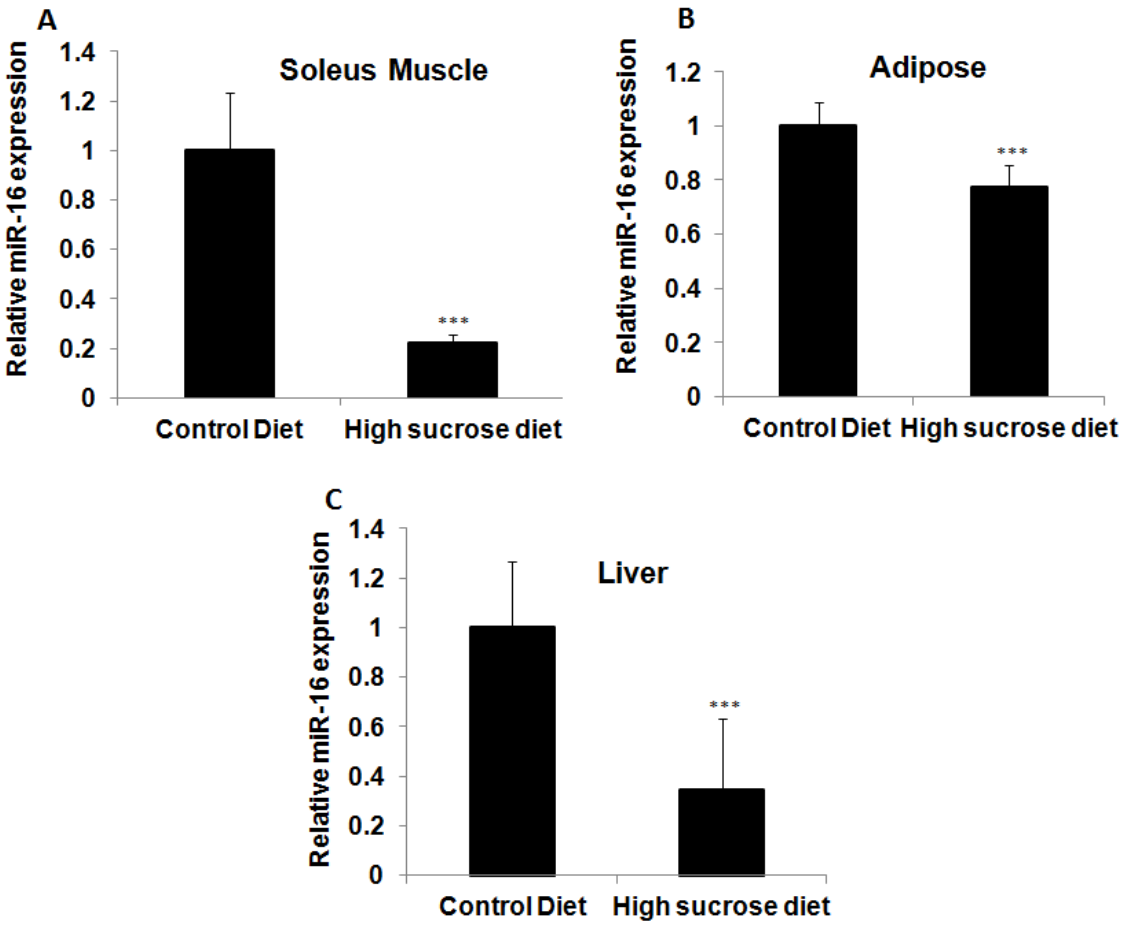


Figure 4.6. miRNA-16 expression is down-regulated in HSD fed rat tissues. Tissues from control diet and high sucrose diet were lysed in TRIzol and miR-16 expression was analyzed in (A) Soleus muscle, (B) Adipose tissue and (C) Liver using qPCR. Values were normalized with U6 and expressed relative to control diet which was set to 1. Data are represented as Mean ± SEM (n=3).

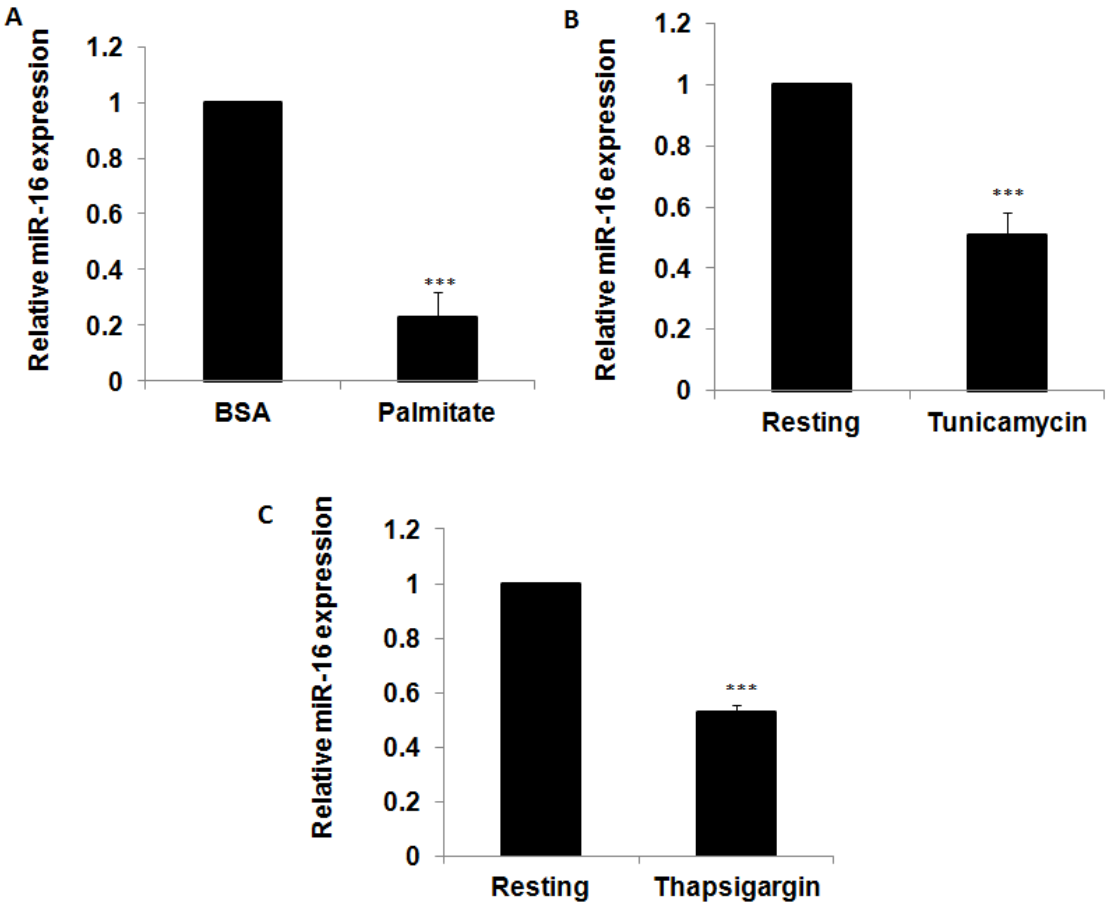


Figure 4.7. miR-16 expression is down-regulated in skeletal myoblasts treated with palmitate, tunicamycin and thapsigargin. C2C12 cells were treated with (A) 500µM of palmitate, (B) 5µg/mL of tunicamycin and (C) 300nM of thapsigargin for 24 h were lysed in TRIzol and cDNA was prepared. cDNA was used for determining miRNA expression and data was normalized to control U6. Data shown are a representative of three independent experiments (Mean ± SEM).

4.8. Functional effects of miR-16 on insulin stimulated glucose uptake in L6 myoblasts

To further investigate the role of miR-16 in mediating insulin sensitivity, we determined its functional effects on insulin stimulated glucose uptake. For this, we have ectopically expressed miR-16 in L6 myoblasts and performed 2-NBDG glucose uptake assay and we observed that in comparison to control cells, forced expression of miR-16 improved both basal as well as insulin stimulated glucose uptake suggesting that miR-16 exerts insulin sensitizing effects on myoblasts (Figure 4.8A). Next in order to examine the underlying mechanisms, we studied the expression levels of GLUT4 and MEF2A, key players involved in insulin mediated glucose uptake. The degree of inflammation induced insulin resistance is generally assessed by the rate of glucose disposal from circulation/medium by GLUT4. It is well documented in literature that the levels of GLUT4 are reduced in TNF-α exposed adipocytes. Further, in our earlier experiments we observed that MEF2A levels were also reduced upon TNF-α stimulation (data not shown). Importantly, the expression of GLUT4 is in direct control by MEF2 family of transcription factors with a major contribution of MEF2A. In addition, in our current data, we observed that basal glucose uptake levels were also enhanced upon miR-16 over-expression. Considering all this, we analyzed the expression of GLUT4 and MEF2 (MEFA and C) upon miR-16 over-expression in L6 myoblasts. We noted that GLUT4 and MEF2A expression levels are significantly up-regulated (Figure 4.8B, 4.8C), in miR-16 over-expressing myoblasts; however no significant change was noticed in the expression of another isoform of MEF2 (Figure 4.8D), MEF2C, demonstrating the specificity of the miR-16. Further, no change

was also observed in the expression pattern of PHLPP1 (Figure 4.8E), a crucial player in regulating Akt driven insulin signaling cascade in peripheral tissues. In parallel, miR-16 over-expression in L6 myoblasts was validated using qPCR analysis (Figure 4.8F). Collectively our data establishes the role of miR-16 in regulating M (LPS+IFNγ) macrophages driven inflammatory responses and positions it as an important player in enhancing insulin sensitivity in tissues such as skeletal muscle.

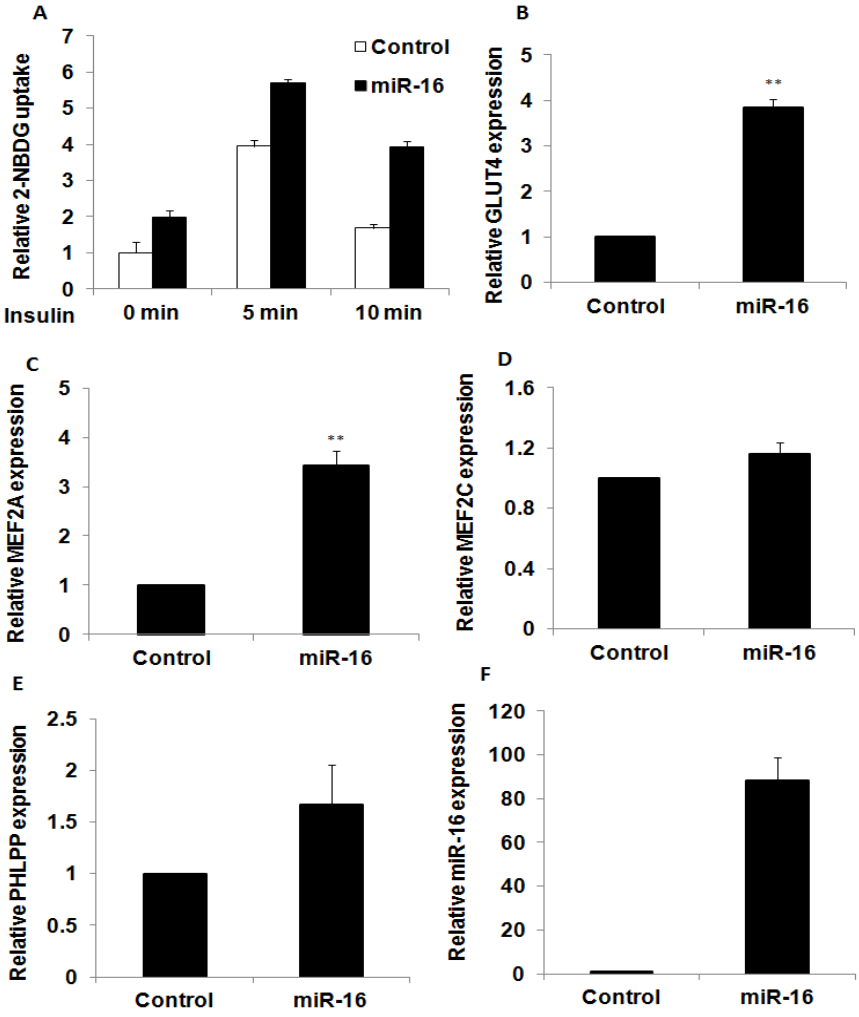


Figure 4.8. Ectopic expression of miR-16 enhances insulin stimulated glucose uptake in L6 skeletal myoblasts. (A) Mean basal and insulin stimulated glucose uptake was measured using 2-NBDG by fluorometry in L6 myoblasts transfected with control or miR-16 mimic. Values are normalized with the corresponding protein content and expressed relative to basal control cells which was set as 1. Data (mean ± SD) shown are a representative of three independent experiments. mRNA expression levels of rattus (B) GLUT4, (C) MEF2A, (D) MEF2C, (E) PHLPP expression in miR-16 mimic over-expressed L6 myoblasts was analyzed by qPCR. Data shown are a representative of three independent experiments. (F) In parallel, we validated the over-expression of mature miR-16 by qPCR.

4.9. Summary

In this study we have identified that miR-16 expression is down-regulated in inflammatory conditions in macrophages and metabolic tissues of HSD fed rats. We have showed that forced expression of miR-16 suppresses macrophage mediated inflammatory cytokine production and their downstream inhibitory effects on insulin signaling pathway. Furthermore ectopic expression of miR-16 improved insulin mediated glucose uptake in L6 myoblasts via up-regulation of GLUT4 and MEF2A. Collectively our data highlight the important role of miR-16 in ameliorating inflammation induced insulin resistance.

Chapter 5

Discussion and future perspectives

Macrophages are regulatory cells which play a vital role in maintaining tissue homeostasis. Broadly there are two categories of macrophages; M (LPS+IFNγ) and M (IL-4+IL-13) depending on their activation signals and functional outcomes and any imbalances in their functions leads to disruption of immunoregulatory functions. Macrophages have specifically evolved to control inflammation which is an essential process in mediating immunomodulation, de-regulation of which exerts deleterious effects [1]. Although several canonical factors controlling macrophage mediated inflammation have been identified such as Kruppel like factors (KLF), Peroxisome proliferating activated receptors (PPARs), TSC-mTORC, IRF, PTEN, epigenetic regulation etc., microRNAs are gaining importance because of their increasing or active participation in regulation of biological processes [20, 92, 94, 102, 103, 277]. Hence we were interested in identifying the regulatory role of microRNAs in the control of macrophage polarization (activation) and their effect on myoblast insulin resistance. In this study we have identified for the first time, two potential microRNAs; miR-712 and miR-16 which were involved in dampening macrophage pro-inflammatory responses and their inhibitory effects on skeletal myoblast insulin sensitivity (negative regulators of inflammation).

miR-712 regulates macrophage mediated inflammatory responses and insulin sensitivity in skeletal myoblasts

In the present study we aimed to demarcate the role of microRNAs in inflammation induced insulin resistance because microRNAs have become the potential therapeutic agents in treating a wide variety of diseases. Using microRNA microarray profiling, we

obtained the signature of miRNAs that are differentially modulated in polarized macrophages. We noted that among several microRNAs, miR-712 was one of the most significantly down-regulated microRNAs in LPS+IFNγ polarized macrophages. Further we observed similar decreasing trend in its expression under various inflammatory conditions such as palmitate exposed and *M.bovis* infected macrophages.

Computational analysis revealed that several genes involved in inflammation may be the targets of miR-712 and few genes known to participate in metabolism and cancer were also predicted to be targeted by miR-712. Of all the pathways predicted to be targeted by miR-712, we observed that 62 pathways were specifically associated with macrophage functions. These pathways importantly constituted genes involved in infections (bacterial, viral, parasitic), immune responses (innate and adaptive), inflammatory diseases (Cardiovascular, Alzheimer's, Parkinson's, Huntington's disorders), metabolic functions (insulin, fatty acid, sphingo, glycan, inositol metabolism etc) and cytoskeletal organization (Endocytosis, lysosomes, phagocytosis, transport molecules etc.). Based on these observations we hypothesized that miR-712 is strongly associated with macrophage mediated inflammation and insulin resistance (metabolic disease). We next analyzed the functional effects of miR-712 by performing gain of function experiments where we observed that over-expression of miR-712 caused regression of pro-inflammatory responses (decrease in iNOS, TNF-α, IL-6 and IFN-β expression) highlighting its potential anti-inflammatory role. In striking contrast to our results, Son *et al.* in the year 2013 have portrayed miR-712 as a pro-atherogenic microRNA responsible for inducing atherosclerosis [156]. This observed discrepancy could be due to differences in the cell types used, as they have reported its pro-inflammatory role in endothelial cells of mice.

Besides, it is a well known fact that the expression of miRs is tissue specific and thus their functions may vary from one cell to the other. Although they share common targets in every cell due to their conserved seed sequence, depending on the stimuli they receive and the type of gene expression in the cell, their targets may vary and thus they may exert different functional effects. For instance, Liu *et al.* in the year 2012 have showed that forced expression of miR-155 into cardiomyocytes inhibited their migratory function while another study performed by Li *et al.* in the same year have showed that silencing of miR-155 inhibited migration of renal cancer cells emphasizing the contrasting roles of miR-155 in different cell types [278, 279]. Similarly Pancratz *et al.* in the year 2015 have showed that miR-155 exhibited divergent roles in different forms of vascular growth in endothelial cells where it promoted pro-arteriogenesis whilst inhibiting angiogenesis; as both of them differ greatly with regard to external stimuli and downstream regulatory factors [280]. In line with the above facts we have further observed that unlike in macrophages, the expression levels of miR-712 in myoblast cells are up-regulated in response to pro-inflammatory insults such as palmitate, tunicamycin and thapsigargin and its role in these cells requires further elucidation. Furthermore in our studies, we have showed that miR-712 over-expression in macrophages displayed inhibitory effects on inflammation induced insulin resistance in skeletal myoblasts. However we still do not know whether the effect is because of an autocrine action of miR-712 on skeletal myoblasts, as miRs are recently shown to be secreted out to perform effector functions or is it a consequence of the reduced paracrine effects of LPS+IFNγ polarized macrophages. Because of its distinct expression levels across different cells, it may exhibit diverse functions in various cell types, thus over-expression of miR-712 directly into skeletal

muscle may have opposite effects on insulin sensitization in comparison with over-expression into macrophages. For instance, Liu *et al.* in the year 2012 have showed that though the expression levels of miR-221/222 were high, the biological functions of miR-221 and miR-222 in vascular walls were cell specific and exhibited opposite effects on proliferation, migration and apoptosis in vascular smooth muscle cells (VSMCs) and endothelial cells (ECs) [281]. Similarly independent studies reported that biological expression of microRNAs were different across cancer cell types, where independent studies showed that miR-199a-3p and miR-99b-3p expression levels where up-regulated in ovarian and breast cancer while the expression was down-regulated in hepatocellular carcinoma though the mechanism of action is unclear [282]. Hence there is a necessity to understand the complex nature of such type of microRNAs. Next we attempted to study the effect of miR-712 on IL-4 polarized macrophages where our data showed that miR-712 did not influence any markers associated with anti-inflammatory macrophages indicating that it is specifically positioned to control macrophage mediated pro-inflammatory responses.

Next we wished to probe into mechanistic facts on how miR-712 dampens pro-inflammatory responses for which we shortlisted a few targets of miR-712 based on computational analysis. We observed that ectopic expression of miR-712 into macrophages reduced LRRK2 expression levels in un-stimulated cells both at transcript and protein levels. LRRK2 is a serine / threonine protein kinase and it is well known to be associated with inflammatory diseases such as Crohn's and Parkinson's disease [283, 284]. Kuss et al. in the year 2014 have documented that IFNγ induces LRRK2 via activation of ERK5 kinase in macrophages [285]. Also LPS alone was shown to induce

LRRK2 expression [275]. However the effect of LPS in combination with IFNγ which mimics the exact disease state on LRRK2 expression needs to be further determined. On the other hand Rovida et al. in the year 2008 have reported that ERK5 is highly activated during CSF-1 induced macrophage proliferation (usually seen in neoplasia) but not during LPS or IFNγ induced macrophage activation [286]. This incongruity could be probably because of different types of cells and experimental conditions used in the study. Hence we feel that there is a necessity to study the role of LRRK2 in LPS and IFNγ co-treatment because LPS and IFNγ trigger target gene expression by different mechanisms. Additionally our data suggested that miR-712 attenuated pro-inflammatory responses by reducing phosphorylation of p38 and ERK1/2 in LPS+IFNγ polarized macrophages. Bioinformatics analysis has shown that miR-712 may putatively target Pde7a which is recently shown to have a causative role in inflammatory diseases such as Asthma, COPD, RA and Lupus [287]. Due to limitations involved in drug designing, inhibiting Pde7a is currently not possible. Hence in such cases miR-712 over-expression would be of therapeutic interest. Similarly in silico analysis has revealed leptin receptor (Lr) as a target of miR-712 where the deficiency of this receptor is known to induce extreme hunger and consequently leading to obesity and insulin resistance [288]. In line with these observations, our data indicated that during pro-inflammatory conditions (palmitate, tunicamycin and thapsigargin treatment) miR-712 levels were increased in skeletal myoblasts and this could be the possible mechanism for decreased expression of leptin receptor in obese or insulin resistant mice and depletion of miR-712 expression levels in such cases may help us in restoring the normal physiological functions. Since our data provides the first line of evidence on the role of miR-712 in glucose metabolism,

further evaluation is required to implicate its role in physiology and disease. Exploring the physiological role of miR-712 in insulin mediated glucose uptake may further help us in better understanding of T2D progression and treatment.

miR-712 being a mouse specific microRNA, although cannot be directly used for human applications, can still primarily be used as model to study various diseases associated with it. However in diseased conditions where its functional homolog miR-205 expression is compromised, replacement with miR-712 may have beneficial effects. Or under certain circumstances, miR-712 can exert potential functional effects compared to its homologs; hence if we can identify such conditions we can apply them for human benefits. Since anti-miR-712 nanoparticles have been designed to prevent atherosclerotic lesions in mouse, similar strategy can be employed in designing miR-712 mimic or antagonist to treat diseases in a tissue or a cell targeted manner.

Based on miRSystem pathway analysis we speculate that miR-712 in addition to controlling inflammatory responses, may have an important role in regulating cancer development as several genes chiefly involved in regulating cell proliferation, apoptosis and several types of cancers were predicted to be miR-712 targets. Tumours are frequently associated with different activation states of macrophages (TAMs, atypical and pro-inflammatory macrophages) where they are shown to execute dual roles by promoting and regressing tumours. They are shown to be associated with all the stages of cancer starting from tumour initiation to cancer progression including metastasis [25]. Thus we strongly presume that miR-712 may have a key role in driving macrophage mediated inflammatory diseases such as insulin resistance and cancers and determining its role in such cell types may be informative for therapeutic benefits.

In summary our work demonstrated that the restoration of miR-712 expression in M (LPS+IFNγ) macrophages may ameliorate insulin sensitivity which could be beneficial during the treatment of T2D.

miR-16 regulates macrophage mediated inflammatory responses and insulin sensitivity in skeletal myoblasts

Secondly we have chosen miR-16 for further analysis because although being versatile in its expression and functions, its role in inflammation induced insulin resistance is poorly defined. Consistent with many related studies, our data showed that miR-16 expression is notably down-regulated in inflammatory macrophages, myoblasts and metabolic tissues of HSD fed rats suggesting its adaptable role in different cells. On the contrary, several reports in the existing literature have documented that miR-16 expression is increased during inflammation which repeatedly suggests that the function of miRs is complex and context dependent.

Further in our profiling results, we noted that miR-16 expression was moderately reduced in M (LPS+IFNγ) macrophages. Although the changes in their expression levels are small in magnitude they are likely to have significant effects on cellular physiology. For instance, microRNA profile in polarized human monocyte derived macrophages (MDMs) also displayed small magnitude changes in their expression levels despite robust functional responses to MDM polarizing conditions. More specifically, treatment of MDMs or differentiated THP1 with LPS and IFNγ increased the levels of miR-29b by less than 2 fold, nevertheless over-expression of miR-29b significantly increased the expression of TNF-α, IL-6 and CXCL9 in a dose dependent manner. Further, we have

recently showed that PHLPP, a serine / threonine phosphatase is reduced by about 50% in LPS treated immune cells (RAW 264.7 macrophages, THP1, BMDMs, and PBMs) however reducing cellular levels of PHLPP to about 50% by siRNA robustly enhanced iNOS expression reiterating the fact that small changes need not necessarily be inconsequential. Although the changes in miRs in our present studies and in Graff *et al.* are small, they may exert significant effects because miRs may act in coordination with other miRs belonging to same family or other family (which are also stimulus responsive) and the final outcome may be a balanced effect of all the players involved [236, 266]. Supporting this possibility, Moon et al. in the year 2014 have showed that co-transfection of miR-16 and miR-15a mimics reduced the phagocytic activity of BMDMs, while in our study, no significant effect on phagocytosis was noted upon miR-16 over-expression alone because a synchronized effect of miR-15a and miR-16 may be possibly required for influencing phagocytosis [252]. Since miR-16 is a ubiquitous microRNA, it may probably regulate several physiological processes such as cell proliferation/death. Thus it may be possible that the change in its expression level is desirably maintained low (~2-3 fold) across different cells under inflammatory conditions and therefore any huge alterations in its expression may be detrimental to the cell.

Subsequently we analyzed the functional effects of miR-16 by performing gain of function experiments where we noted that as observed earlier, over-expression of miR-16 caused regression of LPS+IFNγ induced pro-inflammatory responses (decrease in TNF-α, IL-6 and IFN-β expression), suggesting that it may exert anti-inflammatory effects in diseases linked to inflammation. Next we determined the effect of miR-16 on M (IL-4) polarized macrophages where we observed that miR-16 promoted IL-4-stimulated

arginase-1 expression but showed no effect on other M (IL-4) markers such as MRC-1, IL-10 and YM1. These observations restate the fact that miRNA specifically regulate signaling cascades in M (LPS+IFNγ) or M (IL-4) polarized macrophages. Next we have observed in this study that the addition of conditioned supernatants of miR-16 transfected and M (LPS+IFNγ) skewed macrophages on to L6 myoblasts, partially restored insulin stimulated glucose uptake. Interestingly, over-expression of miR-16 directly into L6 myoblasts improved insulin sensitivity of cells by up-regulating GLUT4 and MEF2A expression. Our observations are in line with the findings of Jette *et al.* where they noted that miR-16 expression was reduced in L6 myoblasts cultured under high glucose conditions and in skeletal muscles of T2D patients [273]. Till date although miR-16 targeting AU rich elements is well illuminated, the functional role of miR-16 in macrophage polarization induced by LPS and IFNγ and inflammation induced insulin resistance (IR) in skeletal muscle is not well studied [289]. Hence our data portrays for the first time, the insulin sensitizing effects of miR-16 in skeletal myoblast. Other aspects of the study such as the expression patterns of miR-16 in HSD rats (a model for inflammation induced insulin resistance) are novel as well. Further, the findings of this study are in line with the observations of Ye *et al.* where they reported that miR-16 was decreased under high glucose conditions in retinal endothelial cells (RECs) of human, while ectopic expression of miR-16 significantly improved the phosphorylation of insulin receptor (IR) with a concomitant decrease in inflammatory cytokine TNF-α [290].

How miR-16 regulates pro-inflammatory responses of macrophages and enhances insulin sensitivity in myoblasts at molecular level? Besides mRNA destabilizing effects of miR-16, miR-15a/16 were recently shown to target TLR4 expression by regulating PU.1

transcription factor upon LPS or bacterial stimulation leading to enhanced phagocytosis and ROS production and increased expression of IL-1β, IL-6 and IL-21 cytokines [252, 289]. In addition, several putative targets of miR-16 identified by computational tools such as TargetScan likely explain the effects of miR-16 on M (LPS+ IFNγ) macrophages. These putative targets include TRAF3, IRAK2, TAB3, LITAF and MAPK pathway players (MEK1, ERK1 and Raf1) which have a very important role in TLR4/NFkB signalling [291-293]. Further, although speculative, other possibility could be that miR-16 may be down-regulating CCDN1 and Bcl2 expression where CCDN1 regulates Cdk4 and Cyclin D/E activity [294, 295]. Recent studies reported that Cdk4/cyclin D/E complex is involved in the regulation of inflammatory responses and glucose metabolism [296, 297]. Bcl2, an anti-apoptotic protein, is also shown to associate directly with insulin receptor substrates (IRS1/2), which has the potential to alter the insulin signalling [298]. Further Kwon *et al*. proposed that miR-16 may have a role in insulin/PI3K-Akt signaling cascade [299]. Exploring the physiological role of miR-16 in insulin mediated glucose uptake may further help us in better understanding of T2D progression and treatment.

In summary our work demonstrated that the restoration of miR-16 expression in M (LPS+IFNγ) macrophages or in skeletal muscle may improve insulin sensitivity which could be beneficial for treatment of T2D.

Chapter 6

Materials and Methods

6.1. Materials

The reagents, chemicals and solvents used for the experiments are listed in the following tables.

Table 6.1. Recombinant proteins or enzymes and biochemicals required for treatments and assays

Recombinant proteins/ Biochemicals	Company	Catalogue
E.coli LPS-0127:B8	Sigma-Aldrich (St. Louis, MO, USA)	L4516
Fatty acid free BSA	Sigma-Aldrich (St. Louis, MO, USA)	A8806
Palmitate	Sigma-Aldrich (St. Louis, MO, USA)	P5585
Insulin (Bovine pancreas)	Sigma-Aldrich (St. Louis, MO, USA)	I0516
Recombinant mouse IFNγ	PeproTech (Rocky Hill, NJ, USA)	315-05
Recombinant IL-4	PeproTech (Rocky Hill, NJ, USA)	214-14
Recombinant IL-13	PeproTech (Rocky Hill, NJ, USA)	210-13
Recombinant mouse M-CSF	e-Bioscience, Inc. (San Diego, CA, USA)	14-8983-80
Escherichia coli (K-12 strain) BioParticles fluoresceine conjugate	Molecular Probes (Invitrogen, Carlsbad, CA, USA)	E2861
D-Luciferin	Gold Biotechnology (Olivette, MO, USA)	LUCK-250
Coelentrazine	Gold Biotechnology (Olivette, MO, USA)	C-25

Table 6.2. Reagents required for molecular analysis of biomolecules such as DNA, RNA, cDNA etc.

Reagents for molecular analysis	Company	Catalogue
Carboxy-H2DCFDA	Molecular Probes (Invitrogen, Carlsbad, CA, USA)	C400
Enhanced chemiluminescence	GE Healthcare Life Sciences (Piscataway, NJ, USA)	RPN2232
2-NBDG	Molecular Probes (Invitrogen, Carlsbad, CA, USA)	N13195
TRIzol	Ambion (Ambion, Austin, TX, USA)	15596018
DNase I	Ambion (Ambion, Austin, TX, USA)	AM2222
Power SYBR Green PCR Master Mix	Ambion (Ambion, Austin, TX, USA)	4367659
DNTPs	Invitrogen (Invitrogen, Carlsbad, CA, USA)	18427088
Random hexamers	Merck Millipore (Billerica, MA, USA)	61069247007A
Taq polymerase	Merck Millipore (Billerica, MA, USA)	11615010
KOD polymerase	Merck Millipore (Billerica, MA, USA)	710863
T4 DNA Ligase	Invitrogen (Invitrogen, Carlsbad, CA, USA)	15224017

Table 6.3. MicroRNA mimics used in our studies

MicroRNA mimics	Company	Catalogue
miR-16-5p mirVana miRNA mimic (MC10339)	Ambion (Ambion, Austin, TX, USA)	4464066
miR-712-5p mirVana miRNA mimic (MC11561)	Ambion (Ambion, Austin, TX, USA)	4464066
mirVana microRNA mimic, Negative Control #1	Ambion (Ambion, Austin, TX, USA)	4464058

Table 6.4. Reagents used for cell culture experiments

Cell culture reagents	Company	Catalogue
DMEM	Thermo Fischer SCIENTIFIC (Waltham, MA, USA)	11995073
FBS	Thermo Fischer SCIENTIFIC (Waltham, MA, USA)	10082147
Pen Strep	Thermo Fischer SCIENTIFIC (Waltham, MA, USA)	1514012
Trypsin-EDTA	Thermo Fischer SCIENTIFIC (Waltham, MA, USA)	15400-054
Nucleofector Solution V	Lonza, Allendale, NJ, USA	VVCA1003 KT
Lipofectamine 2000	Invitrogen (Invitrogen, Carlsbad, CA, USA)	11668027

Table 6.5. Antibodies used in our studies for western blot analysis

Antibodies	Company	Catalogue
Anti-iNOS	BD Biosciences (San Jose, CA, USA)	610329
Anti-LRRK2-MJFF2 (c41-2)	Abcam (Kendall Square, Cambridge, UK)	ab133474
Anti-β-actin	Santa Cruz Biotechnology (Santa Cruz, CA, USA)	sc-1615
Anti-phospho p44/42 MAPK(ERK1/2)(Thr202/Tyr204)	Cell SignalingTechnology (Cell Signaling Technology, Inc., Beverly, MA, USA).	9101
Anti-p44/42MAPK(ERK1/2) antibody	Cell SignalingTechnology (Cell Signaling Technology, Inc., Beverly, MA, USA).	9102
Anti-phospho p38 MAPK(Thr180/Tyr182)	Cell SignalingTechnology (Cell Signaling Technology, Inc., Beverly, MA, USA).	9211
Anti-p38 MAPK antibody	Cell SignalingTechnology (Cell Signaling Technology, Inc., Beverly, MA, USA).	9212
Anti-mouse HRP	Genscript (Genscript, Piscataway, NJ, USA)	A00160
Anti-rabbit HRP	Genscript (Genscript, Piscataway, NJ, USA)	A00098

Table 6.6. Kits used for cytokine analysis and gene expression studies

Kits	Company	Catalogue
TNF-α ELISA kit	R&D systems (Minneapolis, MN, USA)	510-RT-010
IL-6 ELISA kit	PeproTech (Rocky Hill, NJ, USA)	900-k50
IP-10 ELISA kit	PeproTech (Rocky Hill, NJ, USA)	900-M153
NCode VILO miRNA first strand cDNA synthesis kit	Invitrogen (Invitrogen, Carlsbad, CA, USA)	A11193050
SuperScript III reverse transcriptase kit	Invitrogen (Invitrogen, Carlsbad, CA, USA)	18080044

Table 6.7. Chemicals used for cell lysis and biomolecular analysis

Chemicals / reagents	Company	Catalogue
Trypan Blue Solution	Sigma-Aldrich (St. Louis, MO, USA)	T8154
Sodium dodecyl sulfate (SDS)	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	194821
Acrylamide	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	14824
Bisacrylamide	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	134985
Ammonium persulfate (APS)	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	148134
Protein ladders	Bio-Rad Laboratories (Hercules, CA, USA)	1610374
TEMED	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	202788
Tris HCL	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	84073
Disodium-EDTA	USB (Affymetrix, Santa Clara, CA, USA)	15701
Bovine serum albumin (BSA)	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	140299
Bradford reagent	Sigma-Aldrich (St. Louis, MO, USA)	B6916
sodium orthovanadate ($Na_3VO_4.2H_2O$)	Sigma-Aldrich (St. Louis, MO, USA)	450243

Aprotinin	AMRESCO (Cleveland, OH, USA)	E429
Leupeptin	AMRESCO (Cleveland, OH, USA)	M180
Glycine	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)	074933
Thapsigargin	Cayman CHEMICAL (Ann Arbor, MI, USA)	10522
Tunicamycin	Cayman CHEMICAL (Ann Arbor, MI, USA)	11445
Triton X-100	Sigma-Aldrich (St. Louis, MO, USA)	T8787
DNA ladders (50bp, 10kb)	Merck Millipore (Billerica, MA, USA)	-

Table 6.8. General chemicals and solvents used in our experiments

Chemicals / Solvents/ reagents	**Company**
Chloroform	Rankem (Okhla industrial area, New Delhi, India)
Methanol	
Hydrochloric acid (HCL)	
Sulfuric acid (H_2SO_4)	
Ethanol (Molecular grade)	AMRESCO (Cleveland, OH, USA)
Glycerol	Sisco Research Laboratories (SRL-Andheri east, Mumbai, India)
Sodium hydroxide (NaoH)	
Sodium chloride (Nacl)	
Potassium chloride (Kcl)	
Disodium phosphate (Na_2HPO_4)	
Mono potassium phosphate (KH_2PO_4)	
Dithiotreitol (DTT)	
Luria Bertani agar	
Luria Bertani broth	
Isopropanol	
Bromophenol blue	
Agarose	
Ethidium bromide	
Sodium pyrophosphate ($Na_4P_2O_7$)	
Sodium fluoride (NaF)	

6.2. Methods

6.2.1. Cell line maintenance and differentiation

RAW 264.7, HEK 293T and L6 cells were cultured in DMEM and maintained in 10% heat-inactivated foetal bovine serum (FBS) while C2C12 cell line was grown in DMEM containing 20% FBS. All the cells were grown in 1% streptomycin-penicillin containing medium. RAW 264.7 cells were maintained by lifting the cells gently using scrapers while HEK 293T, L6 and C2C12 cells required trypsinization. Cells were provided with 5% CO_2 and 37°C for maintenance and treatments. All the cells were grown as monolayer adherent cells in polystyrene coated culture dishes (Corning, NY, USA).

C2C12 differentiation was performed using 2% horse serum (HS) where cells were grown to 90% confluency and were incubated in HS media for 48 h. They were further incubated in fresh HS containing media for another 24 h and post 3 days of differentiation, cells were given respective treatments for desired time points.

Cell preservation was achieved by re-suspending any type of cells in 90% FBS, 5% to 10% DMSO containing DMEM medium followed by gradual freezing at -1°C/minute. After cells have attained -80°C, were then shifted to liquid nitrogen for final storage.

6.2.2. Culture of murine BMDMs

BMDMs were derived from mice as described earlier. Briefly, around four month old mice were sacrificed, and the long bones (Humerus, radius, ulna, tibia and femur) were excised carefully using scissors from fore limbs and hind limbs. Bones which are intact from both the ends were harvested and stored in cold DMEM medium. Inside the sterile culture hood, surplus mass surrounding the bones was neatly removed. The ends of the

intact bones were cut and the bone marrow cells were flushed into a falcon tube containing pre-warmed DMEM medium using 1 mL syringe. The flushing was repeated multiple times with medium until the red marrow turned into white. The bone marrow cells were then centrifuged at 200g for 3 minutes at 4°C and the cell pellet was carefully resuspended in DMEM containing 10% FBS (0.1X Penstrep was used, if needed) and were seeded into 100mm culture dishes. The marrow cells per mouse were plated into four 100mm dishes and these cells were ex vivo differentiated into freshly prepared macrophage differentiation medium (DMEM+10% FBS+0.1XPenstrep+20ng/ml M-CSF; 1.5µl of M-CSF stock (100ng/ml) to 7.5 ml medium plated in each100mm dish). On 4th day of culturing, another 4 ml of macrophage differentiation medium was added to the same plate containing cells. On 7th day of differentiation, around 80-90% cells were observed to be adherent and were considered as differentiated macrophages; Bone Marrow Derived Macrophages (BMDMs). Adherent macrophages were carefully separated from non-adherent cells by gently scraping them. The collected cells (~10 million BMDMs from single mouse) were centrifuged at 200g for 5 minutes at RT and re-seeded into culture dishes before they were used in experiments.

6.2.3. Cell stimulations

RAW 264.7 cells are treated with 1µg/mL of LPS and 100ng/mL of IFNγ for indicated time points. Similarly they were stimulated using 10ng/mL of IL-4 and IL-13. LPS, IFNγ, IL-4 and IL-13 were obtained as lyophilized powders and they were re-constituted in plain DMEM medium. Tunicamycin and thapsigargin were resuspended in DMSO as per manufacturer's instructions and C2C12 cells were treated at 5µg/mL and 300nM

concentrations, respectively. RAW 264.7 and C2C12 cells were treated with palmitate as discussed below.

6.2.4. Palmitate preparation and treatment

The stock solution of 50 mM palmitate (C16:0) was prepared by dissolving palmitate in pre-heated (70ºC) 0.1N NaOH. The solution was further diluted 5 fold in pre-warmed 25% BSA solution to give a final stock of 10mM. Resting or transfected cells were then treated with 500µM of palmitate for 24 h. Cells were serum starved for minimum of 3 h prior to stimulation.

6.2.5. Nucleofection

RAW 264.7 cells were cultured in 10% fetal bovine serum (FBS) containing DMEM medium at 37°C in 5% CO_2 environment. All transfections were carried out using the Solution V (Lonza, Allendale, NJ, USA) of nucleofection apparatus (Lonza, Allendale, NJ, USA) according to manufacturer's instructions. Briefly, 12 X 10^6 cells were nucleofected with 300 nM of control or miR-16 mimic after gently resuspending them in 100µl of nucleofector solution V. Post nucleofection, 500µl of pre-warmed medium was added to the transfected cells and were then transferred to the plate containing 10ml of FBS containing DMEM. Plates were then incubated for minimum of 16 h at 37°C and transfected cells were then seeded at equal density and treated as mentioned in figure legends.

6.2.6. Lipofection

HEK 293T and L6 myoblasts were transfected using Lipofectamine 2000 as per manufacturer's instructions. The final concentration of miRNA mimics used for L6 myoblasts transfection was 100nM. The concentration of miRNA mimics used for HEK

293T transfections for luciferase assay was about 250nM. Post 24 h of transfection, all the cells were utilized for experiments.

6.2.7. Cell lysis and western blotting

RAW 264.7 cells post LPS and IFNγ stimulations for respective time points were washed in phosphate buffered saline (PBS). Subsequently, cells were lysed in TN1 lysis buffer containing protease inhibitors. The total protein was estimated by Bradford assay and protein-matched lysates were denatured and resolved on 10% SDS PAGE. The proteins separated by electrophoresis were next transferred on to PVDF membrane using wet transfer apparatus and the membrane was incubated with preferred primary antibody for overnight at 4°C. The membrane was then washed with TBST buffer (Tris buffered saline with 0.1% Tween20) for minimum of three times and was then incubated with horse radish peroxidase (HRP) conjugated secondary antibody for 1 h at room temperature. Post secondary antibody incubation, the membranes were washed again and the protein of interest was then detected using enhanced chemiluminescence (ECL).

6.2.8. Real Time PCR analysis

Post stimulation cells were carefully washed with PBS and total RNA was extracted using Trizol according to manufacturer's instructions. The qualitative and quantitative assays for total RNA was performed using NanoDrop and agarose gel analysis. The reverse transcription for detecting microRNAs and other gene expression was performed using NCode VILO miRNA and Superscript III first strand cDNA synthesis kits respectively. Reverse transcription was performed according to manufacturer's instructions. The principle of microRNA cDNA synthesis using NCode VILO miRNA cDNA synthesis kit is illustrated in the figure 6.1. The kit provides universal reverse

primer which can be commonly used for all the microRNAs and genes, however miR / gene specific forward primers needs to be designed and the primers for miRs which we have chosen to work are listed in table 6.9. The reaction mixture and the program used for the cDNA reaction are listed in the table 6.10 and table 6.11 respectively. qPCR was performed on Step One Plus instrument (Applied Biosystems, Foster City, CA, USA). Data were normalized to respective control genes (18S, actin for mRNA and RNU6 for microRNA) and the results were analyzed using comparative delta C_T method.

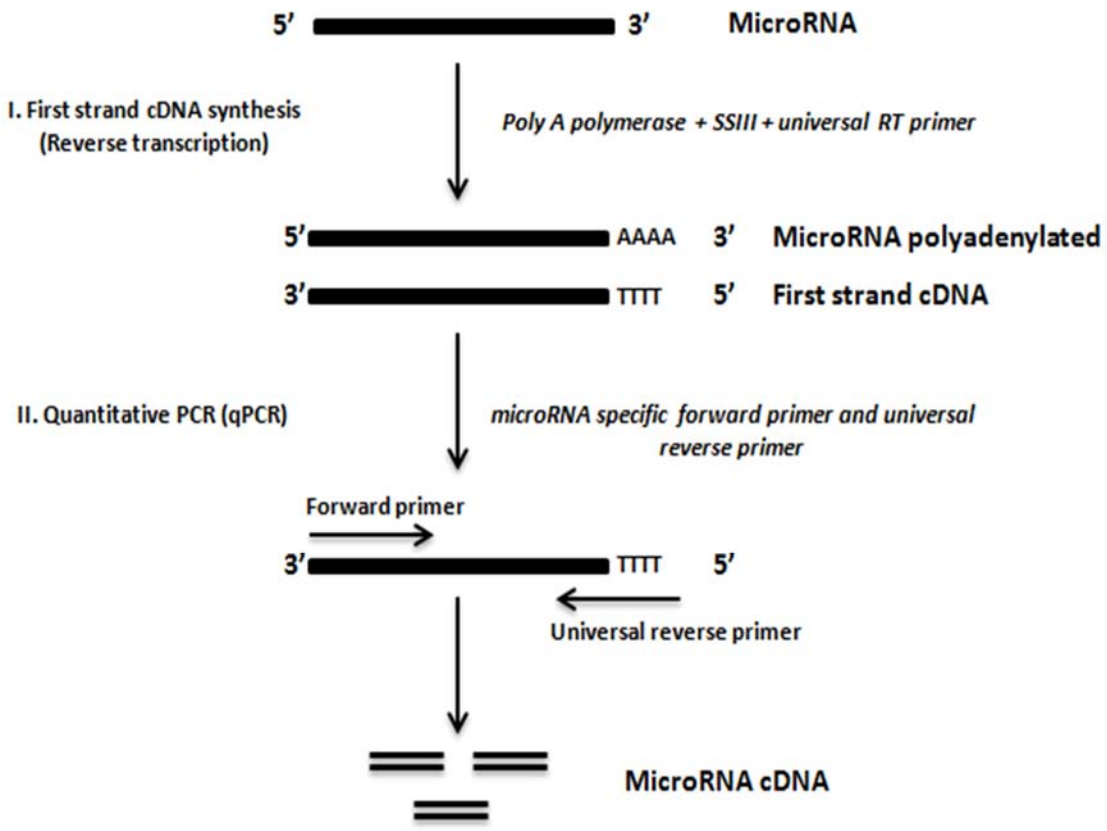


Figure 6.1. MicroRNA cDNA synthesis using NCode VILO miRNA kit. The kit enables poly A tailing of all RNA molecules followed by first strand cDNA synthesis using SuperScript III and universal RT primer. The microRNA / gene specific forward primer and universal reverse primer (provided in the kit) mediate cDNA synthesis. The figure is adapted from Invitrogen NCode VILO miRNA cDNA synthesis kit manual.

Table 6.9. Forward primers and qPCR conditions used for microRNA cDNA synthesis

MicroRNA	Forward primer	Annealing temperature	Amplicon length
miR-712	GTGGCCCCTGCGCAAGGATG	60°C	~ 75-85*
miR-16	CGGTAGCAGCACGTAAATATTGG CGA	60°C	~ 75-85*
RNU6 (Control gene for small RNA)	GTGGCCCCTGCGCAAGGATG	60°C	~ 100*

* indicates the approximate size of the amplicon calculated by us based on the amplicon size detected on the gel, as the length of poly A tail during cDNA synthesis is unspecified. *Amplicon length = Amplicon size - length of the mature microRNA.*

Table 6.10. Reverse transcription mix using NCode VILO microRNA cDNA synthesis kit

S.No	RT reaction mix	Volume
1	cDNA	2µl
2	miR specific forward primer-10µM	0.2µl
3	Universal reverse primer-10µM	0.2µl
4	DEPC water	12.6
5	SYBR with pre-mixed ROX dye	5µl
	Total	**20µl**

Table 6.11. Poly A tailing and first strand cDNA synthesis are performed in a single reaction using following parameters

Factors	Incubation	Termination
Temperature	37 °C	95°C
Time	60 minutes	5 minutes

6.2.9. 3' UTR reporter cloning

A genomic fragment encompassing 0.58 kb 3' UTR of mouse LRRK2 (NCBI nucleotide database) was PCR amplified using RAW 264.7 cell genomic DNA. Then the amplified product was non-directionally cloned into pGL3 promoter vector using XbaI restriction sites. Positive clones selected by ampicillin resistance were screened by restriction digestion and colony PCR and clones were finally confirmed by DNA sequencing analysis. The primers and PCR conditions used for 3'UTR LRRK2 amplification are listed in table 6.12.

Table 6.12. Primers and PCR conditions for LRRK2 3'UTR amplification

MicroRNA	Forward primer	Annealing temperature	Amplicon length
mmu-LRRK2-F.P	CGCTCTAGAAAGACATCAGGCAGCT	54°C	0.58 Kb
mmu-LRRK2-R.P	CGCTCTAGATTTCCTGAGCTAACAAAAATC		

6.2.10. Luciferase assay

HEK 293T cells were transfected with control or miR-712 mimic in combination with PGL3 promoter-luciferase plasmids or PGL3 cloned with LRRK2 3' UTR and renilla luciferase constructs and were incubated for 24 h. Then the cells were lysed in passive lysis buffer and the lysates were mixed with required volumes of luciferase assay buffer (LAB) and the firefly luciferase activity was measured using Sirius Luminometer (Berthold Detection Systems GmbH, Germany). Further renilla luciferase activity was measured using renilla luciferase assay buffer (RLAB). The data was plotted by normalizing firefly luciferase values to the renilla luciferase values.

6.2.11. ELISA for cytokine analysis:

RAW 264.7 cells were polarized using LPS and IFNγ for 10 h and the culture supernatants collected were centrifuged at 14,000 rpm for 3 minutes at 4°C to remove any cell debris present. The amount of TNF-α, IL-6 and IP-10 in the supernatants were analyzed using ELISA kits as per manufacturer's instructions. IFN-β in the supernatants was measured as previously described [300]. All the cytokines were analyzed at 450nm using Wallac 1420 Victor Multimode Plate Reader (Perkin-Elmer, Waltham, Massachusetts, USA).

6.2.12. Microarray microRNA profiling

RAW 264.7 macrophage cells were stimulated with above indicated concentrations of LPS+IFNγ or IL-4+IL-13 for 24 h in duplicates and treated samples were completely lysed in TRIzol. The processed samples were carried in dry ice and were outsourced for Affymetrix chip based microRNA analysis (Chip version-Affymetrix miRNA 2.0). The quality of RNA was assessed by using Agilent Bioanalyzer system (Agilent

Technologies, Santa Clara, CA, USA) where they have measured RNA integrity number (RIN > 8 indicates good quality RNA) and ribosomal RNA ratios (28S:18S ratio of 2:1 indicates good quality RNA). The qualities of our samples are mentioned in the table 6.13. The microRNA analysis was performed at iLife Discoveries (Gurgaon, Haryana, India) where they have hybridized the processed samples on to the chip containing miRNA probes for 17 h at 45°C. The raw data sets were analyzed using GeneSpring GX 12.0 software for determining differential gene expression and clustering analysis.

Table 6.13. Qualitative assessment of RNA using Agilent Bioanalyzer

S. No	Sample ID	RNA concentration	RIN value	28S/18S ratio
1	Resting-1	643 ng/µl	9.2	1.7
2	Resting-2	732 ng/µl	9.2	1.7
3	LPS+IFNγ-1	762 ng/µl	9.2	1.7
4	LPS+IFNγ-2	803 ng/µl	9.1	1.6
5	IL-4+IL-13-1	511 ng/µl	9.2	1.6
6	IL4+IL-13-2	712 ng/µl	8.5	1.7

6.2.13. Animal treatment

These experiments were conducted at Institute of Science, Nirma University, Ahmedabad, India. Adult Wistar albino rats which were healthy and weighing 150 ± 10g were maintained in polypropylene cages were fed with either regular diet (control group) or high sucrose diet (65% treated group) for 60 days. Animals were sacrificed according to the guidelines provided by the committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA). The procedure involving animals was reviewed and approved by the Animal Ethical Committee of Institute of Science, Nirma University, Ahmedabad, India (Protocol No. IS/BT/FAC-13-1009).

6.2.14. Phagocytosis measurements

RAW 264.7 cells phagocytic efficiency was measured using fluorescent *E.coli* bacteria K-12 strain. The lyophilized bacteria were resuspended in PBS as per manufacturer's instructions and RAW 264.7 cells transfected with control or miR-16 mimic were infected with or left un-infected at a multiplicity of infection (MOI) of 10. The infected cells were then centrifuged at 650g for 2 min for establishing optimal contact between bacteria and macrophages. The infection was performed for 3 h at 37°C. Then the bacterial supernatant was removed and external fluorescence was quenched by using 500µg/mL of trypan blue of pH 4.4 (set with citrate buffer). The cells were then carefully washed with ice-cold PBS, lysed in 0.1% Triton X-100 and the uptake of fluorescent bacteria by RAW 264.7 cells was determined in the cell lysates using multiplate reader (Perkin-Elmer, Waltham, Massachusetts, USA) with excitation at 494nm and emission at 518 nm.

6.2.15. Reactive oxygen species measurement

Post transfection of miR mimics, RAW 264.7 cells stimulated with LPS and IFNγ for 24 h or left un-stimulated (Resting, R) were incubated with 5µM DCFDA dye for 30 min in dark. Then cells were carefully washed using ice-cold PBS, were lysed in 0.1 % Triton X-100. Amount of ROS in the cell lysates was measured using multiplate reader (Perkin-Elmer, Waltham, Massachusetts, USA) with excitation at 485nm and emission at 535 nm.

6.2.16. Glucose uptake assay

Post nucleofection of microRNA mimics, RAW 264.7 macrophage cells were stimulated with LPS (1µg/mL) and IFNγ (100ng/mL) for 10 h. Supernatants from activated macrophages were harvested and pre-cleared cultured media was transferred to L6 rat

myoblasts for 18 h. As a control, L6 myoblasts were incubated with plain DMEM medium instead of supernatants from inflamed macrophages (control). Cells were serum starved for minimum of 6 h followed by starvation in glucose free medium for 30 min. Next cells were primed with insulin for 5 and 10 min and were incubated with 50μM of 2-NBDG for 15 min. Finally reaction was terminated by washing the cells using ice cold PBS and were lysed in 0.1% Triton X-100. Fluorescence was measured at 485nm excitation and emission at 535 nm on Wallac 1420 Victor Multimode Plate Reader (Perkin-Elmer, Waltham, Massachusetts, USA).

6.2.17. Computational analysis

All the nucleotide sequences of the genes required for expression analysis studies were obtained from NCBI Nucleotide. The microRNA sequences required for our studies were acquired from miRBase. The microRNA pathway analysis was performed using miRSystem analysis tool (http://mirsystem.cgm.ntu.edu.tw/). The microRNA target prediction was done by using TargetScan, Miranda, MiRDB, PITA and DIANA softwares. The gene and miRNA interacting structures were obtained by using RNAhybrid software. The precursor structures of microRNAs were drawn by using RNAfold software (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

6.2.18. Statistical Analysis

Numerical data were expressed as mean ± SEM of 3 independent experiments unless indicated otherwise and statistical significance was calculated using unpaired Student's *t*-test where $*p<0.05$, $**p<0.01$, $***p<0.001$ and $****p<0.0001$ are considered as significant and NS indicates non-significant.

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Appendix