THE ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASE-2 (MKP-2) IN MACROPHAGE DEVELOPMENT AND GENE EXPRESSION

A thesis presented by

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Abstract

Mitogen-activated protein kinase phosphatase-2 (MKP-2) is a type 1 nuclear dual specific phosphatase (DUSP4) and an important immune regulator. It specifically dephosphorylates the MAP kinases ERK and JNK to influence pro- and anti-inflammatory cytokine production. MKP-2 has recently been shown to play a significant role in controlling *Leishmania mexicana* infection (Al-Mutairi *et al*., 2010) primarily by influencing macrophage activity. However, information on the effect of MKP-2 deletion at the molecular level on macrophage development and function is limited.

This project utilised a novel DUSP4 gene knockout mouse and investigated the effects of MKP-2 deletion on M-CSF induced MAPK signalling and macrophage development as well as macrophage gene expression. Experiments in bone marrow derived macrophages demonstrated that in response to M-CSF macrophage, proliferation was reduced following to MKP-2 deletion. This was correlated with ERK phosphorylation, the expression of CD115 and CD34 on macrophage progenitors as well as the induction of genes related to macrophage differentiation and proliferation, colony stimulating factor-2 (*Csf2*) and monocyte to macrophage differentiation associated (*Mmd*) genes.

In addition a comparative microarray gene expression analysis was conducted on MKP-$2^{-/-}$ and MKP-$2^{+/+}$ macrophages following (LPS) or (IL-4) activation. As demonstrated previously, and associated with a role for MKP-2 in antimicrobial activity, arginase-1 expression was up-regulated in MKP-$2^{-/-}$ compared with MKP-$2^{+/+}$ macrophages. Surprisingly, and in contrast, we found that other alternative activation markers *Ym1 (Chi3l3)* and *Fizz1/Retnla (Relm-α)* were significantly reduced in MKP-$2^{-/-}$ macrophages when compared with their wild-type counterparts. As both Ym1 and Fizz1 have been implicated to play a major role in extracellular matrix disposition this suggests a significant role for MKP-2 in wound

healing. Collectively, the findings in the current study have established that MKP-2 plays an important role in macrophage development and immune function.

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Abbreviations

AAM	Alternatively activated macrophages
ALI	Acute lung injury
AP-1	Activating protein 1
APC	Antigen presenting cells
APS	Ammonium Persulfate
ARE	AU-rich elements
Arg1	Arginase-1
ASK1	Apoptosis signal-regulating kinase 1
β-ME	B-mercaptoethanol
BCR	B-cell antigen receptors
BMDM	Bone marrow-derived macrophages
Bmp	Bone morphogenetic proteins
BSA	Bovine serum albumin
C5a	Complement component 5a
CAM	Classically activated macrophages
CCL-	Chemokine (C-C motif) ligand
CCR-	CC-chemokine receptor
CD	Common domain
CD115	Cluster of differentiation 115, Transmembrane tyrosine kinase receptor (M-CSFR)
cDNA	Complementary DNA
CDP	Common dendritic cell precursors
CHI3L3	Chitinase-3 like molecules 3
CL100	MKP-1 human homologue
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
Col1a1	Collagen type I alpha 1
Col1a2	Collagen type I alpha 2
Col3a1	Collagen type III alpha 1
Cox2	Cyclooxygenase 2
CREB1	Cyclic-AMP-responsive-element-binding protein 1
cRNA	Complementary RNA
CSF	Colony stimulating factors
CSF-1R	Colony stimulating factor-1 receptor
CSF-2	Colony-stimulating factor-2

CX3CR	CX3C-chemokine receptor 1
DC	Dendritic cells
DC-SIGN	Dendritic Cell-specific intercellular adhesion molecule-3-grabbing nonintegrin
DMEM	DulBeccos Modified Eagle Medium
DTT	Dithiothreitol
DUSP	Dual-specificity phosphatases
EAE	Experimental autoimmune encephalomyelitis
ECL	Enhanced chemiluminescene
Edn1	Endothelin-1
Eef2	Elongation factor 2
EIF2B2	Eukaryotic translation initiation factor 2B
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
FCS	Fetal Calf Serum
G-CSF	Granulocyte colony stimulating factor
G3PDH	Glyceraldehyde-3-phosphate dehydrogenase
Gdf2	Growth differentiation factor 2
gDNA	Genomic DNA
GM-CSF	Granulocyte/macrophage colony stimulating factor
GMP	Granulocyte/macrophage progenitors
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GPCR	G-protein-coupled receptors
HPS	Haematopoietic stem cells
HSP25/27	Heat-shock proteins
IAP1	Inhibitor of apoptosis protein 1
Ibsp	Integrin binding sialoprotein
IFN-γ	Interferon-γ
IGF-I	Insulin-like growth factor-I
IKK	IκB kinase
IL-	Interleukin-
INM	Innate activated macrophages
iNOS	Inducible nitric-oxide synthase
IRAK4	IL-1 receptor-associated kinase 4
IRF-	IFN regulatory factor -
JAK-STAT	Janus kinase/signal transducers and activators of transcription
JIP	JNK-interacting proteins
JNK-1	C-Jun-NH2 terminal kinase 1
KAR	Kinase suppressor of Ras-1

KIM	Kinase-interacting domain
LCM	L929-conditioned medium
LCMV	Lymphocytic choriomeningitis virus
Lif	Leukaemia inhibitory factor
lincRNAs	Long intergenic non-coding RNAs
LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
Ly-6C	Lymphocyte antigen
M-CSF	Macrophage colony-stimulating factor
MAL	MyD88 adaptorlike
MAPK	Mitogen activated protein kinase
MK2	MAP kinase-activated protein kinase 2
MAPKKK	MAPK kinase kinase
MCP-1	Monocyte chemoattractant protein-1
MDM	Monocyte-derived macrophages
MDP	Macrophage/dendritic cell progenitors
MEF	Mouse embryonic fibroblasts
MEKK3	MAPK/ERK kinase kinase 3
MEP	Megakaryocyte/erythrocyte progenitors
MHC-II	Major histocompatibility complex II
MIQE	Minimum Information for Publication of Quantitative Real-Time PCR Experiments
MKB	MAP kinase-binding
MKK	MAP kinase kinases
MKKK	MAPK kinase kinase
MKP	MAP kinase phosphatases
MMD	Monocyte to macrophage differentiation
MP1	MEK partner1
MPS	Mononuclear phagocyte system
MRC-1	Mannose receptor 1
MS	Multiple Sclerosis
MSK	Mitogen- and stress-activated kinases
multi-CSF	Multipotential CSF
MyD88	Myloid differentiation primary- response gene88
NCBI	National Centre for Biotechnology Information
NES	Nuclear export signal
NFATc1	Nuclear Factor of Activated T-cells c1
NFκB	Nuclear factor kappa beta
NK	Natural killer cells
NK-T	Natural killer T-cells

NLS	Nuclear localization signal
NO	Nitric oxide
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositide 3-kinases
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
Poly I:C	polyinosinic:polycytidylic acid
Postn	Periostin
PRR	Pattern recognition receptors
QARS	Glutaminyl tRNA synthetase
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction amplification
RA	Rheumatoid arthritis
RBC	Red blood cells
rmM-CSF	Recombinant mouse M-CSF
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RPL6	Ribosomal protein L6
RQI	RNA quality indicator
RT	Reverse Transcription
RTK	Receptor tyrosine kinase
SAPK	Stress-Activated Protein Kinases
SARM	Sterile α- and armadillo-motif–containing protein
SAv-HRP	Streptavidin-horse radish peroxidase
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SFK	Src family kinase
siRNA	Small interfering RNA
SR	Scavenger receptors
SRP14	Signal recognition particle 14kDa
T-ERK	Total ERK
T-JNK	Total JNK
TAB1	TAK1-binding protein 1
TAK-1	Activating kinase-1 binding protein
TARC/CCL17	Thymus and Activation-Regulated Chemokine
TCR	T-cell antigen receptors
TEMED	Tetramethylenediaamine
TF	Tissue factor

TGF	Transforming growth factor
TGFR	Transforming growth factor receptor
Th	T helper
TIR	Toll-IL-1 receptor
TIRAP	TIR-domain–containing adaptor protein
TLR	Toll-like receptors
TNF-α	Tumor necrosis factor alpha
TPL2	Tumour-progression locus 2
TRAF6	TNF receptor-associated factor 6
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain–containing adaptor protein-inducing IFNβ
TTP	Tristetraprolin
UBC	Ubiqiutin C
UBC13	Ubiquitin-conjugating enzyme 13
UTR	Untranslated regions
VRK1	Vaccinia-related kinase 1
WBC	White blood cells
WT	Wild-type

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1. GENERAL INTRODUCTION

1.1 The immune system

1.1.1 Introduction

The immune system is one of the most important physiological functions that protect the host from diverse pathogenic microorganisms as well as to clear toxic and allergenic substances (Chaplin, 2010). This defence process does not include host antigens in order to avoid host tissue damage (Janeway, 2001, Chaplin, 2010). Two important immune mechanisms are used by the host to eradicate invading pathogens and eliminate foreign molecules: the innate and adaptive immune responses. Innate immunity is an immediate response that rapidly detects pathogen-associated molecular patterns (PAMPs) on different antigens through a group of cell surface pattern recognition receptors (PRRs). Whereas, the adaptive immune response is more specific in recognizing antigens through antigen-specific receptors expressed on the surfaces of T- and B-lymphocytes. Adaptive responses are initiated by antigen presenting cells (APCs) of the innate immune system (Janeway, 2001, Chaplin, 2010). Although innate and adaptive immunity are represented as two separate arms of the host response, synergy between both systems is necessary for a fully effective immune response (Chaplin, 2010). The next section compares in detail innate and adaptive immunities.

1.1.2 Innate versus adaptive immune systems

The innate immune response is considered the first line of defence within the host. It is characterized by a rapid kinetic response to antigens but it lacks immunological memory (Borghesi and Milcarek, 2007, Chaplin, 2010). The innate immune system is reinforced by immune cells called phagocytes such as macrophages, neutrophils and dendritic cells (DCs), which respond within minutes to hours of infection, engulfing and killing the pathogen (Akira, 2011, Borghesi and Milcarek, 2007). Innate immunity uses germ line-

encoded PRRs expressed on various innate cells to detect target molecules expressed by different infectious agents such as lipopolysaccharides (LPS), phosphoantigens, lipids, and double-stranded RNA (Medzhitov and Janeway, 1997, Akira *et al.*, 2006, Borghesi and Milcarek, 2007). It was previously thought that innate responses were non-specific in antigen recognition, however Toll-like receptors (TLRs), which afford sufficient specificity to distinguish host tissue from foreign molecules, have been discovered. These are conserved receptors that represent the first line of defense against wide range of invading microorganism and the key to the innate immune system (Doyle and O'Neill, 2006). Currently, 13 members of TLRs have been identified in mammals (Uematsu and Akira, 2006). TLRs are discussed in detail in section 1.2.4.2. Activation of TLRs triggers downstream signalling pathways to produce pro-inflammatory cytokines and chemokines important for the elimination of pathogens (Akira and Takeda, 2004). The innate immune response initiates a rapid, effective response to infections, but in some cases it fails to eradicate microbes completely and this is achieved through the adaptive immune response (Akira, 2011).

The adaptive immunity is the second-line immunity system of a host and exhibits relatively late phase of responsiveness (Borghesi and Milcarek, 2007). This part of the immune system is triggered by APCs of the innate immune system resulting in a stronger and long lasting defence mechanism (Gonzalez *et al.*, 2011). Adaptive immunity depends on antigen specific receptors on T- and B-lymphocytes called T-cell antigen receptors (TCRs) and B-cell antigen receptors (BCRs) respectively (Janeway *et al.*, 2001) to detect complex antigenic molecules in highly specific manner (Borghesi and Milcarek, 2007). An important feature of adaptive immunity is that it generates immunological memory specific to the first encounter of specific pathogen in order to enhance the response to subsequent attacks by the same pathogen (Janeway *et al.*, 2001).

1.1.3 Cells of the immune system

An intact immune response needs the contributions from many subsets of immune cells called white blood cells (WBCs) or leukocytes. Each subset performs a specific job to

detect or clear the pathogen and all immune cells work in cooperation to mediate the body's immune response (Geissmann *et al.*, 2010, Janeway *et al.*, 2001). Mature WBCs originate from haematopoietic stem cells (HSCs) in bone marrow and differentiate in response to internal and external signals to have distinct functions and physical characteristics. HSCs also give rise other cellular blood elements including red blood cells (RBCs) and platelets (Figure 1.1) (Janeway *et al.*, 2001, Chaplin, 2010, Kondo, 2010). At first, HSCs generates two main lineages: common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), which further differentiate to other types of blood cell (Janeway *et al.*, 2001, Kondo, 2010).

Lymphoid progenitors are the source of the B- and T-lymphocytes (B-cells and T-cells), natural killer (NK) and natural killer T-cells (NK-T cells) (Chaplin, 2010). When B-cells are activated they differentiate into plasma cells which secrete antibodies. B-cells and T-cell are defined by the B-cell and T-cell receptor expression respectively (Akashi *et al.*, 2000, Janeway *et al.*, 2001). NK cells are lymphocyte-like granular cells that recognize and kill tumour cells or virus-infected cells (Jonsson and Yokoyama, 2009, Chaplin, 2010). NK-T cells exhibit the functions of both NK and T-cells (Chaplin, 2010).

Myeloid progenitors are another lineage, which originates from HSCs and gives rise to megakaryocyte/erythrocyte progenitors (MEPs) or granulocyte/macrophage progenitors (GMPs) (Akashi *et al.*, 2000, Janeway *et al.*, 2001, Chaplin, 2010). MEPs can differentiate in to platelets and erythrocytes (RBCs). Whereas, GMPs drive the production of monocytes, mast cells precursors and granulocytes including basophils, eosinophils, and neutrophils. Mast cells enter tissues and complete their maturation their. Monocytes differentiate to macrophages or DCs, the antigen presenting cells (APCs) that connect the innate and the adaptive immune systems (Janeway *et al.*, 2001, Chaplin, 2010).

Recently, a new subset of cells has been identified which shares many characteristics with CMPs and is termed macrophage/dendritic cell progenitors (MDPs) (Fogg *et al.*, 2006).

MDPs generate monocytes and common dendritic cell precursors (CDPs) (Varol *et al.*, 2007). For a graphic representation see Figure 1.1.

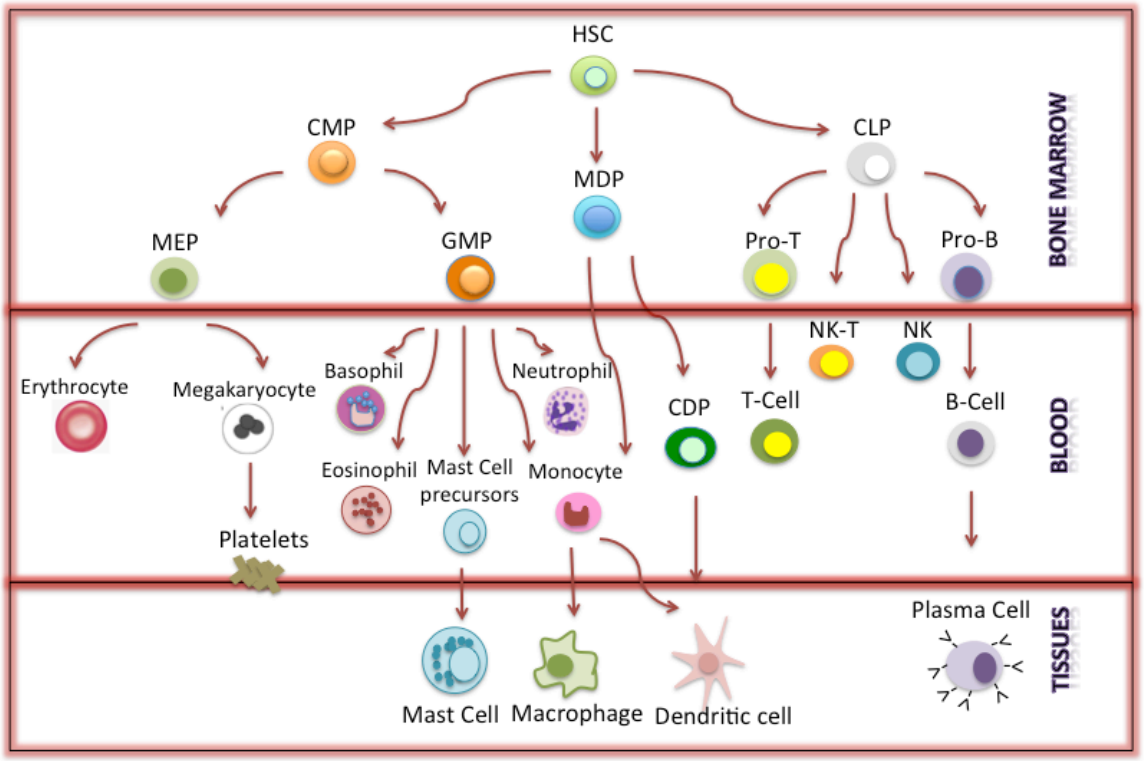


Figure 1.1: Differentiation of immune cells. Haematopoietic stem cells (HSCs) differentiate in the bone marrow into common lymphoid progenitors (CLPs) and common myeloid progenitor cells (CMPs). CLPs give rise to NK, NK- T cells, B-cells and T-cells. CMPs differentiate to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs). GMPs give rise to monocytes that differentiate in peripheral tissues in to different subsets of macrophages and DCs. GMPs also give rise to different types of granulocytes including eosinophils, neutrophils, basophils and mast cells. MEPs differentiate to erythrocytes and megakaryocytes which give rise to platelets. Dendritic cells are also derived from common DC precursors (CDPs), which are originated from HSCs in bone marrow.

1.1.4 Mononuclear phagocytes (MPs) and dendritic cells

The mononuclear phagocyte system (MPS) is a part of the immune system which was first proposed in 1969 as new classification of highly phagocytic mononuclear cells and their precursors (van Furth *et al.*, 1972). These cells consist of bone marrow-derived myeloid cells including the promonocytes and their precursors in the bone marrow, blood circulating monocytes and tissue macrophages (Geissmann *et al.*, 2010). Murine MPs uniquely express CD115 (the receptor for macrophage colony stimulating factor (CSF-1R/M-CSFR)), which is used to define this population (Auffray *et al.*, 2009, Francke *et al.*, 2011).

Monocytes are MPs and important immune effector cells which originate from a common myeloid progenitor that is shared with other granulocytes. They are released into the blood, where they circulate for several days before they enter the tissues to differentiate in to other cell populations (Figure 1.1). Monocytes migrate to tissues during infections following stimulation of their chemokine and adhesion receptors (Geissmann *et al.*, 2010). Studies from the past 20 years have provided insights into the fate and function of human and mice monocytes and their subsets. Monocytes were initially identified by the expression of CD14 (which is part of Lipopolysaccharide (LPS) receptor). However, the identification of other antigenic markers showed that monocytes in peripheral blood are heterogeneous, thus are divided in to subsets and exert different physiological activities. Mice monocytes particularly were first identified by the expression of two markers F4/80 and CD11b (Gordon and Taylor, 2005). However, subsequent specific classification of murine monocytes divided these cells into two categories: inflammatory and resident monocytes. This is based on their expression of CC-chemokine receptor 2 (CCR2), CX3C-chemokine receptor 1 (CX3CR1) and the myeloid marker lymphocyte antigen (Ly-6C) (Auffray *et al.*, 2009, Taylor and Gordon, 2003, Gordon and Taylor, 2005b). Inflammatory monocytes are Ly-6C-CCR2-positive cells but express low amounts of ($CX3CR^{low}$). They recruit to the inflammatory site in response to the production of Chemokine (C-C motif) ligand 2 (CCL2) also called monocyte chemoattractant protein-1 (MCP-1) (Gordon and Taylor, 2005) to secrete cytokines, reactive oxygen intermediates

(ROIs), nitric oxide (NO) (Serbina *et al.*, 2008), tumor necrosis factor alpha (TNF-α) and IL-1 (Auffray *et al.*, 2009). They also ingest apoptotic cells and toxic molecules by phagocytosis (Auffray *et al.*, 2009). Inflammatory monocytes have also been implicated in the pathogenesis of chronic inflammatory diseases such as arthritis and atherosclerosis (Nahrendorf *et al.*, 2007, Ingersoll *et al.*, 2011). Resident monocytes are Ly-6C-negative monocytes express CX3CR1 at high levels but do not express CCR2 (Taylor and Gordon, 2003, Auffray *et al.*, 2009). Resident monocytes have been proposed to supply tissue with macrophage and DC populations (Auffray *et al.*, 2009, Geissmann *et al.*, 2010).

In 1939, Ebert and Florey observed that monocytes from blood vessels developed into macrophages in the tissues (van Furth and Cohn, 1968). Macrophages are another MPs and major phagocytic cells found in all body tissues and play an important role in the innate immune response to pathogens as well as helping to initiate the adaptive immune response (Mosser and Edwards, 2008, Gordon and Taylor, 2005b). Section 1.1.5 is discussing in detail the macrophages with their functions and models of activation.

Another type of immune cells derived from monocytes is the dendritic cells. Dendritic cells were first recognised in 1973 as a novel cell population in mouse spleen (Steinman and Cohn, 1973). They play a critical role in the innate and adaptive immune systems and this depends on their maturation status. Immature DCs are highly active phagocytes, whilst on maturation they become important cytokine-producing cells (Banchereau and Steinman, 1998, Mellman and Steinman, 2001). DCs are activated when they detect antigens through PRRs. An activated DC is potent antigen presenting cells characterized by increased expression of Major histocompatibility complex II (MHC-II) and co-stimulatory molecules such as CD40, CD80 and CD86 (Boggiatto *et al.*, 2009, Brandonisio *et al.*, 2004). They transport antigens captured in peripheral tissues to prime naive specific T-cells in the lymph nodes to initiate an adaptive immune response (Banchereau and Steinman, 1998, Banchereau *et al.*, 2000).

1.1.5 Macrophages

Macrophages are major phagocytic cells that differentiate from monocytes. They are heterogeneous effector cells that perform different functions at different anatomical locations. These functions range from maintaining tissue homeostasis to playing central roles in tissue immune defence against infections and the resolution of inflammation (Mosser and Edwards, 2008, Gordon and Taylor, 2005). Macrophages are found in all body tissues, they are known in the bone as osteoclasts, in the central nervous system as microglial cells, in the connective tissue as histiocytes and in the liver as Kupffer cells. Macrophages are also found in alveoli, the gastrointestinal system, spleen and peritoneum (Kumar and Jack, 2006, Janeway *et al.*, 2001). Within the category of MPs, macrophages are often distinguished by differential expression of surface makers such as F4/80, CD11b, CD14 and CD68 (Murray and Wynn, 2011a), see Table 1.1 for details.

As macrophages located throughout the body, they maintain tissue homeostasis by clearing dead cells and debris generated from tissue remodelling without triggering immune-cell signalling (Kono and Rock, 2008). This housekeeping process is mediated by scavenger receptors, phosphatidyl serine receptors, the thrombospondin receptor, integrins and complement receptors (Mosser and Edwards, 2008, Erwig and Henson, 2007). Macrophages also remove necrotic debris resulting from trauma or stress and markedly affect the expression of surface proteins to produce pro-inflammatory mediators. This alteration in the expression of surface proteins can be used to identify biochemical markers specific to a particular macrophage population (Zhang and Mosser, 2008). The response to these endogenous signals is mediated by TLRs, as part of the innate immune response (Mosser and Edwards, 2008, Kono and Rock, 2008). Macrophages are also responsible for iron processing, clearing approximately 2×10^{11} erythrocytes each day to recycle iron and haemoglobin for the host to reuse (Mosser and Edwards, 2008, Ganz, 2012).

In addition to maintaining healthy tissues, macrophages play an important role in the immune responses and fighting infections. They constantly exert immediate response to

any danger signals and phagocytose and/or detecte these signals by cell surface receptors. In response to the many different endogenous and exogenous stimuli that activate macrophages, they display a unique ability to change their physiology and function. This plasticity allows them to be involved in both innate and adaptive immune responses (Stout and Suttles, 2004, Gordon and Taylor, 2005b, Stout *et al.*, 2005). According to the stimuli that activate macrophages, they are classified into: innately activated macrophages, classically activated macrophages, alternatively activated macrophages and regulatory macrophages (Figure 1.2) (Gordon, 2003, Gordon and Taylor, 2005, Gordon, 2007). Because of the high interference in surface marker expression between macrophage subsets and other MPs, exploring gene expression profiles of macrophage phenotypes using cytokines or microbial stimulation has been a useful approach to macrophage characterization (Murray and Wynn, 2011). The following sections describe in detail the four activations pattern of macrophages.

Table 1.1: Common macrophage cell surface markers.

Marker Name	Comments
CD11b	Expressed on all myeloid lineage cells, including macrophages.
F4/80	Expressed on most tissue macrophages in the mouse.
CD68	Expressed on all macrophages. Useful for immunohistochemistry (IHC).
CSF1R/CD115	Expressed on all monocytic cells, including macrophages and osteoclasts.
CD163	Expressed on most macrophages. Useful for immunohistochemistry (IHC).
CD14	Main receptor for LPS and is expressed predominantly on the surface of monocytes and macrophages.

1.1.6 Macrophage activation classes

1.1.6.1 Innate activation

Following to microbial stimuli such as bacterial LPS, macrophages quickly respond and activate through TLRs to exhibit an inflammatory phenotype called innate activated macrophages (IAM). These macrophages are able to produce nitric oxide (NO), reactive oxygen species (ROS) as well as pro-inflammatory cytokines and mediators such as IL-6, IL-12, TNF-α, (Gordon and Taylor, 2005, Menzies *et al.*, 2011). In addition to the production of pro-inflammatory cytokines, IAM maintain their phagocytic activity. When a pathogen has been eradicated, apoptotic cells are cleared through scavenger receptors (SRs), which induce phagocytosis and inhibit pro-inflammatory cytokine secretion, thus switching off macrophage activation and protecting the tissues from further destruction and the persistence of inflammation. However, in chronic inflammation, the innate immune response fails to eliminate the pathogen and inflammation persists. Therefore adaptive immune cells such as T helper (Th) cells are primed and further differentiate by APCs in to Th1 and Th2 to drive either classical or alternative macrophage activation (Gordon, 2003, Gordon, 2007).

1.1.6.2 Classical activation

The term classically activated has been assigned to macrophages that are produced during cell-mediated immune responses. Studies characterised macrophage activation found two signals can classically activate the macrophages and they are: interferon-γ (IFN-γ) produced from Th1 cells or neutral NKs together with TNF-α or a TNF-α inducing agent e.g. LPS (Edwards *et al.*, 2006, Martinez *et al.*, 2009, Mosser, 2003). These cells can be identified by the up-regulation of surface molecules such as MHC class II and CD86 which enhances their ability to present antigens and kill intracellular pathogens (Mosser, 2003). Also, they are characterized by enhanced microbicidal and tumoricidal properties and the release of high levels of pro-inflammatory cytokines, chemockines and inflammatory mediators. Classically activated macrophages (CAM) induce IL-6, IL-12, IL-1, IL-23, oxygen and nitrogen radicals and superoxide anions for efficient killing

ability (Mosser, 2003, Mosser and Edwards, 2008). This is mediated by a combination of signalling pathways such as mitogen activated protein kinase pathway (MAPK), Janus kinase/signal transducers and activators of transcription (JAK-STAT) pathway and nuclear factor kappa beta (NFκB) signalling pathway (O'Shea and Murray, 2008b). Indeed, participation of CAM in chronic inflammatory and autoimmune diseases has been well studied. These cells have been shown to be an essential component of the defence against various bacterial, protozoal or viral pathogens (Filipe-Santos *et al.*, 2006, Gordon and Taylor, 2005, Gordon, 2007). For example, stimulating macrophage with IFN-γ and TNF-α prior to *Leishmania* spp infection produced a population of macrophages that efficiently kills the parasite (O'Shea and Murray, 2008). However, the activity of CAM must be controlled to avoid tissue damage caused by excessive inflammation. This explains the involvement of CAM in the immunopathology of autoimmune diseases such as rheumatoid arthritis (RA) (Mosser and Edwards, 2008, Szekanecz and Koch, 2007).

1.1.6.3 Alternative activation

In contrast to pro-inflammatory responses generated by CAM, alternatively activated macrophages (AAM) display potent anti-inflammatory activity and have important roles in wound healing and fibrosis. Numerous studies have identified this population as driven by IL-4 and/or IL-13 produced by Th2 cells (Mosser and Edwards, 2008, Gordon, 2003, Mosser, 2003, Martinez *et al.*, 2009). Granulocytes also serve as sources of innate IL-4 production (Mosser and Edwards, 2008). These AAM produce minimal amounts of pro-inflammatory cytokines and are less efficient at killing pathogens when compared to CAM (Mosser and Edwards, 2008). Instead this cell population participates in wound healing and tissue remodeling as it secretes elements of the extracellular matrix including arginase (Kreider *et al.*, 2007a), which converts arginine to orthinine, a precursor for polyamines and collagen (Edwards *et al.*, 2006). Alternatively activated macrophages are supported by up-regulation of mannose receptors (MRs) to clear debris to reinstate tissue homeostasis (Mosser and Edwards, 2008). Although AAM are anti-inflmmatory, several studies have determined roles for these cells in promoting allergic responses driven by IL-4 and IL-13. However, their function in allergy remains controversial between promoting

and suppressing allergic inflammation (Murray and Wynn, 2011). A recent study suggested that AAM are exaggerating lung inflammation caused by Sendai virus by secreting IL-13 (Kim *et al.*, 2008). In another review, AAM induced during rhinovirus infection have also been shown to recruit eosinophilic airway inflammation site by producing the chemokine (CCL11) (Nagarkar *et al.,* 2010). However other reports have also identified a suppressive role for AAM in allergy and asthma. This is by phagotcytosing fungal conidia, thus inhibiting asthma symptoms by fungal infection (Bhatia *et al.,* 2011). In addition, Chitinases expressed by AAM have also been proposed to suppress allergic inflammation by degrading chitin, which is a highly abundant allergen in the airway (Reese *et al.,* 2007).

The characterization of murine AAM was achieved using experimental helminth and nematode infections. This suggests that AAM play an important role in immunity to these infections (Nair *et al.*, 2005). These studies have identified reliable markers for this type of macrophage (Table 1.2), including chitinase and chitinase-like molecules such as CHI3L3/YM1 and YM2, acidic mammalian chitinase and stabilin-interacting chitinase-like protein as well as resistin-like molecule alpha (Relmα/Fizz1)(Raes *et al.*, 2002).

Chitinases were originally thought to degrade the chitin-coated surfaces of some parasites and fungi. However, chitinases failed to show chitin-degrading activity. Later, chitinases were shown to exhibit matrix-binding ability, providing evidence for their role in extracellular matrix disposition (Raes *et al.*, 2002b, Nair *et al.*, 2005, Mosser and Edwards, 2008). Likewise, Fizz1 has also been shown to be involved in the wound-healing functions of type-2 cytokine-activated macrophages (or AAM) (Nair *et al.*, 2005).

The expression of other markers driven by IL-4 was identified by many studies (Table 1.2). The CC chemokine Thymus and Activation-Regulated Chemokine (TARC/CCL17) was found to be induced by IL-4 in peritoneal macrophages. It has shown that CCL17 attracts T-cells and macrophage into allergic inflammation sites (Liddiard *et al.*, 2006). IL-4 was also able to upregulate the IL-27 receptor alpha chain WSX-1 IL-27Rα chain (Rückerl *et al.*, 2006), dectin-1 and mannose receptor expression (MRC-1) (Menzies *et al.*,

2011) in mouse bone marrow macrophages (BMDMs) and CCL22 in human blood monocytes and macrophages (Mantovani *et al.*, 2002). Macrophage-derived insulin-like growth factor-I (IGF-I) mRNA and protein were also expressed following to IL-4 or IL-13 treatment in mouse BMDMs. IGF-I has long been involved in the pathogenesis of the lung diseases and pulmonary fibrosis by stimulating fibroblast proliferation and promote collagen matrix synthesis (Wynes and Riches, 2003).

Like autoimmunity produced by classical activation, AAM can be harmful to the host. Uncontrolled activation of wound-healing macrophages is involved in the pathology of chronic schistosomiasis characterized by excessive tissue fibrosis (Hesse *et al.*, 2001). A study utilized schistosome lung granuloma mice model; demonstrate the expression of Arg-1 in vivo by AAM also by Schistosome eggs. The maintenance in Arg-1 expression developed fibrotic granulomas due to arginase continuous activation of fibroblasts(Hesse *et al.*, 2001).

Table 1.2: Common markers for alternatively activated macrophages.

Marker Name	Comments
Relm-α (Fizz1, Retnla), mouse	Promote deposition of extracellular matrix, Highly induced by IL-4 and IL-13.
Chi3l3 (Ym1), mouse marker	Chitinase-like protein that can bind to extracellular matrix, Highly induced by IL-4 and IL-13.
CCL17	Attracts T cells and macrophages to allergy sites, induced by Il-4 and suppressed by IFN-γ.
IL-27Rα	Inhibits pro-inflammatory cytokine production, upregulated by IL-4.
IGF1	Stimulates fibroblast proliferation, induced by IL-4.
CCL22	Attracts Th2 cells, induced by IL-4.
MRC-1	Clear debris to reinstate tissue homeostasis, induced by IL-4 and IL-13.
Arginase-1	The hepatic isoform of arginase, induced by IL-4 and IL-13.

1.1.6.4 Regulatory macrophages

Similarly to the macrophage populations described earlier, regulatory macrophages can be induced by a number of different agents. This includes IL-10 (Martinez *et al.*, 2008), prostaglandin E2 (PGE2) (Strassmann *et al.*, 1994), transforming growth factor (TGF)-β (Feinberg *et al.*, 2000), glucocorticoids and apoptotic cells (Erwig and Henson, 2007) acting in combination with a second stimulus such as TLR ligation (Mosser and Edwards, 2008). Regulatory macrophages have anti-inflammatory properties: overproduction of IL-10 by these cells blocks the production IL-12 (Mosser and Zhang, 2008, Gerber and Mosser, 2001). The role of this macrophage population is to inhibit the immune response and limit the inflammation. Unlike AAM, these regulatory macrophages do not produce extracellular matrix elements. They highly express co-stimulatory molecules (CD80 and CD86) to present antigens to T cells (Edwards *et al.*, 2006). Regulatory macrophages can be generated in response to some parasites, bacteria and viruses producing the above mentioned signals thus supporting the survival of these pathogens (Mosser and Edwards, 2008).

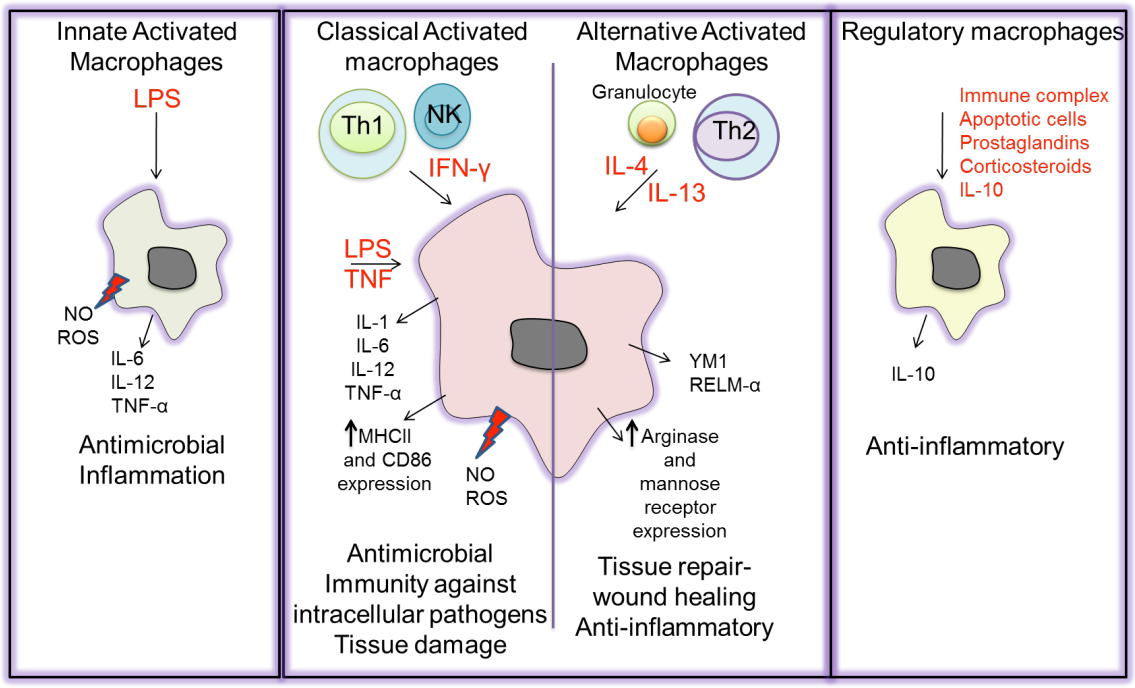


Figure 1.2: Macrophage phenotypes. Microbial stimuli such as TLR agonist LPS produce innate activated macrophages. Classical activation is the result from interferon-γ (IFN-γ) and tumor necrosis factor (TNF) produced by innate immunity. Interleukin-4 (IL-4) and Interleukin-13 (IL-13) can activate macrophages alternatively. Various types of stimuli, including immune complexes, prostaglandins, apoptotic cells, corticosteroids and interleukin-10 (IL-10) can generate regulatory macrophages.

1.1.7 The origin and maintenance of macrophages

As discussed earlier in section 1.1.4, monocytes are released into the bloodstream and migrate into extravascular tissues to differentiate in to tissue-resident macrophages (Gordon and Taylor, 2005). During inflammation monocytes also give rise to inflammatory macrophages and migration of monocytes from the bloodstream to the tissues is dramatically increased (Mosser and Edwards, 2008). Under inflammatory conditions, monocytes and neutrophils differentiate from their bone marrow precursors with the help from several factors of such as granulocyte colony stimulating factor (G-CSF) and chemokines including CC-chemokine ligand (CCL2) and (CCL5). Also, stimulation of TLR and IL-1 receptor (IL-1R) pathways induces haematopoietic cytokines such as G-CSF and granulocyte/macrophage colony stimulating factor (GM-CSF, also called CSF-2), to encourage bone marrow precursors to produce neutrophils and monocytes and more macrophages (Murray and Wynn, 2011).

Although it is well known that monocytes are precursors of macrophages and DCs, the in vivo development and differentiation process is still not clear. However it is known that regulation of haematopoiesis, the process of developing mature blood cells from bone marrow precursors is controlled by two interacting systems. First, the stromal marrow cells regulate the number of the stem cells and the generation of progenitors. Second, the coordinated interaction of haematopoietic growth factors stimulates differentiation of progenitors as well as the proliferation and maturation process necessary to produce mature blood cells (Metcalf, 1991).

Reports studied *in vitro* macrophage models illustrate that monocytes and macrophages requires macrophage colony-stimulating factor (M-CSF) for their growing and differentiation. In the mouse, bone marrow-derived cells stimulated with M-CSF give rise to macrophages whereas culturing with GM-CSF and IL-4 produces DCs (Lacey *et al.*, 2012). The following section describes in detail the importance of M-CSF to the development of macrophages and the signalling pathways involved in this process.

1.1.8 Colony stimulating factors (CSFs)

Colony stimulating factors are haematopoietic-cell growth factors that bind to receptor proteins on the surfaces of HSC to activate intracellular signaling cascades important for these cells to differentiate and proliferate. The CSF family comprising four glycoprotein growth factors, which regulate the production, maturation, survival and functions of granulocytes and monocyte-derived macrophages. Two haemopoietic CSFs are capable of stimulating the production of both macrophages and granulocytes from murine spleen and marrow cells, granulocyte/macrophage CSF (GM-CSF also called CSF-2) and Multipotential CSF or interleukin-3 (multi-CSF or IL-3) (Morstyn and Burgess, 1988, Rowe and Rapoport, 1992, Wadhwa and Thorpe, 2008). GM-CSF also controls the survival and function of several cells, e.g. neutrophils, and dendritic cells (Wadhwa and Thorpe, 2008, Caux *et al.*, 1992). Granulocyte colony stimulating factor (G-CSF) or (CSF-3) is a third CSF specific for granulocytic. Whereas, M-CSF (CSF-1) stimulates the generation of macrophages in preference to granulocyte (Morstyn and Burgess, 1988, Rowe and Rapoport, 1992, Wadhwa and Thorpe, 2008).

1.1.8.1 Macrophage colony stimulating factor (M-CSF)

M-CSF also called CSF-1 is the specific regulator of the differentiation, proliferation, survival and function of macrophages. It is a 70kD type I transmembrane protein cleaved into a disulphide-linked dimer with each monomer containing four alpha-helical bundles (Chitu and Stanley, 2006). This protein is secreted by several cells including endothelial cells, smooth muscle cells, bone marrow stromal cells, fibroblasts, uterine cells, hepatocytes and cells of the monocyte/macrophage lineage (Wadhwa and Thorpe, 2008). It affects several functions of macrophages and monocytes including cytotoxicity, phagocytosis and chemotactic activity (Chitu and Stanley, 2006). More importantly, it mediates proliferation of monocyte and their differentiation into mature macrophages. M-CSF influences myeloid precursors to differentiate into monoblasts, then pro-monocyte leads to monocyte and finally macrophage production (Tushinski *et al.*, 1982, Wadhwa and Thorpe, 2008). A study utilised M-CSF-deficient mice showed reduced numbers of

blood monocytes and tissue macrophages, and this defect was reversed by administration of exogenous M-CSF (Stanley *et al.*, 1997). Bone marrow-derived macrophages (BMDMs) cultured in M-CSF are frequently used to study macrophage function and signalling (Cunnick *et al.*, 2006).

M-CSF exerts its effect through binding with the cell surface M-CSF receptor (M-CSFR/CSF-1R/CD115), which is a transmembrane protein encoded by the protooncogene c-fms (Wadhwa and Thorpe, 2008, Pixley and Stanley, 2004). The M-CSFR belongs to receptor tyrosine kinase (RTK) family expressed mainly on cells of monocyte and macrophage lineage. It is composed of an extracellular Ig-like domain, juxtamembrane domain, and intracellular domains required for its catalytic activity including an ATP-binding domain, a kinase insert domain, and a major kinase domain (Pixley and Stanley, 2004). M-CSF stimulation causes M-CSFR dimerization, autophosphorylation of seven specific tyrosine residues (544, 559, 697, 706, 721, 807 and 974) and ubiquitination leading to its degradation. Phosphorylated residues are recognized by different tyrosine kinases such as the Src family (Capurso *et al.*, 2012), which further phosphorylates several intracellular signaling including phosphatidylinositide 3-kinases (PI3K/Akt) , MAPK and STAT family resulting in cell differentiation, proliferation and survival (Hamilton, 1997). Studies on CSF-1R signaling showed that association of the CSF-1R with downstream signaling molecules is dependent on the phosphorylation of tyrosine residues on the receptor. The effect of mutations on these tyrosine residues on proliferation and differentiation has been also examined. Mutations in the c-fms gene can activate the kinase to produce growth-stimulatory signals in the absence of M-CSF. Oncogenic activation of the c-fms gene causes the removal of a negative regulatory tyrosine residue that simulates a conformational change induced by CSF-1 binding. The v-fms oncogene inhibits factor-dependent growth and causes tumorigenicity in factor-dependent myeloid cell lines, which develop proliferative disorders of multiple haematopoietic lineages when introduced into murine bone marrow progenitors (Rettenmier *et al.*, 1988, Pixley and Stanley, 2004).

As mentioned above, the haematopoietic growth factor M-CSF controls the development, proliferation and function of macrophages through several signaling pathways. The following section illustrates in detail the M-CSF signaling pathways that regulate macrophage development.

1.1.8.2 M-CSF signaling pathways that regulate macrophage numbers

The M-CSF signaling pathways that regulate macrophage differentiation/proliferation are poorly understood. This section summarises the pathways involved in M-CSF signaling in macrophages. Activation of PI3K has shown to be involved in CSF-1-regulated macrophage survival (Hamilton, 1997, Murray *et al.*, 2000). This involves the activation of p110α, which is mediated by binding PI3K p85 to kinase insert site in CSF-1R and activation of the upstream Src family kinase (SFK) (Lee and States, 2000). The upstream Src family kinase (SFK) is also required ERK phosphorolylation in M-CSF regulated blood monocytes and primary macrophages survival (Capurso *et al.*, 2012). Jaworowski et al., (1996) reported that M-CSF activates Raf/MEK/ERK. Active ERKs further phosphorylate several proteins and transcription factors which causes immediate early genes expression required for macrophage proliferation (Jaworowski *et al.*, 1996). M-CSF is also able to activate the c-Jun-NH2 terminal kinase 1 (JNK-1) and p38 MAP kinases (Valledor *et al.*, 2008). A study by Novak et al., (1995), reported M-CSF activation of janus kinase, Tyk2, which further phosphorylate transcription factors STAT1 and STAT3 in BMDMs (Novak *et al.*, 1995).

1.2 Mitogen activated protein kinases (MAPKs) family

The MAP kinases represent a well-recognised pathway of highly conserved serine/threonine protein kinases. They are involved in a wide range of cellular activities including, proliferation, differentiation, survival, apoptosis and immune responses (Kyriakis and Avruch, 2001, Cargnello and Roux, 2011). In mammals, three major subfamilies of MAPKs have been identified and are classified on the basis of sequence similarity, differential activation by agonists and substrate specificity. These are: the extracellular signal-regulated kinases including ERKs-1/2 and big MAP kinase (ERK5), c-Jun N-Terminal kinases (JNK-1/2/3) and isoforms of p38 MAP kinase (p38-α/β/γ/δ) (Gupta *et al.*, 1996, Gupta *et al.*, 1995, Pearson *et al.*, 2001). All share the **TXY**- sequence, where T and Y are threonine and tyrosine, and X is glutamate, proline or glycine, in ERK, JNK or p38 MAP kinase respectively (Ahn *et al.*, 1991, Payne *et al.*, 1991, Robbins *et al.*, 1993). These are activated by wide range of stimuli such as serum, growth factors for example, PDGF and EGF, environmental stresses such as osmotic shock, UV light and cytokines such as TNF-α (Kyriakis and Avruch, 2001, Torii *et al.*, 2004). These stimuli trigger membrane receptors to activate MAPK pathways such as; G-protein-coupled receptors (GPCRs, transforming growth factor β receptor (TGFR), receptor tyrosine kinases (RTKs), Toll-like receptors, cytokine receptors, or via protein kinase C (PKC) isoforms, calcium, or stress stimuli (Schramek, 2002).

The mechanism by which MAPK function includes: activation of the MAPK by upstream kinases, nuclear translocation of MAPKs, binding and phosphorylation of MAPK downstream transcription factors for targeted gene expression. MAPK are activated by phosphorylation on both tyrosine and threonine residues in the Thr–X–Tyr sequence by a family of dual-specific MAP kinase kinases (MKKs or MEKs), which are in turn phosphorylated and activated by a MAPK kinase kinase (MKKKs or MEKKs) (Turjanski *et al.*, 2007, Chang and Karin, 2001) (Figure 1.3). In mammalian cells, 7 MKKs have been identified and these are highly specific to their substrates whereas the 14 known

MKKKs show more variant structure and can be activated by different of upstream signals (Garrington and Johnson, 1999).

Once, activated MAPKs further phosphorylate several substrates (Widmann *et al.*, 1999, Turjanski *et al.*, 2007) which in turn can regulate gene expression including nuclear transcription factors such as ELK-1, c-Jun, ATF2, and CREB (Derijard *et al.*, 1994, Turjanski *et al.*, 2007) and cytosolic proteins such as MAPKAP kinase-2/3 (Stokoe *et al.*, 1992). This is efficiently regulated by the contribution of a signalling complex consisting of several scaffold proteins, which work as organizers of the MAPK pathway such as; kinase suppressor of Ras-1 (KAR) and MEK partner1 (MP1), JNK-interacting proteins (JIPs) and β-Arrestin 2 (Morrison and Davis, 2003, Whitmarsh, 2006). Downstream transcription factors are responsible for the formation of dimers, which bind to DNA to activate MAPK-dependent genes. For example c-Jun interacts with c-fos in the formation of the AP-1 complex (Derijard *et al.*, 1994).

The molecular events included in MAPK pathway show both discrete and overlapping regulation of the transcription factors by kinases (Pearson *et al.*, 2001). For example, c-Jun was identified as a substrate for both JNKs and ERKs. C-Jun binds to JNKs within the N-terminus to phosphorylate Ser^{63} and Ser^{73} whereas, phosphorylation of ERK is occurring within a C-terminal inhibitory site (Derijard *et al.*, 1994). The resulting activities of the substrates reflect a combination of multiple protein kinases involved and overall output of the pathway. In addition, the MAP kinase pathways form complexes to assist their activation and localization, specificity, and substrates. The involvement of scaffold proteins and adaptor or linker molecules has been found for some of the kinase modules, which facilitate activation (Pearson *et al.*, 2001, Turjanski *et al.*, 2007). However, the regulation of complex formation by these intermediates also provides another site for cross talk between MAPK signaling pathways, which further complicate the signaling process.

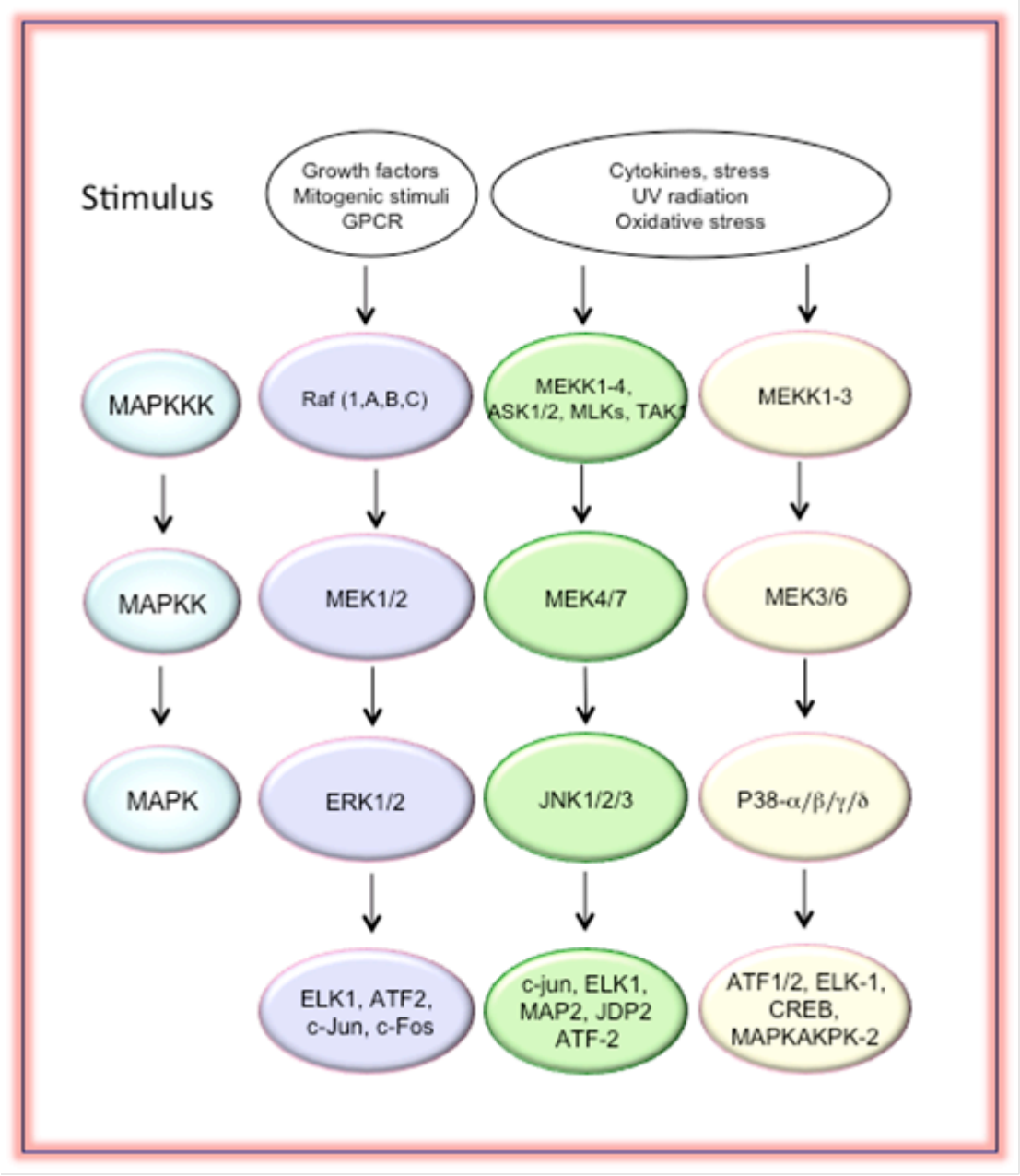


Figure 1.3: MAPK pathways. The mitogen activated protein kinase pathway is highly conserved and plays an important role in a variety of cellular processes. It involves triple kinase system (MAPKKK, MAPKK and MAPK) and is phosphorylated following receptors activation. Three subfamilies of MAPKs are identified including ERK1/2, JNK1/2/3 and p38 isoforms.

1.2.1 Extracellular signal-regulated kinases (ERK) family

The ERK family is the most well-known and best characterized of the MAPK family, consisting of isoform family members (ERK1–5 and ERK7/8) (Boutros *et al.*, 2008). ERK1 and ERK2 (p44/p42 MAPK) were the first mammalian MAP kinase isoforms identified and the most studied along with ERK5. Both ERK1/2 isoforms share 83% amino-acid identity and are regulated by comparable factors and conditions (Boutros *et al.*, 2008, Bogoyevitch and Court, 2004). Nevertheless, some studies have reported that differences between these isoforms in term of substrate specificities (Seger and Krebs, 1995).

ERK1/2 signalling is initiated at the plasma membrane tyrosine kinase receptor, GPCRs and channels (Schramek, 2002). Following to receptor activation, the small G-protein Ras becomes activated which phosphorylates the upstream MKKKs (the Raf proteins), causing phosphorylation of the MKKs (MEK1/2)(Boutros *et al.*, 2008, Pullikuth and Catling, 2007), and the subsequent dual phosphorylation of threonine^{183} and tyrosine^{185} residues within the Thr-Glu-Tyr (T-E-Y) motif (Turjanski *et al.*, 2007).

Once activated, ERK can then target cytosolic substrates (CD120a, calnexin), which controls cytoskeletal remodelling (Pullikuth and Catling, 2007) and it can also translocate to the nucleus to target nuclear substrates including the activating protein 1 (AP-1) family, such as c-Jun and c-Fos as well as Elk-1, NF-AT, SRC-1, and STAT3 (Roux and Blenis, 2004, Pearson *et al.*, 2001). The physiological responses mediated by ERK depend on cell-surface receptor density, the amount of ligand, the duration of signalling, the cell type under consideration, and the subcellular distribution of ERK itself (McKay and Morrison, 2007).

The ERK1/2 signalling has been shown to play a vital role in a number of cellular functions including growth, proliferation, and survival (Hommes *et al.*, 2003, Krens *et al.*, 2006). Mice lacking ERK1 or 2 have been used to identify the functions of these isoforms.

Several reports found ERK1-null mice have no overt phenotype, develop normally, and are fertile (Pages *et al.*, 1999, Hommes *et al.*, 2003). However, these mice showed defective thymocyte maturation (Pages *et al.*, 1999, Hommes *et al.*, 2003, Nekrasova *et al.*, 2005). Another study found ERK-1 deficient mice exhibit increased locomotor activity (Mazzucchelli *et al.*, 2002). In the other hand, deletion of ERK2 led to embryonic lethality due to impaired placental and trophoblast formation and mesoderm differentiation (Gerits *et al.*, 2007, Yao *et al.*, 2003). Over all, this suggests that ERK1 and 2 may play different functional roles during development in different cell types.

1.2.2 c-Jun N-Terminal Kinases /Stress-Activated Protein Kinases (JNKs/SAPKs)

In the early 1990s, the JNK family was first identified as a kinase activated *in vivo* in respone to cycloheximide treatment (Gupta *et al.*, 1996). To date three isoforms are known, JNK1, JNK2, and JNK3, and all are activated by stress, cytokines, and growth factors (Roberts and Der, 2007, Raman *et al.*, 2007). Both JNK1 and JNK2 show ubiquitous tissue distribution, whereas JNK3 is found in the brain, heart, and testis (Boutros *et al.*, 2008, Bode and Dong, 2007). The JNK signalling module comprises MKKKs, including MLK1–4, leucine zipper-bearing kinase, TAK-1, ASK1, and zipper sterile-α-motif kinase followed by the MKK (MEK4/7). MEK4/7 further activates the JNKs phosphorylation of threonine^{183} and tyrosine^{185} residues within the Thr-Pro-Tyr (T-E-Y) motif (Roberts and Der, 2007, Raman *et al.*, 2007, Bogoyevitch and Kobe, 2006).

The JNK family members are divided into splice forms. Both JNK1 and 2 have α1 and β1 forms exist for (p46) and α2 and β2 forms exist for (p54), whereas JNK3 has only the α1 and α2 forms for (p46) and (p54). The α1 and β1 forms (p46) differ from each other by alternative exon usage, leading to replacement between kinase domains IX and X, which assign substrate specificity. The difference between the α2 and β2 forms is identifies by the same exon usage. The p46 and p54 forms differ from each other by the C-terminal region that is alternatively spliced (Waetzig and Herdegen, 2005, Davis, 2000). Among

the current literature, very few reports compare the effects of the 10 splice forms in a single biological system (Gupta *et al.*, 1996, Tsuiki *et al.*, 2003, Yang *et al.*, 1998).

Similar to other MAP Kinases, JNK can phosphorylate several substrates, which mediate the action of the kinase. These include transcription factors such as c- jun, JDP2, ELK-1, p53, ATF2 c-Myc, Paxillin, MAP2 and γ-H2AX. In addition, a number of cytosolic substrates have been identified, including Bcl2, Bim, Bax and akt (Wada and Penninger, 2004, Weston and Davis, 2002, Manning and Davis, 2003). Also, the arrestin proteins have shown to interact with JNK to allow the re-localisation of JNKs from the nucleus to the cytosol (Song *et al.*, 2006). In addition, scaffolding proteins JNK-interacting proteins (JIPs) have shown to sequester and assign subcellular localization and functions of JNKs (Raman *et al.*, 2007)**.** The JNK signalling regulates different cellular processes that include apoptosis, cell proliferation, tumour development (Davis, 2000, Lin and Dibling, 2002), cell migration (Ventura *et al.*, 2006) and inflammatory responses (Hommes et al., 2003).

1.2.3 p38 MAP kinase Family

The p38 MAPK family is the third group of the MAPKs consists of four isoforms (α, β, γ, and δ) (Raman *et al.*, 2007). These isoforms share only 60% amino-acid identity, which demonstrates their different functions and distribution. The two isoforms p38α and p38β are ubiquitously expressed, whereas p38γ is found in skeletal muscle, and p38 δ MAPK is specific to the small intestine, pancreas, testis, and kidney (Cuenda and Rousseau, 2007, Mayor *et al.*, 2007). All isoforms of p38 MAP kinase are activated in response to cytokines, growth factors, and stress such as UV light and osmotic shock (Kyriakis and Avruch, 2001, Roberts and Der, 2007). These stimuli activate p38 signalling cascade through the MKKKs (MLK2 and MLK3, dual leucine zipper-bearing kinase, ASK1, map three kinase-1, and TAK-1). These kinases further activate MKKs (MEK3/4/6) (Raman *et al.*, 2007, Roberts and Der, 2007, Han and Sun, 2007), which activate p38 MAPK through phosphorylation within the Thr-Gly-TYR (T-G-Y) motif (Cuenda and Rousseau, 2007). The MKKK TGFβ-activating kinase-1 binding protein (TAK-1), has been shown to

control the activation/repression of the p38α isoform (Cuenda and Rousseau, 2007). Both MEK3 and 6 are specific for p38 MAP kinase, however, MEK3 can only activate three of the P38 isoforms (α, γ, and δ), whereas MEK6 can activate all p38 isoforms, including p38β (Han and Sun, 2007, Li *et al.*, 1996). The JNK scaffolding proteins JIPs bind to p38 isoforms to affect subcellular localization (Raman *et al.*, 2007). Once activated, p38 MAP kinase can further phosphorylate several cytosolic or nuclear transcription factors, including cytosolic phospholipase A2, heat-shock proteins (HSP25/27), MAP kinase-activated proteins (MAPKAP-K2), CREB, ATF1/2/6, p53, Elk-1, c-Fos, and NFκB(Raingeaud *et al.*, 1996, Tan *et al.*, 1996, Nick *et al.*, 1999).

Isoform specific knockout mice and pharmacological inhibitors have been used to study the association of the p38 MAP kinase with inflammation, cell proliferation, survival and death (Hommes et al., 2003). Of note is the role of p38 pathway in the regulation of a wide range of immunological responses, due to its presence in many immune cellular components such as macrophages, neutrophils and T cells (Ono and Han, 2000). For example, activation of p38 has been implicated in inflammatory disorders such as inflammatory bowel disease, as p38α expression was enhanced in gut lamina propria macrophages and neutrophils (Waetzig *et al.*, 2002).

1.2.4 MAP Kinases in Innate immune responses

1.2.4.1 Introduction

Over the last decade tremendous efforts have been made to understand signal transduction mechanisms and gene regulation involved in immune responses. This helped the discovery of novel therapeutic compounds for treating inflammatory disorders. One of the best-studied signalling cascades is the MAPK signalling pathway, which plays a significant role in immune mediated inflammatory responses (Dong *et al.*, 2002, Zhang and Dong, 2005). In this section, the current data of the role MAP kinase pathway in immune function is reviewed.

In innate immune cells, MAPK activation has been mostly deliberated in the context of TLR stimulation (Zhang and Dong, 2005). TLRs are conserved receptors considered to be the first line of defense against the verity of invading microorganisms and also the key activator of innate immune responses. They recognize (PAMPs) that are conserved among microbial microorganisms to activate a variety of signalling pathways to express genes for pro-inflammatory cytokines, chemokines, and other inflammatory mediators (Zhang and Dong, 2005, Arthur and Ley, 2013, Kawai and Akira, 2010, Uematsu and Akira, 2006).

TLRs are type-1 membrane glycoproteins that recognise PAMPs via extracellular domains comprised of leucine-rich repeats (LRRs) responsible for recognition of different types of PAMPs, a transmembrane spacing component and a cytoplasmic portion which is similar to that of the interleukin-1 receptor (named Toll-IL-1 receptor domain abbreviated to TIR) required for downstream signaling transduction (Takeda *et al.*, 2003). To date, 10 TLRs have been described in humans, whilst some 13 TLRs have been recognized in mice, reviewed by (Uematsu and Akira, 2006). TLR 1, 2, 4, 5, and 6 are located on cell surfaces that can recognise molecules from bacteria, fungi, and protozoa. TLR3, 7, 8, and 9 are located within cell endocytic compartments and can respond to nucleic acids derived from viruses or bacteria (Uematsu and Akira, 2006, Akira *et al.*, 2001, Kumar *et al.*, 2009). The functions of TLR10 in humans as well as TLR11-13 in mice remain unknown (Kawai and Akira, 2010).

1.2.4.2 TLR activation of MAPKs

Studies using knockout mice and co-expression studies in cell lines such as HEK cells have been valuable in understanding the TLR signaling pathway and coupling of these receptors to the interior (Akira and Takeda, 2004). TLR signalling starts from the cytoplasmic domains Toll and IL-1 receptor (TIR), which interact with various TIR domain-containing adaptor proteins following signal stimulation. These include myloid differentiation factor 88 (MyD88), TIR-domain–containing adaptor protein-inducing IFNβ (TRIF), also known as (TICAM-1), TIR-domain–associated protein (TIRAP), also called MyD88 adaptorlike (MAL), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo-motif–

containing protein (SARM) (Uematsu and Akira, 2006; O‟Neill and Bowie, 2007; Kumar *et al*., 2009a; Kawai and Akira, 2011; Takeuchi and Akira, 2010).

TLRs signals are mostly mediated via the adaptor molecule myeloid differentiation primary-response protein 88 (MyD88), except for TLR3. TLR3 requires TIR domain-containing adaptor protein-inducing IFNβ (TRIF), while TLR4, which uses both MyD88 and TRIF (Kawai and Akira, 2010, Arthur and Ley, 2013, Newton and Dixit, 2012). Signals through MyD88 results in proinflammatory cytokine production, including the expression of TNFα, IL-6, IL-12, and IL-1β (Akira *et al.*, 2006), whereas, a TRIF-dependent pathway induces proinflammatory cytokines and type-1 interferon by the activation of IFN regulatory factor 3 (IRF3) and 7 (IRF7). Another adaptor molecule, TRAM, is only utilised by TLR4 to induce the TRIF-dependent pathway (Oshiumi *et al.*, 2003). Recruitment of these adaptor molecules further activates several transcription factors and signalling pathways, including nuclear factor-κ B (NFκB) and mitogen-activated protein kinases (MAPKs) (Kumar *et al.*, 2009, Kawai and Akira, 2010).

In the MAPK pathway, IL-1 receptor-associated kinase 4 (IRAK4) is recruited to MYD88, which then combine with IRAK1 and IRAK2, as well as the E3 ubiquitin ligase TNF receptor-associated factor 6 (TRAF6) and the E2 ubiquitin-conjugating enzyme 13 (UBC13; also known as UBE2N). These lead to the formation of K63-linked polyubiquitin chains on both TRAF6 and IRAK1, which leads to the activation of the MAPK pathway (Figure 1.4) (Kawai and Akira, 2010, Newton and Dixit, 2012, Arthur and Ley, 2013). Another adaptor molecule, TRAF3, can be recruited to MYD88, which can be degraded by K48-linked ubiquitylation, which is mediated by the E3 ubiquitin ligases inhibitor of apoptosis protein 1 (IAP1; also known as BIRC3) and IAP2 (also known as BIRC2). Degradation of TRAF3 also activates MAPKs to influence proinflammatory cytokine production but not type-I interferon (IFN) production (Tseng *et al.*, 2010).

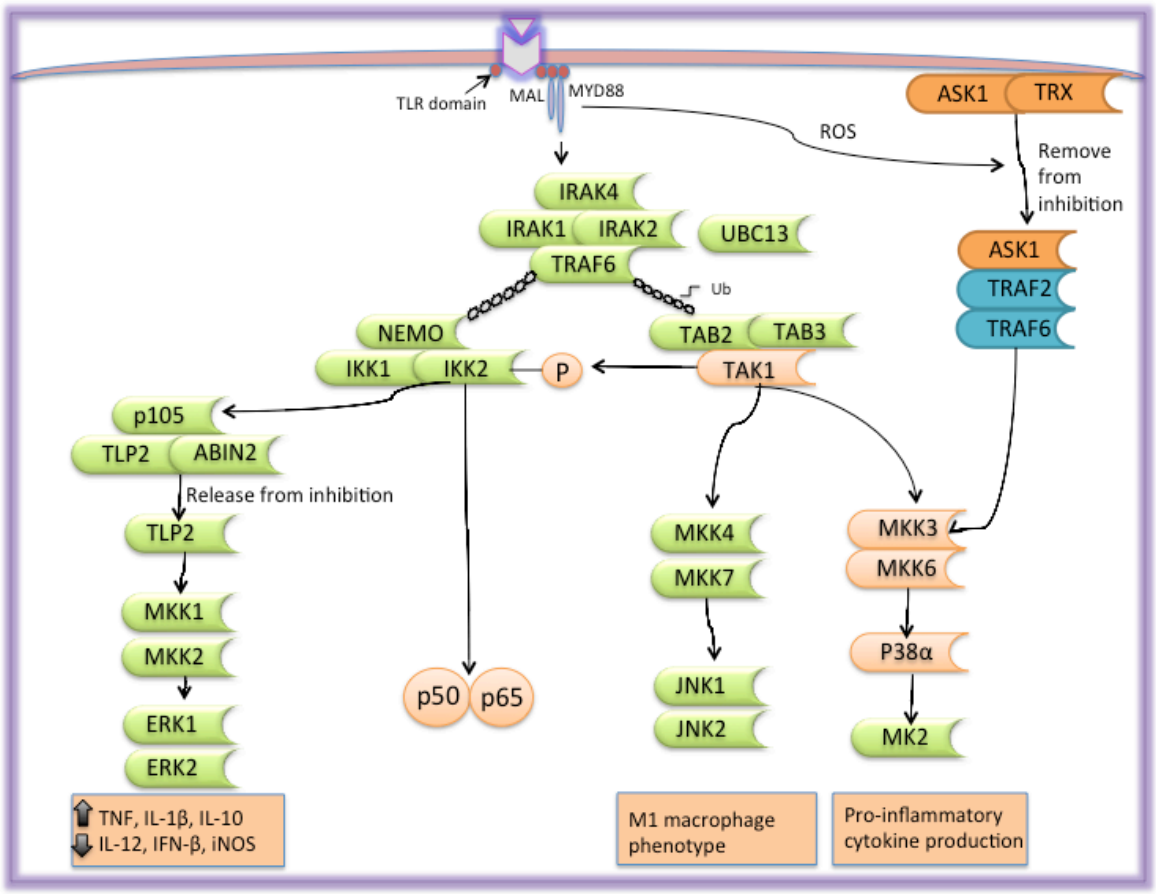


Figure 1.4: TLR activation of MAPKs (Adapted from Aurther and Ley, 2013)

1.2.4.3 MAP3K regulation of TLR-induced MAPK activation

Following to the recruitment of TIR-domain–containing adaptor molecules to TLRs, the next stage is the activation of the MAPK kinase kinase (MKKK) followed by the activation of the MKK. Both p38α and JNK and their prior MKKs are activated by the MAP3K7 TGFβ-activated kinase 1 (TAK) in vitro. TAK1 binds with TAK1-binding protein 1 (TAB1) and the ubiquitin-binding proteins TAB2 and TAB3. Both TAB2 and TAB3 associate TAK1 to the K63-ubiquitylated TRAF6 complex to trigger TAK1 activity (Figure 1.4) (Wang *et al.*, 2001). *In vitro* studies have indicated the important role of TAK1 in IL-1R and TNFR via forming a complex with TAB1 and TAB2 (Shibuya *et al.*, 1996, Takaesu *et al.*, 2000, Takaesu *et al.*, 2003). In response to TNF-α and IL-1β**,** TAK1 expression was essential for JNK and p38 activation as well as for IL-6 production in TAK1-deficient MEFs (Sato *et al.*, 2005). The function of TAK1 in TLR signalling has been suggested in KO studies. In response to LPS and nonmethylated CpG DNA (a ligand for TLR9), expression of TAK1 in $TAK^{-/-}$ B-cells was found to be important for JNK, p38α activation as well as for ERK1/2 via the MAP3K tumour-progression locus 2 (TPL2/MAP3K8) (Sato *et al.*, 2005). However, a study that covered TAK1 specific deficiency in myeloid cells showed that deletion of TAK1 in peritoneal macrophages has no effect on LPS-mediated activation of MAPKs. Instead, TAK1 deletion enhanced MAPK activation in neutrophils, thus increased LPS-induced TNF-α and IL-6 production (Ajibade *et al.*, 2012). Nevertheless, in agreement with Sato and colleagues (2005), in $TAK1^{-/-}$ BMDMs, in which the kinase domain is truncated, TAK1 was indispensable for JNKs activation following LPS. The production of IL-1β and TNF-α was increased in response to LPS whereas IL-10 production was decreased suggested the augment effect on both IL-1β and TNF treatment (Eftychi *et al.*, 2012). The phenotypical differences can be related to difference in macrophage type (peritoneal versus BMDMs) or the compensation by another MAP3K in case of complete deletion of the TAK1.

Another MKKK is apoptosis signal-regulating kinase 1 (ASK1 or MAP3K5). ASK1 forms a complex with thioredoxin in steady state, which is dissociated following TLR4 stimulation and the production of ROS. Once ASK1 is released, it undergoes

autophosphorylation and activation (Noguchi *et al.*, 2005). Also, ASK1 binding to TRAF2 and TRAF6, promotes ASK1 oligomerization and activation (Fujino *et al.*, 2007). This kinase is required for p38α activated by LPS in DCs as well as for the induction of TNF-α, IL-1 and IL-6 (Matsuzawa *et al.*, 2005). Mice deficient in ASK1 are resistant to LPS-induced septic shock and K/BxN-induced rheumatoid arthritis (Mnich *et al.*, 2010).

The MAPK/ERK kinase kinase 3 (MEKK3 or MAP3K3) can bind to TRAF6 and mediate the activation of p38α and JNKs by IL-1β in mouse embryonic fibroblasts (MEFs) (Huang *et al.*, 2004). In primary immune cells, the role of MEKK3 in p38α and JNK activation has not been elaborated. However, studies utilising (siRNA)-mediated knockdown of MEKK3 have shown a slight decrease in LPS-induced activation of p38α and ERK1/2 in RAW264.7 cells (Kim *et al.*, 2007).

The TLRs-induced ERK activation in primary macrophages is mediated by the MKKK, TPL2 (Gantke *et al.*, 2012). In the steady state, TPL2 is inhibited by the NF-κB subunit precursor protein p105 (Beinke *et al.*, 2003). Following to TLR stimulation, p105 is phosophorylated by IκB kinase (IKK) complex and released (Beinke *et al.*, 2004). TPL2 can then activate MAPK kinase 1 (MEK1) and MEK2, which further phosphorylates ERK1/2. In addition, TPL2 can be phosphorylated by IKK2 also on Ser400, which is essential for LPS-induced ERK activation in macrophages (Robinson *et al.*, 2007).

1.2.4.4 The role of JNK in the immune response

As mentioned in section 1.2.2, JNK1 and JNK2 are widely distributed, whereas JNK3 is specifically found in the brain, heart, and testis. In macrophages, several stimuli have been shown to activate JNK1 and 2, including cytokines, LPS, and oxidative stress (Rincon and Davis, 2009). Selective deletion of JNK1 and 2 in mice macrophages protects against insulin resistance following a high-fat diet due to reduced tissue infiltration by these innate immune cells. In addition, JNK-deficient macrophages show reduced classical activation as compared with wild-type (WT) counterparts. Following

IFN-γ and LPS, JNK$^{-/-}$ BMDMs show a reduction of classical M1 marker genes (*Ccr7* and *Cd11c*), chemokines (Ccl2 and Ccl5), and cytokine genes (*Il1b, Il6, and Tnfa*) (Han *et al.*, 2013). Also, JNK2$^{-/-}$ macrophages have shown impairment in uptake and degradation of lipoproteins due to lacking JNK2-mediated phosphorylation of the scavenger receptor A (SR-A) for internalization of lipids (Ricci *et al.*, 2004). In human DCs, the JNK inhibitor SP600125 blocked the upregulation of CD80, CD83, and CD86 suggesting an important role of JNK in DC maturation (Iijima et al., 2005, Nakahara et al., 2004).

In adaptive immunity, studies utilising JNK1 and JNK2 double knockout mice established important role for JNK in CD4+ T-cell differentiation into effector T-helper 1 (Th1) and Th2 cells (Dong *et al.*, 2001, Zhang and Dong, 2005). JNK1-deficiency enhanced the Th2 response, in which mice failed to heal skin lesions and exacerbated *Leishmania* infection. In contrast, JNK-2$^{-/-}$ mice showed attenuation of IFN-γ production (Constant *et al.*, 2000, Dong *et al.*, 2001). The JNK1-negative regulation of Th2-cytokine expression was mediated by phosphorylating and inhibiting the transcription factor Nuclear Factor of Activated T-cells c1 (NFATc1) and increasing ubiquitination and degradation of JunB, which accumulates in Th2 cells (Rincon and Davis, 2009, Li *et al.*, 1999, Chow *et al.*, 2000, Zhang and Dong, 2005). T-cells derived from JNK1 and JNK2 double knockout mice cause a preferential differentiation in the Th2 lineage characterized by increased production of Th2 cytokines (Rincon and Davis, 2009).

1.2.4.5 The role of p38 in the immune response

At the level of innate immune cells, the p38 pathway plays a specific role in the production of proinflammatory factors, such as IL-12, IL-6, TNF-α, cyclooxygenase 2 (Cox2), and inducible nitric-oxide synthase (iNOS). Pre-treatment of peritoneal macrophages with p38 inhibitors (including SB 203580, SB 202190, and SB 202474) led to 40–70% reduction in LPS-induced IL-12 production (Lu *et al.*, 1999). In the same cells, the production of IL-6 and TNF-α is also inhibited by the p38-MAPK inhibitor (SB 202190). In MEK3-deficient mice, the upstream kinase for p38 MAPK, the production of IL-12 was completely abolished in peritoneal macrophages and BMD-DCs (Lu *et al.*,

1999). Specific deletion of p38α in different types of cells, BMDMs, and keratinocytes revealed that p38α has a cell type-specific function. p38α signalling in myeloid cells decreased skin inflammation by inducing anti-inflammatory gene expression (Il10), whereas in epithelial cells it increased the inflammation by inducing leukocyte chemoattractants and other proinflammatory mediators (Kim *et al.*, 2008). The contradiction between the role of p38α in BMDMs in the Kim et al. study and the above-mentioned studies could be due to the general effect of the p38 MAPK inhibitor that inhibits both p38α and β. Also, it was found that the anti-inflamamtory effect of p38α signalling was mediated by the induction of Il-10 through the activation of mitogen- and stress-activated kinases (MSKs), in which MSKs act as negative regulators of TLR signalling (Kim *et al.*, 2008). In dendritic cells, several studies have proposed the involvement of p38 MAPK in maturation of monocyte-derived DCs. In response to LPS, and TNF-α, inhibition of p38 pathway prevents DC maturation derived from human blood monocytes (84–87). The SB203580 inhibitor blocked the up-regulation of CD40, CD80, CD86, and CD83 induced by LPS and TNF-α (Arrighi *et al.*, 2001, Xie *et al.*, 2003). Little is known about the function of p38γ and p38δ proteins in immunity, since the most-commonly used p38 inhibitors do not target these isoforms.

In adaptive immunity, the p38 MAP kinase is known to be important for the production of IFN-γ and Th1 differentiation. Specific p38-inhibition by pyridinyl imidazole compounds blocked the production of IFN-γ by Th1 cells stimulated by IL-12 (Rincon *et al.*, 1998). Also, in vitro-differentiated T-cells in to Th1 cells derived from MEK3-deficient mice show a substantial reduction in IFN-γ in production in relation to wild type cells (Lu *et al.*, 1999). Notably, inhibiting p38 kinases or targeting MEK3 had no effect on IL-4 production by Th2 cells. However, it is unclear whether TCR ligation causes p38 MAPK during the differentiation of Th1 cells or whether IL-12 are key regulators of this process. As with $CD4^+$ T cells, p38 MAPK is also activated in $CD8^+$ T cells. Studies on transgenic mice expressing dominant-negative p38 MAPK revealed that activation of this kinase was required for production of IFN-γ by CD8+ T cells. However, in vivo activation of the p38 MAP kinase pathway induced spontaneous apoptotic $CD8^+$ T-cell death. This apoptosis

process referred to decreased expression of the antiapoptotic protein Bcl-2 (Merritt *et al.*, 2000).

1.2.4.6 The role of ERK in the immune response

Although ERK1/2 is primarily involved in cell growth and proliferation, several inflammatory processes were found to require ERK1/2 activation. Inhibiting ERK1 and ERK2 with the MEK inhibitor PD98059 caused a defect in TNF-α induction in murine peritoneal macrophages stimulated with LPS. Furthermore, peritoneal and BMDMs deficient in TPL2, the upstream activator of ERK1 and ERK2, were protected from LPS/D-Galactosamine–induced endotoxin shock due to a defect in the induction of TNF-α (Dumitru *et al.*, 2000, Kaiser *et al.*, 2009). In the same reports, it was shown that ERK1/2 activation induces IL-1β and IL-10, but negatively regulated the production of IL-12, IFNβ, and iNOS (Dumitru *et al.*, 2000, Kaiser *et al.*, 2009). ERK-1 was found to be crucial for T-cell activation, as thymocyte maturation was reduced by half in ERK1-deficient mice due to decreased expression of α and β chains of the T-cell receptor. This was mediated by the AP-1 family of transcription factors (Pages *et al.*, 1999).

The role of the Ras/ERK pathway in Th2 differentiation has been deliberated in several reports (Jorritsma *et al.*, 2003, Yamashita *et al.*, 1999). The activation of the Ras/ERK pathway was found to be required for IL-4 receptor function and the differentiation of $CD4^+$ Th2 cells. Th2 differentiation was impaired in dominant-negative Ras transgenic T cells (Yamashita *et al.*, 1999). The function of the ERK signaling pathway in the differentiation of naive $CD4^+$ T cells was examined by Jorritsma et al (2003). A weak and transient ERK phosphorylation was obtained from the low affinity peptide, K99R and caused Th2 differentiation from $CD4^+$ T cells. In contrast, a strong ERK activation was gained from the high affinity peptide, MCC, which further induced a strong Th1 differentiation. Reducing ERK by PD98059, in $CD4^+$ T-cells followed by strong peptide stimulation led to dramatic increase in IL-4 expression by naïve CD4+ T-cells and Th2 generation. This suggested the importance in the kinetics of ERK activity in Th1/Th2 differentiation (Jorritsma *et al.*, 2003).

1.2.4.7 Effector kinases downstream of MAPKs

As shown in figure 1.3, several kinases are regulated through phosphorylation by ERK, JNK, and p38 MAP kinase and were found to be essential components in the inflammatory responses. One of the major p38-MAP kinase targets is the MAP kinase-activated protein-kinase 2 (MAPKAP K2 or MK2). Mice lacking MK2 were resistant to LPS-induced endotoxic shock due to a 90% reduction in TNF-α production (Kotlyarov *et al.*, 1999). This defect resulted from impaired phosphorylation of tristetraprolin (TTP or ZFP36), an mRNA-binding protein, which binds to AU-rich elements (AREs) in the 3′ untranslated regions (UTR) of Tnf mRNA to block its translation. Phosphorylation by MK2 decreases TTP interaction with AREs and promotes the binding of human antigen R (HUR or ELAV-like protein 1) to AREs, which stimulates the translation of Tnf mRNA (Ronkina *et al.*, 2007). A significant reduction in NO production was observed in $MK2^{-/-}$ mice after intra-peritoneal LPS injection (Kotlyarov *et al.*, 1999). Furthermore, MK3 has shown to be another p38 MAP kinase target. Using MK2 and MK3 double KO mice, BMDMs showed a further decrease in TNF production as well as expression of p38 and TTP when compared to MK2-deficient counterparts. This suggests that MK3 stabilizes the residual p38 level in the absence of MK2 and that MK3 has a similar role in TTP and TNF regulation (Ronkina *et al.*, 2007).

The nuclear protein kinases MSK1 and MSK2 play key roles in the regulation of gene transcription. ERK1/2 and p38α have shown to phosphorylate MSKs following TLR stimulation (Ananieva *et al.*, 2008). MSKs can then further phosphorylate both the chromatin protein histone H3 and the transcription factor cyclic-AMP-responsive-element-binding protein 1 (CREB1). In macrophages, MSKs play anti-inflammatory roles by the transcriptional induction of IL-10, which can suppress the production of TNF, IL-6, IL-12, and prostaglandins in macrophages. MSK1 and MSK2 deficiency enhanced the levels of these proinflammatory agents by BMDMs in response to LPS (MacKenzie *et al.*, 2013b). Studies demonstrating a mutation at the site of MSK phosphorylation on CREB1 have found that CREB1 is important in the induction of prostaglandin E2 and the regulation of Il-10 transcription (MacKenzie *et al.*, 2013).

The above-mentioned studies highlighted the important proinflammatory role of MK2 and the anti-inflammatory function of MSKs. Specific targeting of these downstream kinases might provide a way to selectively modulate the immune functions of p38α and ERK1/2.

1.3 Regulation of MAPK kinases by protein phosphatases

The previous sections discussed the role of MAPK signal transduction in developing the immune response. However, deactivation of these signals is important to control the potentially harmful effect of the immune system on the host. Also, deactivation of the MAPK pathway regenerates the regulatory cycle, allowing the immune system to respond to subsequent pathogenic challenges (Lang *et al.*, 2006, Li *et al.*, 2009). MAPKs are inactivated totally by dephosphorylation of either or both the tyrosine or threonine residues within the activation loop (Canagarajah *et al.*, 1997). The deactivation process is mediated by many protein phosphatases that can dephosphorylate one or both of the phosphosites in the active site of MAPKs to control the strength and duration of MAPK activity in different cell types (Camps *et al.*, 2000).

Protein phosphatases can be divided into two subfamilies depending on substrate specificity: serine/threonine phosphatases (such as PP2A, PP2C) and tyrosine phosphatases (such as SREP, HePTP and PTP-SL) (Mumby and Walter, 1993, Tonks, 2006, Stoker, 2005). The PTP family of cysteine-dependent protein tyrosine phosphatases is further classified into two subfamilies: tyrosine-specific PTPs that dephosphorylate protein substrates on tyrosine (Theodosiou and Ashworth, 2002) and dual-specificity phosphatases (DUSPs) that dephosphorylate protein substrates on tyrosine, serine, and threonine residues and lipid substrates (Alonso *et al.*, 2004, Tiganis and Bennett, 2007). The MAP kinase phosphatases (MKPs) are DUSPs that dephosphorylate MAPKs on both tyrosine and threonine residues within the T–X–Y activation motif in the kinase domain. These MKPs display different specificities towards MAPKs, and are thus essential negative regulators of MAPK-mediated signalling in various biological processes (Keyse, 2000). The MKPs are reviewed below and summarised in Table 1.3.

1.3.1 Dual specificity MAP Kinase Phosphatases (MKPs) Family

As mentioned above, MKPs family proteins are able to dephosphorylate both the threonine and tyrosine residues of their substrates, thus inactivating the MAP kinases. Structurally, all MKPs share with all DUSPs the phosphatase domain with conserved aspartic acid, cysteine, and arginine residues within the C-terminal, which have been reported to be essential for catalysis activity (Huang and Tan, 2012, Farooq and Zhou, 2004). This domain in MKPs shares sequence similarity with the prototypic VH-1 DUSP encoded by the vaccinia virus, DX26(V/L)X(V/I)HCXAG(I/V)SRSXT(I/V)XXAY(L/I)M, where the D (Asp), C (Cys) and R (Arg) (Denu and Dixon, 1998, Patterson *et al.*, 2009, Huang and Tan, 2012).

The N-terminal region consists of two CDC25 homology domains (also termed CH2 domain) and the MAP kinase-binding (MKB) domain or kinase-interacting motif (KIM), which binds with the common domain (CD) of MAP (Farooq and Zhou, 2004, Huang and Tan, 2012). The MKB domain comprises a cluster of positively charged amino acids that play a role in determining the binding specificity of MKPs towards MAP kinases (Tanoue *et al.*, 2000, Tanoue *et al.*, 2001). A cluster of hydrophobic amino acids together with a cluster of positively charged amino acids is essential for the phosphatase to recognize and regulate specific MAPK isoforms (Tanoue *et al.*, 2002). MAPK binding to the MKB domain has been reported to catalytically activate several MKPs (Denu and Dixon, 1998, Dowd *et al.*, 1998). According to the presence and absence of MKB domain, DUSPs are classified into typical DUSPs (the MKPs) and atypical respectively (Huang and Tan, 2012). At least 16 atypical DUSPs have been identified function independently of their phosphatase activity and instead function as scaffolds in signal transduction pathways. These are listed in Table 1.4.

In mammalian systems to date, around 10 MKP family members have been identified (Kondoh and Nishida, 2007, Patterson *et al.*, 2009, Soulsby and Bennett, 2009). These are further categorized based on their subcellular localization (nuclear, cytoplasmic, or double-located) into Type I, Type II, and Type III (Farooq and Zhou, 2004, Huang and

Tan, 2012) (see Table 1.3). In addition to their subcellular locations, these MKPs vary in their substrate specificity among the MAP kinases, their tissue distribution, and extracellular stimuli by which they are induced (Theodosiou and Ashworth, 2002, Farooq and Zhou, 2004).

Type I MKPs are located within the nucleus, are approximately 300–400 amino acids in length, and contain a nuclear localization signal (NLS) sequence in their N-termini. These MKPs have been shown to play important roles in the feedback control of MAP kinase signalling in the nucleus. MKPs belonging to this group include DUSP-1/MKP-1, DUSP2/PAC-1, DUSP4/MKP-2 and DUSP5/Hvh-3 (see Figure 1.5). All have shown to be induced by various stimuli that activate MAP kinase and display broad substrate specificity to deactivate ERK, p38 MAP kinase, and JNK (Camps *et al.*, 2000, Theodosiou and Ashworth, 2002).

Type II MKPs consist of DUSP6/MKP-3, DUSP7/MKP-X, and DUSP9/MKP-4. All three proteins are cytoplasmic enzymes and exhibit a degree of selectivity towards ERK1/2. This group contains a nuclear export signal (NES) and is distributed in the cytosol; it also shows restricted tissue distribution (see Figure 1.5). (Camps *et al.*, 2000, Keyse, 2000).

Type III MKPs include DUSP8/hVH-5, DUSP10/MKP-5, M3/6, and DUSP16/MKP-7. These are JNK/p38-specific phosphatases that exhibit sizes too large to enter the nucleus by passive diffusion; they shuttle between the cytoplasm and nucleus. MKP-5 from this group has an extended region in the N-terminus with an unknown function, whereas MKP-7 and M3/6 have unknown functions in their C-termini (see Figure 1.5). (Camps *et al.*, 2000, Matsuguchi *et al.*, 2001, Theodosiou and Ashworth, 2002).

Although the MAP Kinases have been shown to be important regulators of the immune response, as reviewed in section 1.2.4.4-5-6, the MKPs have recently joined the limelight as powerful controllers of many different biological processes. Several articles have demonstrated the potential roles of DUSPs in immune response, specifically by their dual dephosphorylating property to inactivate different MAPK isoforms. In the following

section the current knowledge regarding the function of the major MKP family members in the immune response, which have been gained particularly from KO models will be discussed. The general properties of these and other isoforms not discussed are summarised in Table 1.3.

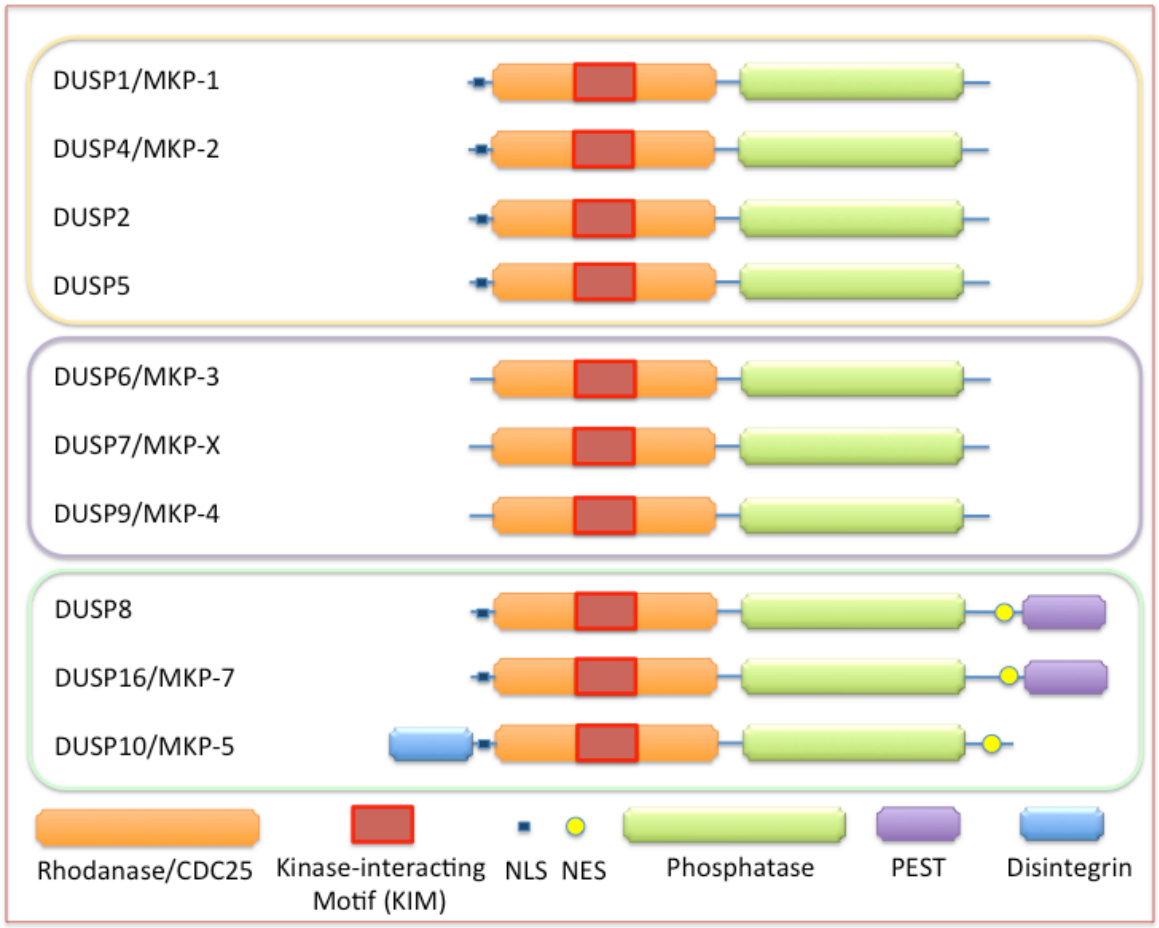


Figure 1.5: The family of dual-specificity phosphatases (DUSPs). Domain structures of the three subgroups of MKPs are shown. PEST is for proline, glutamic acid, serine and threonine. Adapted and modified from (Dickinson and Keyse, 2006, Lawan *et al.*, 2012).

Table 1.3: The MAP kinase phosphatases. Adapted and modified from (Jeffrey *et al.*, 2007, Kondoh and Nishida, 2007, Lawan *et al*., 2012).

DUSP	Other names	Subcellular location	Substrate specificity	MAPK activity in KO mice	Phenotype in KO mice	Tissue distribution
1	MKP1, CL100, hVH1, 3CH134, PTPN10 erp	Nuclear	p38>JNK>>ERK	↑p38 and JNK, no change in ERK macrophages	↑ cytokines (↓ IL-12), ↑ sepsis, Normal development	Heart, skeletal muscle, pancrease, placenta, liver, testes, stomach, lung and brain
2	PAC-1	Nuclear	ERK, p38 > JNK	↓ERK and p38in macrophages and mast cells, ↑ JNK in macrophages and mast cells	↓ Cytokines, PGE2 and NO in macrophages and mast cells, ↓ arthritis, Normal development	lymphoid cells (spleen and thymus)
4	MKP2, hVH2, TYP1, Sty8	Nuclear	JNK~ERK1 > p38	↑ERK, ↓p38, ↓ JNK in macrophages. (Cornell *et al.*, 2010a) ↑p38, ↑JNK, no effect on ERK in macrophages. (Al-Mutairi *et al.*, 2010c)	↓ Cytokines in macrophage, protected against sepsis. ↑Cytokines and arg1 in macrophage, susceptible to *L.mexicana.*	Placenta, skeletal muscle, spleen, kidney and brain, heart, liver, testis, and pancreas.
5	hVH3, B23	Nuclear	ERK	ND	ND	Placenta, liver, heart, lung, brain, kidney, pancreas, skeletal muscle
6	MKP3, Pyst1, rVH6	Cytosolic	ERK > JNK = p38	↑ ERK in embryos	Postnatal lethality, skeletal dwarfism, coronal craniosyno-stosis, hearing loss	Placenta, skeletal muscle, spleen, kidney, and brain, heart, liver, testis, and pancreas.
7	MKP-X, Pyst2	Cytosolic	ERK > p38	ND	ND	Heart, brain, placenta, lung, skeletal muscle, kidney and pancreas
9	MKP4, Pyst3	Cytosolic	ERK > JNK = p38	No change in ERK, JNK and p38 in embryo placentas and ES cells	Placental defects, embryonic lethal	Placenta, kidney, embryonic liver
8	hVH5, M3/6	Nuclear/cytosolic	JNK>p38> ERK	ND	ND	brain, heart, and skeletal muscle

10	MKP5	Nuclear/cytosolic	JNK>p38> >ERK	↑JNKin macrophages and T cells	↑Cytokines in macrophages and T cells, ↓proliferation in T cells	Heart, lung, skeletal muscle, liver, and kidney> brain, spleen and testis.
16	MKP-7	Nuclear/cytosolic	JNK, p38	↑JNK and p38 in macrophages and fibroblast from Dus16$^{-/-}$ mice (Niedzielska *et al.*, 2013)	↑IL-12p40 in macrophages from Dusp1 KO mice died neonatally without displaying any gross anatomical abnormality	Heart, kidney, testis

Table 1.4: The atypical DUSPs. Adapted from (Patterson *et al*., 2009).

Name	Alternative names	Subcellular localization
Laforin	EPM2A (epilepsy, progressive myoclonus type 2A)	Cytoplasm
STYX	Serine/threonine/tyrosine-interacting protein	Not determined
DUSP3	VHR (VH1-related)	Nucleus
DUSP12	YVH1 [protein tyrosine phosphatase (Saccharomyces cerevisiae) orthologue], GKAP (glucokinase- associated phosphatase)	Cytoplasm and nucleus
DUSP13A	TMDP (testis- and skeletal muscle-specific DUSP)	Not determined
DUSP13B	MDSP (muscle-restricted DUSP), SKRP4 (stress-activated protein kinase pathway-regulating phosphatase-4)	Cytoplasm
DUSP14	MKP-L (MKP-1-like protein tyrosine phosphatase), MKP-6	Cytoplasm
DUSP15	VHY (VH1-like member Y)	Cytoplasm
DUSP19	DUSP17 and SKRP1 (stress-activated protein kinase pathway phosphatase 1)	Cytoplasm
DUSP21	LMWDSP21 (low-molecular-mass DUSP21)	Cytoplasm and nucleus
DUSP23	DUSP25, LDP-3 (low-molecular-mass DUSP3), VHZ (VH1-related phosphatase Z)	Cytoplasm
DUSP27	DUPD1 (DUSP and pro-isomerase domain-containing 1)	Cytoplasm
DUSP11	PIR1 (phosphatase that interacts with RNA–ribonucleoprotein complex 1)	Nucleus
DUSP18	DUSP20 and LMWDSP20 (low-molecular-mass DUSP20)	Cytoplasm and nucleus
DUSP22	LMWDSP2 (low-molecular-mass DUSP2), JSP1 (JNK-stimulating phosphatase 1)	Cytoplasm and nucleus
DUSP26	DUSP24, LDP-4 (low-molecular-mass DUSP4) MKP-8, NEAP (neuroendocrine-associated phosphatase), SKRP3	Cytoplasm and nucleus

1.3.2 The role of MKPs in the immune response

1.3.2.1 MKP-1 (DUSP 1)

The mouse MKP-1 cDNA (3CH134) was discovered in the early 1980s as an immediate-early gene induced by mitogens (Lau and Nathans, 1985, Charles *et al.*, 1992). Immediately after, a human homologue (CL100) was recognised as a tyrosine phosphatase gene induced by hydrogen peroxide (Keyse and Emslie, 1992). Both 3CH134 and CL100 show similarity with the vaccinia virus VH1 dual-specificity phosphatase; they are larger in size due to the N-terminal domain (Charles *et al.*, 1993). Because it was the first specific protein phosphatase identified to dephosphorylate both tyrosine and threonine residues in the MAPK, it was named MAP kinase phosphatase (MKP)-1 (Sun *et al.*, 1993).

Evidence from both *in vitro* and *in vivo* studies found that MPK-1 is more selective for p38 and JNK than ERK (Table 1.3) (Liu *et al.*, 1995, Raingeaud *et al.*, 1995, Franklin and Kraft, 1997, Zhao *et al.*, 2005, Chi *et al.*, 2006). Despite its apparent selectivity *in vivo*, the catalytic activity of MKP-1 is enhanced by its binding to all three major MAP kinases through the MKB domain (Slack *et al.*, 2001, Hutter *et al.*, 2000).

MKP-1 is expressed in a wide variety of tissues and cells. The highest levels were observed in the heart, lungs, and liver (Charles *et al.*, 1992, Noguchi *et al.*, 1993). Immunofluorescent microscopy and subcellular fractionation studies revealed that MKP-1 is located in the nucleus, and nuclear translocation is mediated by an N-terminal LXXLL motif (Wu et al., 2005). Expression of MKP-1 is induced in response to growth factors, hormones, LPS, cytokines, osmotic and heat shock, anisomycin, UV, and 12-O-tetradecanoylphorbol 13-acetate (Sun *et al.*, 1993, Dorfman *et al.*, 1996, Saxena and Mustelin, 2000, Tamura *et al.*, 2002).

The physiological functions of MKP-1 in various systems have been extensively studied using KO mice models. Several excellent studies demonstrated the role of MKP-1 in many pathological conditions, including cancer, diabetes and obesity (Wu and Bennett, 2005, Wu *et al.*, 2006, Roth *et al.*, 2009). In the immune system, an important role of

MKP-1 has been suggested by several reports. Because MKP-1 has shown to inactivate p38 and JNK and both have a crucial role in cytokine production, this suggested a link between MKP-1 and cytokine biosynthesis (Chen *et al.*, 2002). This was proven in several studies in which MKP-1 was induced in macrophages following LPS stimulation and overexpression of MKP-1 resulted in reduction of TNF-α and IL-6 production, which was mediated by accelerated JNK and p38 dephosphorylation (Chen *et al.*, 2002). Similarly, overexpression of MKP-1 in peritoneal macrophages caused down-regulation of TNF-α production and endotoxin tolerance via abolishing p38 MAPK activation (Nimah *et al.*, 2005).

Further studies from MKP-1 KO macrophages (alveolar, peritoneal, and bone marrow derived) showed elevated production of several pro-inflammatory cytokines and chemokines in relation to wild-type mice following *in vitro* LPS challenge. This included TNF-α, IL-1β, CCL2, GM-CSF, IL-6, and IL-12 (Zhao *et al.*, 2006, Hammer *et al.*, 2006, Salojin *et al.*, 2006, Chi *et al.*, 2006). These reports and other studies confirmed that in the absence of MKP-1 phosphorylation of p38 as a primary target of MKP-1 was prolonged followed by JNK with no or little effect on ERK1/2 (Chen *et al.*, 2002, Zhao *et al.*, 2005). As with *ex vivo* testing, systemic administration of LPS also significantly increased serum IL-6, IL-12, TNF-α, MCP-1 and IFN-γ. Consequently, MKP-$1^{-/-}$ mice were more susceptible to septic shock and multiple organ failure due to increase NO production in response to LPS challenge (Zhao *et al.*, 2006).

However, MKP-1 deficiency also caused an enhancement in the anti-inflammatory IL-10 production, which was confirmed by a global microarray experiment of MKP-$1^{-/-}$ spleen cells (Hammer *et al.*, 2006). Whilst IL-10 was elevated in $MKP1^{-/-}$ mice, these showed higher incidence of multi-organ failure and mortality compared with wild-type counterparts. One suggested explanation was that IL-10 production was either insufficient or not produced early enough to inhibit the effects of pro-inflammatory cytokines (Howard *et al.*, 1993, Gerard *et al.*, 1993).

In addition to the role of MKP-1 in the innate immune response to LPS, MKP-1 was found to play a critical role in BMDMs stimulated with TLR agents such as CpG, peptidoglycan (Shepherd *et al.*, 2004), zymosan, poly(I:C), and flagellin (Salojin *et al.*, 2006). Activation by these agents in MKP-1 KO macrophages resulted in increased cytokine production and elevated levels of TNF-α, IL-10, as well as CD86 and CD40 due to enhanced p38 activation (Salojin *et al.*, 2006). Of additional interest, it was found that infecting MKP-$1^{-/-}$ mice with *Chlamydophila pneumoniae* was associated with enhanced pulmonary IL-6 production (Rodriguez *et al.*, 2010). Also, in two models of septic peritonitis, serum levels of CCL4, IL-10 and IL-6 together with iNOS mRNA in spleen and liver were increased in MKP-$1^{-/-}$ mice when compared to wild-type (Hammer *et al.*, 2010). In both reports, an increased inflammatory response in the absence of MKP-1 was associated with lethality. In contrast, the absence of MKP-1 had no effect on the survival or bacterial clearance following *Staphylococcus aureus* infection (Wang *et al.*, 2007).

The negative effect of MKP-1 in the innate immune response has also referred to the competition of MKP-1 with the downstream substrates for p38 or JNK pathways (Tanoue *et al.*, 2000). For example, MKP-1 and the downstream kinase MK2 bind to the same C-terminal domain of JNK and p38 (Tanoue *et al.*, 2000) and also in the same acidic domain of p38 (Hutter *et al.*, 2000). This competition allows MKP-1 to interfere with the interactions of MAPKs with the corresponding downstream targets to inhibit signalling transduction.

At the level of the adaptive immune response, MKP-1 deletion increased the severity of collagen-induced rheumatoid arthritis, which correlated to a significant increase in pro-inflammatory cytokines such as TNF-α and IL-6. Because collagen-induced arthritis depends on arthritogenic antibody production against Type-II collagen, which requires T and B cells, this suggests that MKP-1 deficiency could also enhance the adaptive immune response (Salojin *et al.*, 2006).

Taken together, the studies predominantly utilising MKP-1 deficient mice indicated the pivotal role of MKP1 in TLR-mediated pro-inflammatory cytokine release and important

negative feedback control in limiting the activity of p38 and JNK during the innate and adaptive immune responses.

1.3.2.2 MKP-5 (DUSP10)

Another DUSP member which has a key role in the immune function is DUSP10 or MKP-5. This is a member of Type III MKP, was cloned to p38 MAPK in a yeast two-hybrid experiment (Tanoue *et al.*, 1999). MKP-5 consists of an N-terminal MAPK binding domain (150 amino acids) that binds to p38 MAPK and is essential for p38 MAPK dephosphorylation, and two CDC25-like domains (Tao and Tong, 2007). MKP-5 also carries an additional N-terminal domain of unknown function. The MKP-5 gene is found in the heart, lung, liver, skeletal muscle, and kidney but is not expressed in the brain, spleen, or testes (Tanoue et al., 1999). As with other MKPs, expression of MKP-5 is induced by TNF-α, anisomycin, and osmotic stress, but not by UV irradiation or phorbol ester (Jeong *et al.*, 2007, Masuda *et al.*, 2000).

In vitro studies have shown that MKP-5 dephosphorylates both JNK and p38 and negatively regulated cytokine production (Tanoue *et al.*, 1999, Theodosiou *et al.*, 1999). However, in MKP-$5^{-/-}$ peritoneal macrophages stimulated with LPS, JNK activity was increased, but not p38, which enhanced TNF-α and IL-6 production (Zhang *et al.*, 2004). In response to peptidoglycan and polyI:C30, MKP-$5^{-/-}$ peritoneal macrophages produced higher levels of IL-6 and TNF-α when compared to wild type cells (Zhang *et al.*, 2004). Recently, MKP-5 was reported to control p38α inactivation and superoxide generation in neutrophils stimulated with complement component 5a (C5a) (Qian *et al.*, 2009).

In the adaptive immune cells, MKP-5-deficient Th1 and Th2 cells exhibited enhanced levels of JNK activity with no effect upon p38 and thus produced elevated levels of IFN-γ and IL-4, respectively, which indicates that MKP-5 negatively regulates T-cell cytokine expression. However, proliferation of these T cells was impaired and resulted in reduced severity to experimental autoimmune encephalomyelitis (EAE). In contrast, in response to Lymphocytic choriomeningitis virus (LCMV), MKP-$5^{-/-}$ $CD4^{+}$ and $CD8^{+}$ T cells produced significantly increased levels of TNF-α and IFN-γ compared with MKP-$5^{+/+}$ cells, which

led to much more robust and rapidly fatal immune responses to secondary infection with lymphocytic choriomeningitis virus (Zhang *et al.*, 2004).

Taken together, MKP-5 targeting in mice revealed an interesting phenotype with important functions in both innate and adaptive immune responses.

1.3.2.3 PAC-1 (DUSP2)

DUSP2/PAC-1 is another type I MKP was cloned in 1993 as an immediate-early gene from human T cells (Rohan *et al.*, 1993). This nuclear phosphatase shows in transient transfection assays a substrate specificity for p38 and ERK (Chu *et al.*, 1996, Ward *et al.*, 1994). Comprehensive microarray study has reported PAC-1 is highly expressed in activated human mast cells, eosinophils, neutrophils, B and T cells, and macrophages as well as in activated mouse mast cells and macrophages (Jeffrey *et al.*, 2006).

Unlike the effects of MKP-1 and MKP-5 deficiency on the immune response, PAC-1 deficiency positively regulates innate and inflammatory cell signalling. Surprisingly, PAC-1 deletion decreased the activity of ERK and p38 in macrophages and mast cells stimulated with LPS and phorbol 12-myristate 13-acetate (PMA), whereas JNK increased. PAC-$1^{-/-}$ macrophages and mast cells also produced less proinflammatory cytokines including TNF-α, IL-6, and IL-12 as well as chemokines and inflammatory mediators such as PGE2 and NO (Jeffrey *et al.*, 2006, Jeffrey *et al.*, 2007). Its deletion also reduced the activation of Elk-1 and AP-1 as a result of reduced ERK activation. The defect in ERK activation caused by PAC-1 knockout was retrieved by inhibiting JNK. Thus the positive regulatory function of PAC-1 is mostly due to the crosstalk between JNK and ERK. PAC-1 deficiency also caused attenuation in inflammatory responses in KxB/N model of inflammatory arthritis, which is dependent on the function of mast cells and macrophages (Jeffrey *et al.*, 2006).

1.3.2.4 MKP-6 (DUSP14)

MKP-6, also called MKP-L was identified by a yeast-hybrid screen as a CD28 cytoplasmic tail-interacting protein (Marti *et al.*, 2001). MKP-6 can dephosphorylate all

three MAP kinases *in vitro* and is ubiquitously expressed. In the immune system it is found in the thymus, lymph nodes, and spleen. In T cells stimulated through CD28, MKP-6 is rapidly induced, as well as in neutrophils and monocytes (Marti *et al.*, 2001, Salojin and Oravecz, 2007).

Examining CD28 signalling, dominant negative MKP-6 enhanced CD28-induced production of IL-2 as a result of specific enhancement in ERK and JNK activation, but not p38. The reason for the interaction between MKP-6 and CD28 receptor is unclear, but it is possible that CD28 works as anchor protein to keep MKP-6 in close proximity to sites of MAP kinase activation (Marti *et al.*, 2001, Salojin and Oravecz, 2007). In a very recent study, MKP-6 was reported to negatively regulate TNF-α or IL-1-induced NF-κB pathway by dephosphorylating TAK1 (Zheng *et al.*, 2013). Further studies of MKP-6-deficient immune cells such as macrophages will be required to determine the precise role of MKP-6 in immune responses and inflammation.

1.3.2.5 MKP-2 (DUSP4)

Another key type I MKP, which has not been examined to the same extent as MKP-1 is DUSP4/MKP-2. This is an inducible nuclear protein of 394 amino acids (M.W. approx. 43 kDa) was cloned initially from PC12 cells (Misra-Press *et al.*, 1995a). Recently, a novel variant of MKP-2 with a shortened N-terminus has been identified (Cadalbert et al., 2010). MKP-2 represents about a 60% sequence homology, with MKP-1 exhibiting similarities in structure and splicing pattern (Guan and Butch, 1995). Both genes have four exons and three introns. This suggests that they are both derived from a common origin (ancestral gene) (Noguchi *et al.*, 1993, Zhang *et al.*, 2001). However, they differ in their tissue distributions, which may explain different physiological functions for each phosphatase. Unlike MKP-1, MKP-2 is expressed in the brain, spleen, and testes, with the highest expressions in the heart and lung, and with fewest expressions in the skeletal muscle and kidney (Misra-Press *et al.*, 1995, Guan and Butch, 1995, Chu *et al.*, 1996) Nuclear localisation of MKP-2 is determined by triple arginine sequences located within the MKB, and functions as an NLS. The distribution of two putative nuclear localisation

sequences, NLS1/NLS2, is required to alter the cellular localisation of MKP-2 (Chen *et al.*, 2001, Sloss *et al.*, 2005)

According to several studies, MKP-2 was found to be induced by serum, growth factors, a gonadotropin-releasing hormone (GnRH), retinoic acid, the oncogenic gene v-, the tumour suppressor p53, phorbol esters, E2F-1, UV-light, and oxidative stress (Misra-Press *et al.*, 1995a, Guan and Butch, 1995, Zhang *et al.*, 2001, Brondello *et al.*, 1997, Torres *et al.*, 2003, Palm-Leis *et al.*, 2004). *In vitro*, MKP-2 showed specificity for ERK1/2 and JNK more than p38. Nevertheless, the potential regulation of p38 by MKP-2 has been reported in hepatocytes (Berasi *et al.*, 2006). Studies have demonstrated that selectivity between JNK and ERK can vary depending on the cell type (Lawan *et al.*, 2012). In different cells, MKP-2 can regulate either ERK (Misra-Press *et al.*, 1995a, Guan and Butch, 1995) or JNK (Cadalbert *et al.*, 2005, Robinson *et al.*, 2001). For example, MKP-2 can deactivate JNK, but not ERK1/2 in UV-C or cisplatin treated HEK293. In these cells, while JNK translocates to the nucleus, ERK1/2 remains in the cytoplasm, which suggests that MKP-2 may dephosphorylate nuclear JNK, but not cytoplasmic ERK1/2 (Cadalbert *et al.*, 2005). Another study demonstrated that small interfering RNA (siRNA) MKP-2 in HEK 293 resulted sustained JNK activation in response to H_2O_2 treatment (Teng *et al.*, 2007). In addition, over-expression of MKP-2 in PMA-stimulated EAhy926 cells had little effect on ERK phosphorylation despite its translocation to the nucleus, whereas nuclear activation of JNK was completely abolished (Robinson et al., 2001). This contrasts with another study in which MKP-2 transfected in NIH3T3 and HELA cells caused efficient ERK1/2 and JNK dephosphorylation (Chu *et al.*, 1996).

Comparing to MKP-1, MKP-2 is much neglected. However, with the recent development of MKP-2 KO mice in our laboratory as well as by others, MKP-2 has been shown to play an important regulatory role in different physiological and pathological processes including proliferation (Lawan *et al.*, 2011) and apoptosis (Ramesh *et al.*, 2008, Al-Mutairi *et al.*, 2010a) as well as the immune function (Al-Mutairi *et al.*, 2010, Cornell *et al.*, 2010)

MKP-2 deletion studies at cellular and pathological levels have suggested the involvement of this phosphatase in the immune functions. In one study, MKP-$2^{-/-}$ mice were found to be resistant to both LPS-induced endotoxic shock and sepsis. These mice displayed decreased serum levels of TNF-α, IL-1β, IL-6 as well as IL-10 following endotoxin challenge (Cornell *et al.*, 2010). In LPS stimulated MKP-$2^{-/-}$ BMDMs, higher phosphorylation levels of ERK1/2 were obtained in comparison to wild-type cells. The enhancement of ERK activation augmented the MKP-1 expression, which further deactivated p38α and JNK phosphorylation. Authors reported that this feedback loop could explain the reduced TNF-α production by LPS-stimulated MKP-2 deficient macrophages, thus further protecting MKP-$2^{-/-}$ mice from endotoxic shock (Cornell *et al.*, 2010b). In contrast, another study found that MKP-2 negatively regulates pro-inflammatory cytokines production, and that mice lacking MKP-2 are more sensitive to infection by *Leishmania Mexicana* (Al-Mutairi *et al.*, 2010). The susceptibility of MKP-$2^{-/-}$ mice towards *Leishmenia* was correlated to the increased expression of arginase 1 and decreased induction of iNOS. Analysis of MKP-2 deficient BMDMs revealed an increased and prolonged LPS induced JNK and p38 activation, but did not affect ERK1/2 activation. MKP-2 deletion also enhanced LPS-stimulated induction of IL-6, IL-12p40, TNF-α, and prostaglandin E (PGE_2) (Al-Mutairi *et al.*, 2010). Results from a third study utilising MKP-2 deficient mice indicated that MKP-2 suppresses $CD4^+$ T-cell proliferation through regulating STAT5 phosphorylation and IL-2 signalling. Activated MKP-$2^{-/-}$ $CD4^+$ T cells were hyperproliferative due to enhanced CD25 expression and IL-2 production through increased STAT5 phosphorylation (Huang *et al.*, 2012).

The above different phenotypes obtained from MKP-2 deficient studies can be due to different background strains of these independently generated knockout mouse lines or due to experiment strategy variations. However, these studies suggest that the resulted MKP-2 KO phenotype is underpinned by deficit in macrophage function. Since not much work has been done about MKP-2 deletion at cellular level, this suggests importance to further examine the role of MKP-2 in the function of these immune cells.

1.4 Aims and Objectives

Recent studies including work from our laboratory have shown the potential of MKP-2 to play a substantial role in the immune function, though regulating the MAPK pathway in immune cells in particular macrophages. Lack of MKP-2 affects pro-inflammatory cytokine production and further protects MKP-$2^{-/-}$ mice from endotoxic shock or renders them susceptible to *Leishmania Mexicana.* It was hypothesised that the phenotypic outcomes observed *in vivo* following MKP-2 deletion are underpinned by deficits in macrophage function. Given the lack of information on the molecular events that lead to these phenotypic differences following MKP-2 deletion as well as the effects of MKP-2 deficiency on macrophage development, the following will be addressed:

1) The role of MKP-2 on the development of primary mouse bone marrow derived macrophages (BMDMs) isolated from DUSP-4 (MKP-2) deletion mice, having initially characterized the cellular functions of MKP-2 and MAPK mediated signaling in BMDMs, including:
    i. MKP-2 induction.
    ii. Correlating loss of DUSP-4 with changes in MAPK signaling.
    iii. Correlating these effects with macrophage proliferation, development-related gene expression, and the expression of macrophage surface proteins.
2) Determine the transcriptional responses regulated by MKP-2 in macrophages using microarray gene expression analysis following LPS or IL-4 stimulation to identify pro- and anti-inflammatory related gene expressions, respectively. To further confirm differential gene expressions, qRT-PCR assays and ELISA techniques are selected to validate microarray data.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 General Reagents

All materials and reagents used were of the highest commercial grade available and were obtained from the following suppliers.

Sigma-Aldrich Co. LtD (Poole, Dorset, UK)

Bis-Acrylamide, Ammonium Persulphate (APS), Glycerol, Glycine, Kodak X-OMAT LS X-ray film, Methanol, Sodium Chloride, Sodium Dodecyl Sulphate (SDS), N,N,N',N' tetramethylenediaamine (TEMED), molecular grade water, Isopropanol, HCl, Agarose powder, streptavidin-horse radish peroxidase (SAv-HRP), TWEEN-20 and Trizma Base. Lipopolysaccharides (LPS from *Escherichia coli)* (L2880-10MG).

Roche diagnostics GmbH

Dithiothreitol (DTT).

Bio-Rad Laboratories (Hertfordshire, UK)

Pre-stained SDS-Page molecular weight markers.

Boehringer Mannheim (East Sussex, UK)

Bovine serum albumin (BSA, Fraction V).

Gibco Life Technologies Ltd (Renfrewshire, UK)

Tissue culture growth media; DulBeccos Modified Eagle Medium (DMEM), RPMI 1640, antibiotics (Penicillin/Streptomycin), Fetal Calf Serum (FCS), L-Glutamate.

Corning B.V (Buckinghamshire, UK)

All tissue culture flasks, plates, dishes and graduated pipettes.

Whatmann (Kent, UK)

Nitrocellulose Membranes, 3MM blotting paper.

Amersham International Plc (Aylsbury, Buckinhamshire, UK)

ECL detection reagents.

BD Biosciences(Oxford, UK)

Recombinant mouse IL-4.

Round-bottomed flow cytometry tubes.

NUNC Maxisorp plates.

eBioscience,Ltd. (Hatfield, UK)

Recombinant mouse M-CSF protein.

Sterilin Ltd. (Stafford, UK)

90mm triple vent petri dishes.

2.1.2 Microscopy

Merck-Calbiochem (Nottingham, UK)

Mowiol.

Nikon Instruments (New York, USA)

MF-830 Microscope connected to Motic Image Plus 2.0 software.

VWR International Ltd (Leicestershire, UK)

Circular glass 13 or 22 mm diameter coverslips.

0.8-1 mm thick glass microscopy slides.

2.1.3 Antibodies

Jackson ImmunoResearch Laboratories Inc. (PA, USA)

HRP-conjugated goat anti-rabbit IgG.

HRP-conjugated anti-mouse IgG.

Santa Cruz Biotechnology Inc. (CA, USA)

Rabbit polyclonal anti-MKP-2 (S-18).
Rabbit polyclonal anti-T.JNK.
Mouse monoclonal anti-T.ERK.
Rabbit polyclonal anti-T.p38.

Cell Signalling Technology, Inc. (New England Biolabs, UK)

Rabbit polyclonal anti-p-JNK (T183/Y185).
Rabbit polyclonal anti-p-ERK (T202/Y204).

Applied Biosystems, Life Technologies (Paisley, UK).

Rabbit polyclonal anti-p-p38 (44684-G).
AbC™ anti-Rat/Hamster Bead Kit.

2.1.4 Flow Cytometry

eBioscience,Ltd. (Hatfield, UK)

Anti-Mouse CD34-FITC conjugated.

Anti-Mouse CD115 (c-fms)-PE conjugated.

Anti-Mouse MHC Class II-FITC conjugated.

Anti-Mouse F4/80 Antigen-PE conjugated.

Anti-Mouse CD14-PerCP-Cy5.5 conjugated.

Anti-Mouse CD11b-APC conjugated.

Anti-Mouse CD16/CD32 Functional Grade Purified.

2.1.5 Inhibitors

Tocris Bioscience (Bristol, UK)

SP600125 JNK inhibitor.

SB 203580 p38 inhibitor.

U2106 MEK1,2 inhibitor.

2.1.6 Enzyme-linked immunosorbent assay (ELISA)

Peprotech EC Ltd. (UK)

Rabbit anti-mouse Relm-alpha polyclonal IgG antibody (500-P214).

Recombinant murine Relm-alpha standards (450-26).

Rabbit anti-mouse relm alpha biotinylated polyclonal IgG antibody (500-P214Bt).

R&D Systems Ltd. (Abingdon, UK)

Mouse Chitinase 3-like 3/ECF-L monoclonal rat IgG_{2A} antibody.

Mouse Chitinase 3-like 3/ECF-L standard (DY2446).

Mouse Chitinase 3-like 3/ECF-L biotinylated monoclonal gout IgG antibody (BAF2446).

(KPL, Inc., USA)

TMB Microwell Peroxidase Substrate System.

2.1.7 Kits and Products for Molecular Biology

Applied Biosystems, Life Technologies (Paisley, UK)

SuperScript® III First-Strand Kit (synthesis system for RT-PCR), Primers (desalt), SYBR® Select Master Mix, MicroAmp® Fast 8-tube strip and cap strips.

STARLAB, Ltd. (Milton Keynes, UK)

TipOne aerosol-resistant filter tips.

0.5 ml thin-walled PCR tubes, flat cap.

QIAGEN Sample and Assay Technologies, (Crawley - West Sussex, UK)

QIAGEN RNeasy Mini Kit containing RW1 buffer, RPE buffer, RLT buffer, RNase-free water and microcentrifuge spin columns).

QIAshredder columns (disposable cell-lysate homogenizers).

RNase-Free DNase Set.

Promega (Southampton, UK)

GoTaq® Amplification kit.

Cell Signalling Technology, Inc. (New England Biolabs, UK)

100bp DNA ladder and 1 kb DNA ladder.

Agilent Technologies (Wokingham, UK)

SurePrint G3 Mouse GE 8x60K Microarray Kit.

Low Input Quick Amp Labelling Kit, one-colour.

Gene Expression Hybridization Kit.

RNA Spike-In Kit, One-Color.

Gene Expression Wash Buffer Kit.

Hybridization Gasket Slide Kit – 8 microarrays per slide format (8x60).

Bio-Rad Laboratories (Hertfordshire, UK)

Bio-Rad Expression RNA StdSens Reagents and Chips.

2.2 Generation of MKP-2 deficient mice

The deletion of the MKP-2 (DUSP-4) gene was performed by Genoway, Lyon, France using standard procedures. Briefly, the short arm and the long arm flanking both side of the cluster of exon 2-4 of the mouse MKP-2 gene was obtained by PCR using the following primers for the small homology arm: 5'-GTGCCTGGTTCTGTGTGTGTCTGTTCTCC-3' for the forward primer and 5'-TCTTACAGCCCTCTTTCCTCACGGTCG-3' for the reverse primer producing a PCR fragment of 3009 bp. For the long homology arm: 5'-CTTTAGGAGCGACGGCCAGGAACACAGG-3' for the forward primer and 5'-ACCCTGCCACACAGGTTGGAGCAAGG-3' for the reverse primer producing a PCR fragment of 6336 bp. Selected clones obtained from these PCR amplifications were sequenced to minimise the number of point mutation carried through. Both arms were introduced into a pBS vector in either side of the neomycin cassette. The final vector was transfected into 129Sv mouse embryonic stem cells (ES). Only the clones that had homologous recombination events were selected for first by PCR and then using Southern blotting for the short and the long arm on the construct. The screening for the short arm was further used to screen the animal carrying the construct. Four different ES clones were injected into C56Bl/6 blastocystes and reintroduced into OF1 pseudo-pregnant females. Fourteen different male chimeras were obtained and crossed with C57Bl/6 female to obtain the F1 generation. The F1 generation was screened for germ line transmission of the mutation. Three males and 2 females from the F1 generation were heterozygotes for the mutation and were subsequently used for breeding purposes (See figure 2.1.

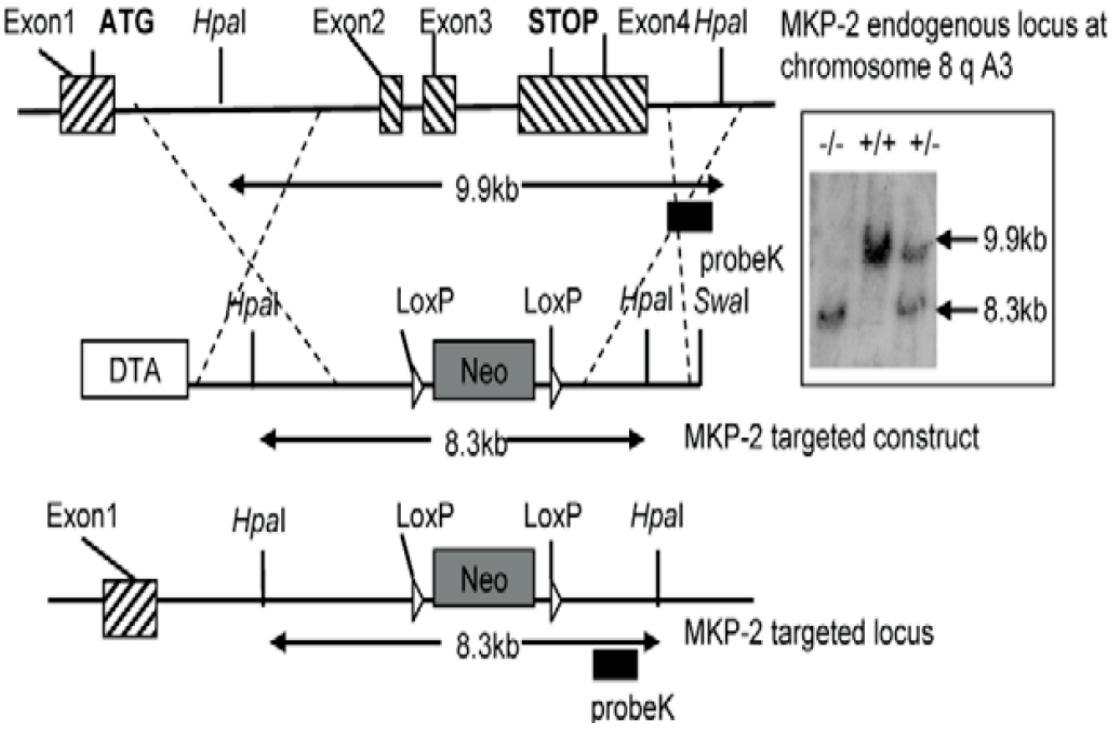


Figure 2.1: Generation of mice lacking DUSP4/MKP-2 gene by targeted homologous recombination: Schematic showing the DUSP4/MKP-2 gene locus, the targeted construct and the resulting targeted allele. Recombination events are indicated by dashed lines and show the replacement of a 8.3kb SwaI DUSP4/MKP-2 genomic fragment containing exon 2-4 by the PGK-Neo cassette. SwaI and HpaI described the restriction sites for the respective enzymes. The pGK-Neo cassette is flanked by LoxP sites. DTA represents the negative selection cassette. Top right panel showed an example of the 3' southern blot analysis of mouse tail tip genomic DNA following digestion with HpaI using an external Probe K as indicated in panel. The autoradiography revealed the 9.9kb (wild type) and 8.3kb (targeted) fragments representing the two different alleles discriminating wild type, heterozygote or homozygote mutant animals.

2.3 DNA preparation for mouse genotyping

2.3.1 DNA extraction

A small section of mouse tail (about 4 mm) was removed using a sterile pair of scissors and placed in 1.5 ml microcentrifuge tubes containing 0.5 ml of lysis buffer, which contained (0.5 µl Proteinase K in (0.2 % (v/v) SDS, 5 mM EDTA, 200 mM NaCl and 10 mM Tris). The samples were incubated at 65°C overnight on a shaker. Samples were spun at 8000 xg for 20 minutes to remove the insoluble material. The supernatant was transferred into new microcentrifuge tubes containing 1/10 vol. 3M NaAc, pH 5.5; then 1⁄2 vol. isopropanol was added and tubes were inverted several times. Samples were spun for 2 minutes at 11,000 xg, the supernatant was discarded before addition of 0.5 ml of ice cold 70 % (v/v) ethanol. Samples were spun once more and the supernatant discarded. The samples were air dried for 30 min at room temperature. The purified mouse genomic DNA was resuspended by adding 50 µl of ultrapure water and heating for 30 min at 65°C. Samples were stored at - 20° C. For genotyping, 1 µl of this extracted genomic DNA was used per PCR reaction.

2.3.2 Polymerase chain reaction (PCR) amplification

The extracted DNA was used directly for PCR using MKP-2 primers for (WT) and (KO). The PCR amplifications were in a volume of 50 µl containing 49 µl of PCR master mix, which composed of (10 µM forward primer, 10 µM reverse primer, 0.2 mM dNTPs, 5x GoTaq buffer, 1.25 U of GoTaq DNA polymerase (Promega, UK) and 35.75 µl dd H_2O.

MKP-2 primers:

WT forward primer 5'-CTTCAGACTGTCCCAATCAC-3'

WT reverse primer 5'-GACTCTGGATTTGGGGTCC-3'

KO forward primer 5'-TGACTAGGGGAGGAGTAGAAGGTGGC- 3'

KO reverse primer 5'- ATAGTGACGCAATGGCATCTCCAGG- 3'

PCR conditions for MKP-2 genotyping: Initial denaturation at 95°C for 2 min. This was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s and

extension at 72°C for 3 min. Then there was a final extension at 72°C for 5 min and the reaction stopped by a 4°C incubation. The PCR products were separated by agarose gel electrophoresis.

2.3.3 DNA detection

The amplified PCR products were separated and detected by using agarose gel (1% w/v) electrophoresis. One gram of agarose and 100 mls of the electrophoresis buffer 1X TAE (consisting of 40 mM Tris, 1 mM EDTA, and 20 mM acetic acid) were mixed and heated for 3-4 min in a standard microwave until the agarose was dissolved. Ethidium bromide (2 μl) was directly added to the agarose gel solution in the fume cupboard. The resulting solution was transferred into the gel cast to allow for solidification, this was then transferred to the electrophoresis chamber containing 1X TAE buffer. Gels were ran at 90-120 volts for 45-90 min. For sample loading, 15 μl of the PCR genotyping reactions were pipetted into the wells of the gel. DNA bands were visualized under UV-light (302nm) in a gel documentation system (InGenius from Syngene). Routinely mice were genotyped by PCR of tail tip DNA and backcrosses of 6 were used (see figure 2.2).

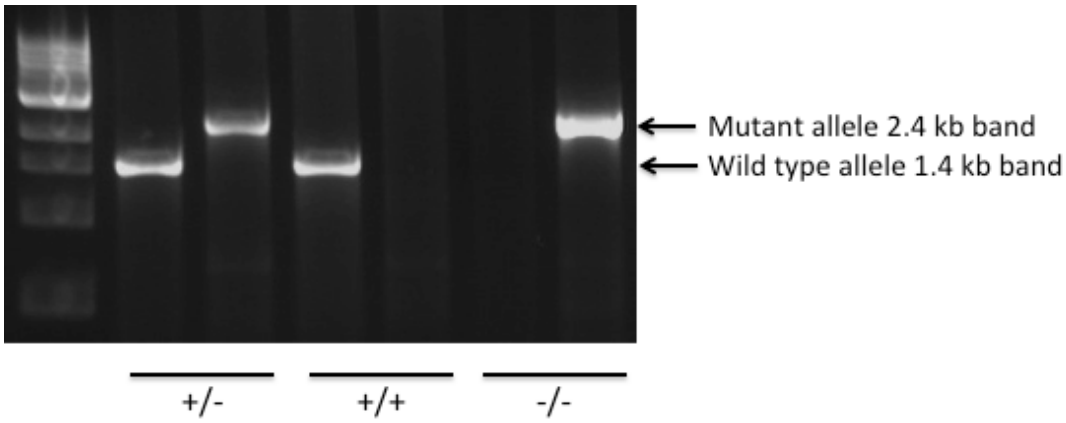


Figure 2.2: **The PCR screening for MKP-2 mice genotype.** Gel image of the three MKP-2 genotypes showing a 1.4 kb amplicon for the wild type allele and the 2.4kb amplicon for the mutant allele. The PCR amplicons were resolved on a 1% (w/v) agarose gel. The size marker was a 1kb DNA ladder (New England Biolabs, UK).

2.4 Cell culture

All cell culture work was conducted in a Class II biological safety cabinet, following strict aseptic conditions. Unless otherwise stated all cells were grown in 90mm petri dishes.

2.4.1 Procedure for L-cells conditional medium

Large quantities of macrophage-colony stimulating factor (M-CSF), required for the culture of bone marrow-derived macrophages (BMDMs), was derived using L929 cells (ECACC). An aliquot of L929 cells was dispersed at 37°C and centrifuged at 200*g* for 5 minutes. The pellet was resuspended in 10 ml of complete DMEM medium containing sodium pyruvate, pyridoxine hydrochloride and high glucose (Invitrogen, Paisley, UK), 10% HI-FCS (Invitrogen, Paisley, UK), 2mM L-glutamine (Cambrex BioScience, Veniers, Belgium), 100 U/ml penicillin (Invitrogen, Paisley, UK) and 100 µg/ml streptomycin (Invitrogen, Paisley, UK). The cells were cultured in a T25 cell culture flask (Greiner Bio-One, Stonehouse, UK) for approximately 4-5 days until confluent. Media was aspirated from the flask and replaced with 5ml cold PBS. After 10 minutes at 4°C, cells were gently scraped using a 30 cm cell scraper (TPP, Switzerland). The resulting cell suspension was centrifuged at 200*g* for 5 minutes and subsequently the supernatant was removed and the pellet was resuspended in 10 ml complete DMEM medium. To each of ten T75 cell culture flasks, 1ml of cell suspension was added. A further 9 ml of complete DMEM medium was added to each flask, which were then incubated in a CO_2 incubator at 37°C / 5% CO_2 until 80-90% confluent. Cells were then scraped; resuspended vigorously with complete DMEM medium to dissolve the cell clumps and 3 ml of cell suspension was distributed into each layer of triple layer flask. A final 200 ml of complete DMEM medium was then added to each triple layer flask. After 7 days of incubation, the supernatant was collected from the flasks, centrifuged at 3000 xg for 5 min, filtered, aliquoted 45 ml into 50 ml tubes and stored at -20°C for later use in the differentiation of macrophages from bone marrow stem cells.

2.4.2 Bone Marrow Isolation (the Generation of Macrophages) and proliferation experiments

Bone marrow-derived macrophages were obtained from 6-8-week-old C57BL/6 mice. Mice were killed by cervical dislocation. Bones of the femurs and tibia were dissected free of adherent tissue and washed with 70% ethanol and sterile PBS (pH 7.4). In cell culture hood, the ends of the bones were cut off and the marrow tissue eluted by irrigation with 5 ml of media per each bone. The media for BMDMs consisted of Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (FCS), 5 mM of L-glutamine, 100 U/ml of penicillin, 10 µg/ml of streptomycin and 30% L-cell conditioned medium as a source of M-CSF, the growth factor for macrophages. Cells were then suspended by flushing using a 21-gauge needle for disaggregation and then by vigorous pipetting to produce a single cell suspension. The cell suspension was then transferred into petri dishes with each one supplemented with 15 ml of BMDMs. Dishes were then incubated at 37°C in a humidified 5% CO_2 for 10 days. After 3 days of incubation, macrophage dishes were supplemented with 10 ml BMD-macrophage media. Furthermore, on day 5 and 7, media were completely replaced with 20 ml BMD-macrophage media. After 10 days, macrophages were confluent. To harvest the cells, the media was removed and replaced by 5ml of cold sterile RPMI 1640 media to detach the cells. Cells were harvested from the dishes and collected in 50 ml tubes. The same step was repeated with 3 ml RPMI to ensure harvesting all the cells. The collected suspension was then centrifuged at 8000 xg for 10 min followed by washing of the cell pellet twice with sterile RPMI 1640 media to remove any traces of DMEM. Lastly, the resulting pellets were re-suspended in 10 ml of complete RPMI that contained 10% fetal calf serum (FCS), 5 mM of L-glutamine, 100 U/ml of penicillin. Cells were then counted from a diluted cell suspension in a haemocytometer and diluted according to the desired cell number and concentration.

For the cell count proliferation assay, bone marrow-derived cell suspension was obtained from 6-8-week-old C57BL/6 mice as described above. Equal number of cells ($3.5x10^6$)

from the bone marrow cell suspension were seeded on the same day of isolation onto petri dishes. Dishes were then incubated at 37°C in a humidified 5% CO_2 for 10 days with each one supplemented with 15 ml of BMD-macrophage media. Cells were fed as above and on day 5, 7, and 10, macrophages were harvested then counted from a diluted cell suspension in a haemocytometer.

For the cell proliferation experiment using haematoxylin staining, macrophages were grown to day 3 and harvested with cold PBS. The macrophages were seeded on coverslips into 24-well plates (5000 cells/well) in 10 % FCS-DMEM, and then allowed to attach for 24 h. Cells were then stimulated for 24, 48, and 72 h (each point performed in triplicate) with 10 ng/ml of recombinant mouse M-CSF. Cultures were then removed and washed twice with cold PBS and then fixed in 70% methanol for 30 min at room temperature. Fixed cells were then washed twice with cold PBS and stained with 1 ml haematoxylin for 20-30 min at room temperature. Excess haematoxylin was then removed with a pipette and rinsed with water until coverslips become transparent. Cell number at each time point was then determined by counting the number of cells in 10 random fields per coverslip using MF-830 Microforge microscope connected to Motic Images Plus 2.0 software.

2.5 Fluorescence-activated cell sorting (FACS)

One million cells from bone marrow cell suspensions were washed with FACS-PBS that contained (PBS, 0.1% BSA, 0.1% NaN_3) and incubated for 10 minutes with 50 µl of a CD16/32-Fc receptor blocker to reduce nonspecific antibody binding. For surface staining, samples were incubated in the dark at 4°C for 40 min with antibodies specific to CD34 a marker for progenitor cells, and CD115, a marker for (M-CSF receptor). Antibodies specific to (CD11c, CD40, CD86 and MHC-II) were used for mature macrophage characterisation experiment. Cells were then washed again and analysed on (FACS CANTO, Becton Dickinson, Oxford, UK) flow cytometer using FACS Diva software. A total of 30,000 events were measured per sample. Appropriate isotypes were used as controls. Flow cytometry compensation beads were used for more consistent and reliable

compensation. One drop of positive flow cytometry compensation beads was added to each compensation test tube. Antibody conjugates that were being used for cell staining were then added. Samples were then incubated for 20 min in dark at 4°C. After that, the beads were washed with FACS-PBS and a drop of negative control beads added. The samples were resuspended in 500 µl of PBS and analysed by flow cytometry. The two components provided distinct positive and negative populations of beads that can be used to set compensation.

2.6 Western blotting

2.6.1 Preparation of Whole Cell Extracts

Cells were grown up to $1x10^6$ cells/ml in 12-well plate for MAPKs or up to $2x10^6$ cells/ml in 6-well plates for MKP-2 and then stimulated with 10 ng/ml of M-CSF for the desired period of time. The plates were then placed on ice to stop the reaction. Cells were immediately washed twice with ice-cold PBS before 150-200µl of pre-heated laemmli's sample buffer (63 mM Tris-HCl (pH6.8), 2 mM $Na_4P_2O_7$, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue) was added. The cells were then scraped from the wells and the chromosomal DNA was sheared by repeatedly flushing through a 21 gauge needle. The samples were then transferred to labelled microcentrifuge tubes and boiled for 3 min for protein denaturation, before storing at -20 °C until reqiured.

2.6.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel kit apparatus was first cleaned in 70% ethanol before assembly, then distilled water added to check the glass plates were flush and not leaking. Resolving gels were prepared containing an appropriate amount (7.5% (w/v), 9% (w/v), 10% (w/v), 11% (w/v) acrylamide: (N, N'-methylenebis-acrylamide (30:0.8), 0.375 M Tris (pH8.8), 0.1% (w/v) SDS and 10% (w/v) ammonium persulphate (APS)). Gels were polymerized by the addition of 0.05% (v/v) N, N, N', N'- tetramethylethylenediamine (TEMED) and gel was

poured between two glass plates assembled in a vertical slab configuration according to the manufacturer's instruction (Bio-Rad). A thin layer of 200 µl 0.1% (w/v) SDS was then added to form a smooth surface on the top of the separating gel. Following gel polymerisation the layer of 0.1% SDS (w/v) was removed and a stacking gel containing (10% (v/v) acrylamide: N, -methylenebis-acrylamide (30:0.8) in 125 mM Tris (pH6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED) was poured on top of the resolving gel and a comb was placed to create the wells. After the loading gel polymerized the comb was removed and the gel was ready for electrophoresis. Polyacrylamide gels were assembled in a Bio-Rad Mini-PROTEAN II™ electrophoresis tank, with both reservoirs filled with electrophoresis buffer (25 mM Tris, 129 mM glycine, 0.1% (w/v) SDS). Aliquots of the denatured protein samples were added to the wells with a syringe. A prestained SDS-PAGE molecular weight ladder of known molecular weights was run concurrently in order to help identify the polypeptide of interest. Samples were electrophoresed at a constant voltage of 120 V, until the bromophenol dye had reached the bottom of the gel.

2.6.3 Electrophoretic Transfer of Proteins to Nitrocellular Membrane

In this step, the separated proteins by SDS-PAGE were transferred to nitrocellulose membranes by electrophoretic blotting following a standard protocol (Towbin et al., 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between two pieces of Whatman 3MM paper and two sponge pads. The cassette was immersed in transfer buffer (25 M Tris, 19 mM glycine, 20% (v/v) methanol) in a Bio-Rad Mini Trans-Blot™ tank and a constant current of 300 mA was applied for 2 hr, whilst the tank was cooled by inclusion of an ice reservoir. The presence of SDS in the resolving gel confers a negative charge on the proteins so the cassette was oriented with the nitrocellulose towards the anode.

2.6.4 Immunological Detection of Proteins

Following transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 2% (w/v) BSA in NaTT buffer (150 mM NaCl, 20 mM Tris (pH 7.4), 0.2% (v/v) Tween-20) for 2 hr with gentle agitation on a platform shaker for antibodies from Santacruz or in 5 % milk for Cell Signalling antibodies. The blocking buffer was then removed and membranes incubated overnight with antiserum specific to the target protein appropriately diluted in NaTT buffer containing 0.2% or 0.5% (w/v) BSA for Santa cruz or Cell Signalling antibodies respectively. On the following day membranes were washed in NaTT every 15 min for 90 min with gentle agitation. The membranes were then incubated for a further 2 hr at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to approximately 1:10000 in NaTT buffer containing 0.2% (w/v) BSA. After six additional washes in NaTT as described before, immunoreactive protein bands were detected by incubation in enhanced chemiluminescene (ECL) reagent for 2 min with agitation. The membranes were blotted and onto a paper towel to remove any excess liquid. The blots were then mounted onto an exposure cassette and covered with cling film, then exposed to X-ray film (Kodak Ls X-OMAT) for the required time under darkroom conditions and developed using X-OMAT machine (KODAK M35-M X-OMAT processor).

2.6.5 Nitrocellulose membrane stripping and reprobing

Used nitrocellulose membranes by Western blotting were stored in sealed dishes and then reprobed for the detection of other cellulose bound proteins. In this step, antibodies were stripped from nitrocellulose membranes by incubating in 15 ml stripping buffer that contained (0.05 M Tis-HCl, 2% SDS, and 0.1 M of β-mercaptoethanol) for 60 minutes at 70°C in an incubator/shaker (Stuart Science Equipment). After incubation, the stripping buffer was discarded in a fume hood sink and the membrane washed three times with NaTT buffer (pH 7.4) at 15 min intervals to remove residual stripping buffer. After washing, membranes were incubated overnight with primary antibody prepared in 0.2%

BSA (w/v) in NaTT buffer. On the following day, membranes were washed with NaTT buffer six times over 90 min. Finally, the blots were ready for the immunological detection protocol mentioned previously.

2.7 Scanning densitometry

All data obtained through immunoblotting were scanned on GS-800 Calibrated Densitometer (Bio-Rad). All quantified values were normalized against control values.

2.8 Polymerase chain reaction (PCR) amplification

2.8.1 RNA Preparation

To ensure high-quality, reproducible, and biologically relevant results, the qRT-PCR assays were performed using the practices laid out in the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009) (See MIQE checklist of experimental parameters in appendix 6.1).

2.8.1.1 Cell harvest and total RNA isolation

Reference: Qiagen's RNeasy Mini Kit Protocol

Two million confluent mature macrophages were seeded in 6-well plates, starved for 18 h, and then stimulated with 100 ng/ml of LPS from *E.coli* or 100 U/ml of mouse rm-IL-4 for 4 h. Total RNA was isolated from the macrophages using the Qiagen RNeasy Mini Kit following the manufacturer's instructions. Adherent macrophages were harvested as follows: Cell-culture medium was aspirated from the wells using a 5 mL pipette. Cells were washed once with warm PBS and then lysed with buffer RLT supplemented with β-mercaptoethanol (β-ME). On-column DNase digestion with the RNase-Free DNase Set (Qiagen, Crawley-West Susex, UK) was performed to remove potential genomic DNA contamination. RNA concentration was then determined with the NanoDrop spectrophotometer. Biological and technical

replicates had N number of 2 for qRT-PCR experiments. Biological replicates had N number of 3 for microarray experiment.

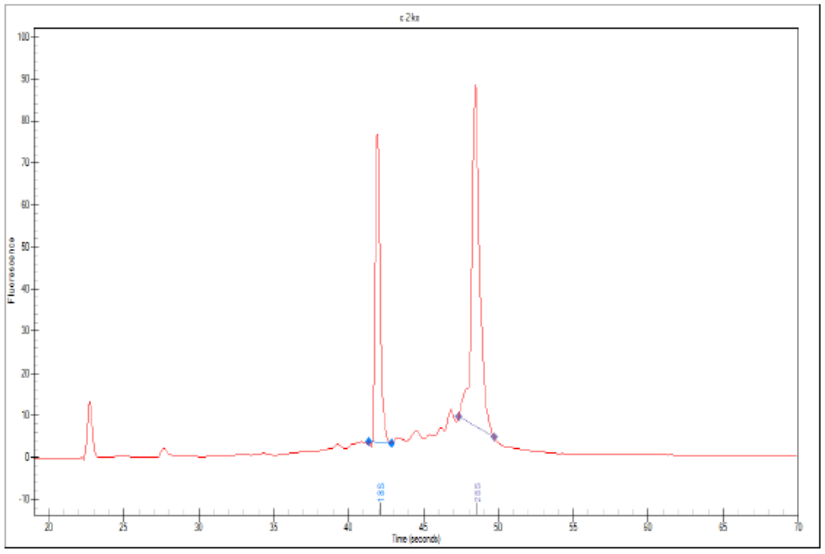
2.8.1.2 RNA concentration

NanoDrop (ND-2000C, Thermo Scientific) (220-750 nm) spectrophotometer was used to measure total RNA concentration. 2µl of RNase-free water was used as a blank. After cleaning the sampling platform, 2µl of a RNA sample was added. The software displays the sample absorption curve as well as the calculated RNA concentration and ratios (A_{260}nm/A_{230}nm; A_{260}nm/A_{280}nm). A ratio at A_{260}nm/A_{230}nm of around 2.0 indicates low salt contamination or carryover and the A_{260}nm/A_{280}nm ratio should also be around 2.0 for pure RNA. All extracted RNA samples had acceptable purity ratios 260/280 and 260/280.

2.8.1.3 RNA integrity determination

The RNA integrity was tested using the Experion Automated Electrophoresis system (Bio-Rad, UK). This is a lab-on-a-chip microfluidic separation technology using fluorescent sample detection. The RNA samples were ran according to the manufacturer's instructions for their StdSens RNA kit. The RNA quality indicator (RQI) feature of Experion software allows estimating the level of degradation in a total RNA sample. The RQI algorithm compares the electropherogram of RNA samples to data from a series of standardized, degraded RNA samples and automatically returns a number between 10 (intact RNA) and 1 (degraded RNA) for each sample. RQI of all samples in the current work was >9.4. The RNA integrity report of a typical sample from this study is shown in figure 2.3.

Well# 12 c 2 ko



12

Well# 12 c 2 ko					
Fragment Number	Fragment Name	Start Time	End Time	Area	% of Total Area
1	18S	41.35	42.85	68.39	19.29
2	28S	47.40	49.70	102.30	28.85

RNA Area: 354.54
RNA Concentration: 169.74 ng/µl
Ratio[28S/18S]: 1.50

RQI: 9.8

Figure 2.3: RNA integrity report.

2.8.2 cDNA synthesis (Reverse Transcription (RT))

To quantify the mRNA transcripts of genes under investigation, total RNA was reverse transcribed to complementary DNA (cDNA) using SuperScript III cDNA Synthesis system (Invitrogen, UK) following the manufacturer's manual. The kit can transcribe a maximum of 5 µg of RNA per reaction. RNA concentrations were normalised between tubes to 2 µg. RNase-free water was added followed by 1 µl of oligo-dT (500 ng/µl) as the first-strand synthesis primer and 1 µl of 10mM dNTP mixture (Bioline, UK). The template-primer mixture was denatured by heating for 5 min at 65°C, and then quick chilled on ice on ice for 5 mins. 10 µl of a reverse transcriptase mastermix was added. These samples were labelled "RT+". To check for the presence of persisting contaminating genomic DNA, an additional reaction tube, labelled "RT-" was set up in parallel for each RNA sample. This reaction contained all of the cDNA synthesis components and total RNA with the exception of the Superscript reverse transcriptase. The reactions were incubated for 50 min at 50°C, and the reverse transcriptase was inactivated by heating to 85°C for 5 min. The resulting cDNA was then used as the DNA template for quantitative real-time PCR (see section 2.2.2.6) for gene expression analysis. PCR carried out using an aliquot of an "RT-" control sample should not generate an amplicon.

Master Mix (1x):

RT buffer (10x): 2 µl

25mM $MgCl_2$: 4 µl

0.1M DTT: 2 µl

RNase OUT: 1 µl

SuperScript III: 1 µl (200U)

2.8.3 Polymerase Chain Reaction (PCR) amplification

The extracted mouse cDNA was used in PCR with specific designed primers. The PCR amplifications were conducted in a volume of 50 µl containing 49 µl of PCR master mix performed with GoTaq® DNA polymerase (Promega, UK).

PCR Master Mixture (1x):

Template (cDNA): 1 µl

Primer Forward: 1 µl (10 pmol/µl)

Primer Reverse: 1 µl (10 pmol/µl)

dNTPs: 1 µl (10 mM)

Buffer (5x): 10 µl

dH_2O: 35.75 µl

GoTaq DNA Polymerase: 0.25 µl (1.25 U)

PCR conditions: initial denaturation at 95°C for 2 min; 35 cycles with each cycle having a denaturation at 95°C for 1 min, annealing at 55°C for 30s and an elongation at 72°C for 30s. There was a final elongation at 72°C for 1 min. The resultant PCR products were analysed by agarose gel electrophoresis.

2.8.4 Quantitative Real Time Polymerase Chain Reaction amplification (qRT-PCR)

The qRT-PCR assay was carried out by placing the samples in sterile PCR fast reaction MicroAmp tubes (Applied Biosystems). Each 20 µl PCR reaction contained: 10 µl of SYBR select Master Mix (Applied Biosystems), 3 µl of 1 pmol/µl of both forward and reverse primers, 3 µl of molecular grade water, and 1 µl of the cDNA template (as described in 2.2.5.2). Two replicates were performed for each experimental point. The thermal cycling and detection was carried out on a StepOne Plus real-time PCR system (Applied Biosystems, UK). The thermal cycle consisted of an initial uracil-DNA glycosylase activation of 2 min at 50°C to control PCR product carryover contamination, the DNA polymerase activation of 2 min at 95°C, followed by 40 cycles of 15 s at 95°C, 60 s at 60°C. A final extension of 5 min at 72°C followed and the reaction was stopped by extended incubation and cooling down to 4°C. As this is a SYBR Green-based chemistry, a melt–curve analysis followed the amplification. As this is a SYBR Green-based chemistry, a melt–curve analysis followed the amplification. This is to confirm that the PCR reaction only generated a single amplicon and not multiple products which

may contribute to the overall signal and complicate gene quantification. For all the genes validated in this thesis, qRT-PCR resulted in a single amplicon according to multi-curve analysis with undetermined Ct values for water and negative controls.

2.9 Quantification method (the relative quantification [ΔΔCt] method)

The quantification method selected to validate microarray results was the relative quantification (ΔΔCt) method (Livak and Schmittgen, 2001). This method compares Ct values between the target gene and the endogenous reference gene to examine the changes in gene expression between control and treated samples. To use this method, it is important to determine the PCR efficiencies of both the target gene and the reference gene primers and ensure they lie within the acceptable range of 90%–110 % (see next section for PCR efficiency). To compare expression levels in samples using this method, the following equations were followed:

1. Calculating the ΔCt between the target gene and the reference gene (for the treated samples and control)

$\Delta Ct = Ct_{target} - Ct_{reference\ gene}$

2. Calculating the ΔΔCt between the treated sample and control

$\Delta\Delta Ct = (Ct_{target} - Ct_{reference})$ control $- (Ct_{target} - Ct_{reference})$ treated

3. Fold change (FC) in the treated sample is then equal to $2^{-\Delta\Delta Ct}$

If the difference in PCR efficiency between primers was >10 %, the following equations can be used:

Ratio = (E_{target}) ΔCt target (control – treated) / $(E_{reference})$ ΔCt reference (control – sample), where $E_{t\ target}$ = PCR efficiency

$\Delta Ct_{target\ (control - sample)} = (Ct_{target})$ in the control $- (Ct_{target})$ in treated

2.9.1 PCR efficiency

A standard curve of a calibrator sample (one of the control untreated samples) was performed to compare the PCR efficiency of the target and control genes (Pfaffl, 2001). Five 5x dilutions of the calibrator sample were prepared. The slope of the standard curve was calculated to obtain the efficiency of the PCR reaction using the following equations:

Exponential amplification = $10^{(-1 / \text{slope})}$

Efficiency = $10^{(-1 / \text{slope})} - 1$

A PCR efficiency of between 90 %–110 % is acceptable (i.e., a slope of between 3.1 and 3.58). The PCR efficiency in the designed primers in table 2.1 ranged from 89.57 to 108.2.

2.9.2 Primer design for SYBR® Select Master Mix assays

The aim here was to design primers that only bind to the selected target. This is to avoid primer dimers and non-specific products in SYBR® assays. Primers were designed with Gene Runner Software and we checked afterward if the primers were unique and specific using BLAST functions of The National Centre for Biotechnology Information (NCBI) Genome Browser ((Johnson et al., 2008); *www.ncbi.nlm.nih.gov/BLAST/)*. The following criteria were followed when designing primers:

1. Amplicons size = ~140-160 bp to obtain a high level of fluorescence without compromising the PCR efficiency.
2. The melting temperature (Tm) of primers kept between 58–60°C; the ΔTm between forward and reverse primers was ≤1°C.
3. Primer length: 18–24 bases.
4. Amplicon GC content: 45 %–60 %
5. Primers comprised sequences of different exons with spanning and flanking the introns. This is to prevent the amplification of contaminating genomic DNA (gDNA).

Primer nucleotide sequences are shown in Table 2.1.

Table 2.1: Nucleotide sequences of the primers used for the analysis of gene expression by Quantitative Real Time Polymerase Chain Reaction amplification (qRT-PCR)

Primer Name	Sequence	PCR Product Size (bp)
QARS	Forward: 5'-GGACTCCAGCTGAGCGCTGCTC-3' Reverse: 5'-GGTGGACTCCACAGCTTCCTCAAT-3'	138
Relnta/Fizz1	Forward: 5'-AGATTCTGCCCCAGGATGCCA-3' Reverse: 5'-GGATTGGCAAGAAGTTCCTTGACCTT-3'	158
Chi3l3/Ym1	Forward: 5'-CAATATAACTTTGATGGCCTCAACCTGG-3' Reverse: 5'-GCAGCCTTGGAATGTCTTTCTCCAC-3'	151
CSF-2	Forward: 5'-TGCCTGTCACGTTGAATGAAGAGG-3' Reverse: 5'-TGTCTGGTAGTAGCTGGCTGTCATGTTC-3'	164
MMD	Forward: 5'-TGGCCGCTACAAACCAACGTG-3' Reverse: 5'-CAAAGGCCCATCCCGTAGATCC-3'	156

2.9.3 Selecting the reference gene

The housekeeping or reference gene is used to normalise differences in the cDNA concentration added in each PCR reaction. Thus, the mRNA expression of this gene must remain stable between samples. Reference genes like glyceraldehyde-3-phosphate dehydrogenase (G3PDH or GAPDH), actins, tubulins, 18S rRNA, and 28S rRNA are commonly used with mice qRT-PCR studies. However, looking at using Ct (crossing threshold) or now know as Cq (quantification cycle under MIQE nomenclature (Bustin et al., 2009)) levels in obtained RT-PCR, some of these commonly used reference genes in macrophages, e.g., B2M and gamma-actin did not show uniform expression between our samples in the microarray data. Also, amplification of gDNA was detected with these genes in negative control samples and genomic products of the same amplicon size were identified in NCBI BLAST with the PCR primers designed. Therefore, a new search for non-commonly used reference genes in mice macrophages that exhibit acceptable variation in microarray data was performed. Genes selected from the literature surveying appropriate mouse reference genes were Eukaryotic translation initiation factor 2B (*EIF2B2),* Glutaminyl tRNA synthetase *(QARS),* Signal recognition practice 14kDa *(SRP14)* (Maess et al., 2010), Ubiqiutin C (*UBC)* (Albershardt et al., 2012), Eukaryotic translation elongation factor 2 (*Eef2),* and Ribosomal protein L6 (*RPL6)* (de Jonge et al., 2007). Primers were designed according to the previous section. UBS, *Eef2*, and *RPL6* all have pseudogenes (Mutimer et al., 1998, Kalyana-Sundaram et al., 2012). Both *EIF2B2* and *SRP14* showed amplification with negative controls. Only *QARS* showed no amplification with negative controls; plus it was found to be stably expressed between the samples from the microaaray data. Based on this, the *QARS* gene was selected as the reference gene for the current project.

2.9.4 Negative controls

To further evaluate the qRT-PCR assay, two negative controls were included and no amplification should be detected in these controls:

1. Non-template control (water control): this contained water, primers and PCR master mix, instead of template and reaction mix. Any amplicons present in these reactions

would suggest DNA contamination of PCR reaction consumables or during the setup of the PCR reaction.

2. No reverse transcriptase control ("RT-" control): no reverse transcriptase was added to the RNA samples during the cDNA synthesis step. Amplicons present in PCRs with these "RT-" samples would suggest genomic DNA carryover in the RNA samples and/or as in (1).

2.10 Microarray expression analysis

Total RNA from treated samples and controls were used to globally analyse gene expression patterns on mouse gene expression Arrays (SurePrint G3 8x60K Mouse expression, Agilent, UK), which includes 39,430 Entrez gene RNAs and 16,251 LincRNAs.

2.10.1 Microarray System

Labelling of high quality RNA, hybridization and scanning were performed using the Agilent Gene Expression system according to the manufacture's instructions.

2.10.2 Labeling Reaction

100 ng of RNA from each sample was amplified and labeled with cyanine 3-CTP using the one-color Low RNA Input Linear Amplification Kit from Agilent. The aim of this is to generate fluorescent cRNA (complementary RNA). The method uses T7 RNA polymerase, which simultaneously amplifies the target material and incorporates cyanine 3-CTP. Generally, the appropriate amount of total RNA was mixed with the diluted volume of the Agilent one-color Spike-In-Mix together with the T7 Promoter primer. Template and primer were denatured at 65°C for 10 min before placing the reaction on ice for 5 min. The cDNA Master Mix was prepared, which contains the AffinityScript reverse transcriptase for reverse transcription of total RNA. Samples were then incubated at 40°C for 2 h. After that the reaction was heated to 65°C for 15 min to inactivate the AffinityScript enzyme. Samples were

placed on ice immediately for 5 min and the Transcription Master Mix was added. It contains T7 RNA Polymerase and the cyanine 3-labeled-CTP. Samples were placed on ice immediately for 5 min and the Transcription Master Mix was added which contains T7 RNA Polymerase and the cyanine 3-labeled-CTP. Samples were then incubated at 40°C for 2h. The resultant labeled and amplified cRNA was purified using Qiagen's RNeasy mini spin columns. Labeling efficiency and cRNA was checked using the NanoDrop spectrophotometer and thus to determine yield and specific activity of each reaction.

2.10.3 Microarray Hybridization

Labeled cRNA with a specific activity of at least 6.0 pmol Cy3 per µg cRNA were fragmented and hybridized on SurePrint G3 8x60K Mouse gene expression (Agilent, UK). The Fragmentation Mix was prepared as follows:

Component	Volume/Mass
Labeled, linearly amplified cRNA	600 ng
Agilent Blocking Agent (10x)	5 µl
Nuclease-free water	Bring volume to 24 µl
Fragmentation Buffer (25x)	1 µl

Samples were incubated at 60°C for no more than 30 minutes in order to fragment RNA and then immediately placed on ice. Hybridization buffer (2x) was then added to stop the fragmentation reaction. The final hybridization mixture for the 8x60 (8 arrays/slide) SurePrint G3 mouse expression microarrays were prepared as follows:

Component	Volume
cRNA from Fragmentation Mix	25 µl
Agilent 2x Gex Hybridization Buffer HI-RPM	25 µl

The sample was spun down and kept on ice until loading onto an array, which was performed as soon as possible. Hybridization on these microarray slides was then carried out at 65°C for 17 h using an Agilent SureHyb chamber and an Agilent hybridization oven. After 17 hours of hybridization at 65°C, slides were washed in Gene Expression Wash Buffer I with 0.005% (v/v) Triton X-102 (Agilent) at room temperature for one minute and then in Gene Expression Wash Buffer II with 0.005% (v/v) Triton X-102 (Agilent, prewarmed to 37°C) for an additional minute. Afterwards slides were air dried and placed in slide holders. Images were scanned immediately using an Agilent DNA microarray C Scanner (Agilent) with the Agilent G3_Gx_1 Color profile (Channel G, Agilent HD, 3µm resolution, 20-bit Tiff), and processed with Feature Extraction Software v10.7 (Agilent) in which information from probe features is extracted from microarray scan data, allowing researchers to measure gene expression.

2.10.4 Data Analysis Using GeneSpring Software

For further analysis, tab-delimited data files in text format obtained from the Feature Extraction were imported to GeneSpring GX 12.0.1 software (Agilent) in order to compare gene expression profiles between various treatment conditions. Data for a given gene were normalized to the median expression level of that gene across all samples. Only more than 2 fold signal changes were used as gene induction or repression (Tarca et al., 2006). Fold change of 2 or more was used for significantly regulated genes using One-way ANOVA (p-value < 0.05). Using a fold change with a cut off of 2.0, custom lists were created by pairing expression profiles of the differently treated macrophages at the given time points for each. Those custom lists display genes that are up- or down-regulated respectively in one condition compared to another condition at identical time points with respect to untreated cells. Processed data was imported into Microsoft Office Excel 2007 for further analysis. In order to validate microarray data, several genes were selected and verified by q.RT-PCR (see section 2.2.6.1).

2.11 Enzyme-linked immunosorbent assay (ELISA)

2.11.1 Cell preparation and treatments

Mature BMDMs were plated out in 24-well plates at $2.5x10^5$ in 500ml of medium. Plates were incubated overnight at 37°C/5% CO_2. The cells were treated with 100 U/ml of IL-4 for the desired time points. Supernatants were collected after centrifugation of plates for 5 minutes at 1000g and stored at -20°C for future use.

2.11.2 Measurement of FIZZ-1/Relm-alpha

The concentration of FIZZ-1 and YM1 was measured in BMDMs supernatant as follows. 96-well plates were coated with rabbit anti-mouse Relm alpha Ab at a concentration of 0.1 mg/ml prepared in Carbonate Buffer (pH 9.6). Carbonate buffer was prepared as follows:

Solution A: 8.5g NaH_2CO_3 (or $NaHCO_3$) in 100ml distilled water (1M)

Solution B: 10.6g Na_2CO_3 in 100 ml distilled water (1M)

Then 45.3ml of A were added and 18.2ml of B to 936.5ml distilled water.

pH was adjusted to pH 9.6 if required.

The plates were coated with 50µl/well overnight at 4°C. The plates were washed next day to remove excess capture antibody using washing buffer (0.05% Tween-20 in PBS pH 7.4) (500 µl tween / litre PBS) for three times and were dried well by hitting on tissues. 200µl of blocking buffer (10% FCS in PBS, pH7.4) was added to each well to cover all un-bound sites on the wells and incubated for 2 h at 37°C. The plates were then re-washed for 4 times with a good drying. Samples were added at 50 µl volume for each well. At the same time, and for standard curve preparation, recombinant murine Relm-alpha was added at a concentration of 100 ng/ml diluted in blocking buffer at a volume of 50µl for each well titrated in doubling dilutions. Samples and standards were incubated overnight at 4°C. After that the plates were washed 5 times and dried as before. Biotin rabbit anti-mouse Relm-alpha Ab) was used as secondary antibody at 0.25 µg/ml concentration diluted in blocking buffer and added as 50µl/well. The plates were incubated for 2 h at 37°C and then washed for 6 times and dried. Streptavidin-horse radish peroxidase (SAv-

HRP) was used as conjugate diluted as 1 in 2000 in blocking buffer and added at a volume of 50µl. Plates were later incubated for 30 minutes at 37°C before a final wash for seven times. After drying, substrate reaction (TMB microwell peroxidase substrate system) was prepared by mixing equal amounts of both system components and 50µl from this mixture was added to each well. The plates were then wrapped with tin foil and incubated for 30 min at 37°C to allow color development. The reaction was finally stopped with 19.15M of H_2SO_4. Absorbance was measured at 450 nm using SPECTRAmax 190 microtiter plate spectrophotometer and Softmax PRO 3.0 software (Molecular Devices, CA, USA).

2.11.3 Measurements of YM1

The ELISA procedure for YM1 was performed as mentioned above using the following reagents:

-Coating: Mouse Chitinase 3-like 3/ECF-L at 0.1 mg/ml in PBS, pH7.4.

-Blocking buffer: PBS, pH7.4.

-Standards: Mouse Chitinase 3-like 3/ECF-L standard at highest concentration 20 ng/ml diluted in 1% BSA/PBS.

-Detection: Biotin labelled anti-mouse Chitinase 3-like 3/ECF-L at 100 ng/ ml in 1% BSA/PBS.

2.12 Data analysis

Statistical analysis was performed using GraphPad Prism Version 6.0, GraphPad Software, California). For the entire experiments in this thesis, values were mean ± SEM for three separate observations. The statistical significance of differences between mean values from control and treated groups were determined by either the student one-tailed or two-tailed unpaired t test. $P < 0.05$ was accepted as significant. For microarray experiment, significantly regulated genes were assigned using One-way ANOVA (p-value < 0.05).

3. THE ROLE OF MKP-2 IN MAPK MEDIATED SIGNALLING AND MACROPHAGE DEVELOPMENT

3.1 Introduction

As outlined in the introduction, MKP-2 has been shown to play a significant role in immune function, particularly in controlling *Leishmania mexicana* infection by influencing macrophage activity (Al-Mutairi et al., 2010). Loss of MKP-2 was found to be associated with enhanced macrophage cytokine production primarily due to JNK and p38 upregulation (Al-Mutairi et al., 2010). MKP-2 has also been shown to regulate the inflammatory responses in sepsis and acute lung injury by modulating the MAPK pathway in macrophages (Cornell et al., 2010).

Previous studies have also demonstrated that activation of the MAPK cascade is essential for macrophages to proliferate in response to M-CSF (Jaworowski et al., 1999, Valledor et al., 2008b, Valledor et al., 2000, Sanchez-Tillo et al., 2007, Comalada et al., 2004). This suggests a potential link between the MAPKs and MKP-2 in regulating macrophage function. M-CSF is a key cytokine that controls macrophage differentiation, proliferation, and survival (Jaworowski et al., 1999, Valledor et al., 2008b, Valledor et al., 2000, Sanchez-Tillo et al., 2007, Comalada et al., 2004). The role of MKP-2 in the regulation of MAPK activity upon stimulation with M-CSF and the resulting effect on macrophage development is completely unknown.

The aim of this chapter is to investigate the importance of MKP-2 in the proliferative response of macrophages to M-CSF. Using a novel DUSP-4 deletion mouse, the

experiments were designed to study MKP-2 induction upon M-CSF stimulation in bone marrow-derived macrophages, to correlate loss of MKP-2 with changes in MAPK signaling and to correlate these effects with macrophage proliferation, development-related gene expression and the expression of macrophage surface proteins.

3.2 Results

3.2.1 MKP-2 deletion negatively affects macrophage proliferation

Initially, the proliferative potential of macrophages following MKP-2 deletion was examined over a period of ten days. This was based on the consistent observations made previously in the laboratory that MKP-$2^{-/-}$ mice produce fewer macrophages compared with wild-type counterparts. An equal number of bone marrow-derived cells were transferred on the same day of isolation into petri dishes. For macrophage differentiation, the culture was supplemented with L929-conditioned medium (L-cell) as a source of M-CSF, which is an important growth factor for macrophages (see method section 2.2.2). To determine macrophage proliferation over time, macrophages were harvested on days 5, 7 and 10 and counted in a haemocytometer. In the absence of MKP-2, macrophage proliferation was reduced by 20.04 %, 21.1 % and 20.1 % at days 5, 7 and 10 respectively in comparison to wild type cells (Figure 3.1). This initial observation suggests that lack of MKP-2 is affecting the growth of macrophages and that MKP-2 might plays an important role in this process.

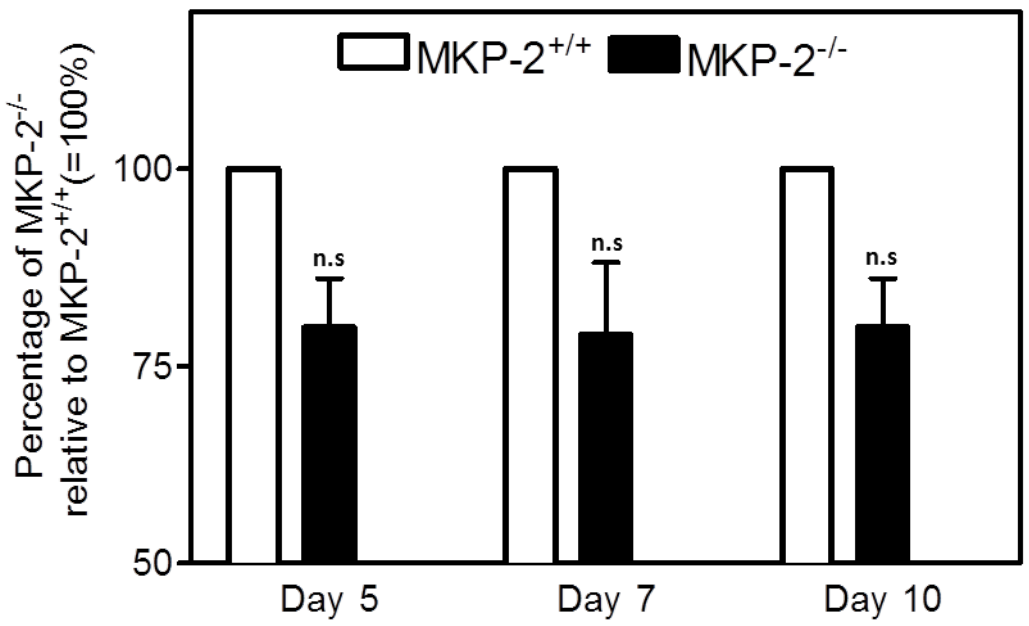
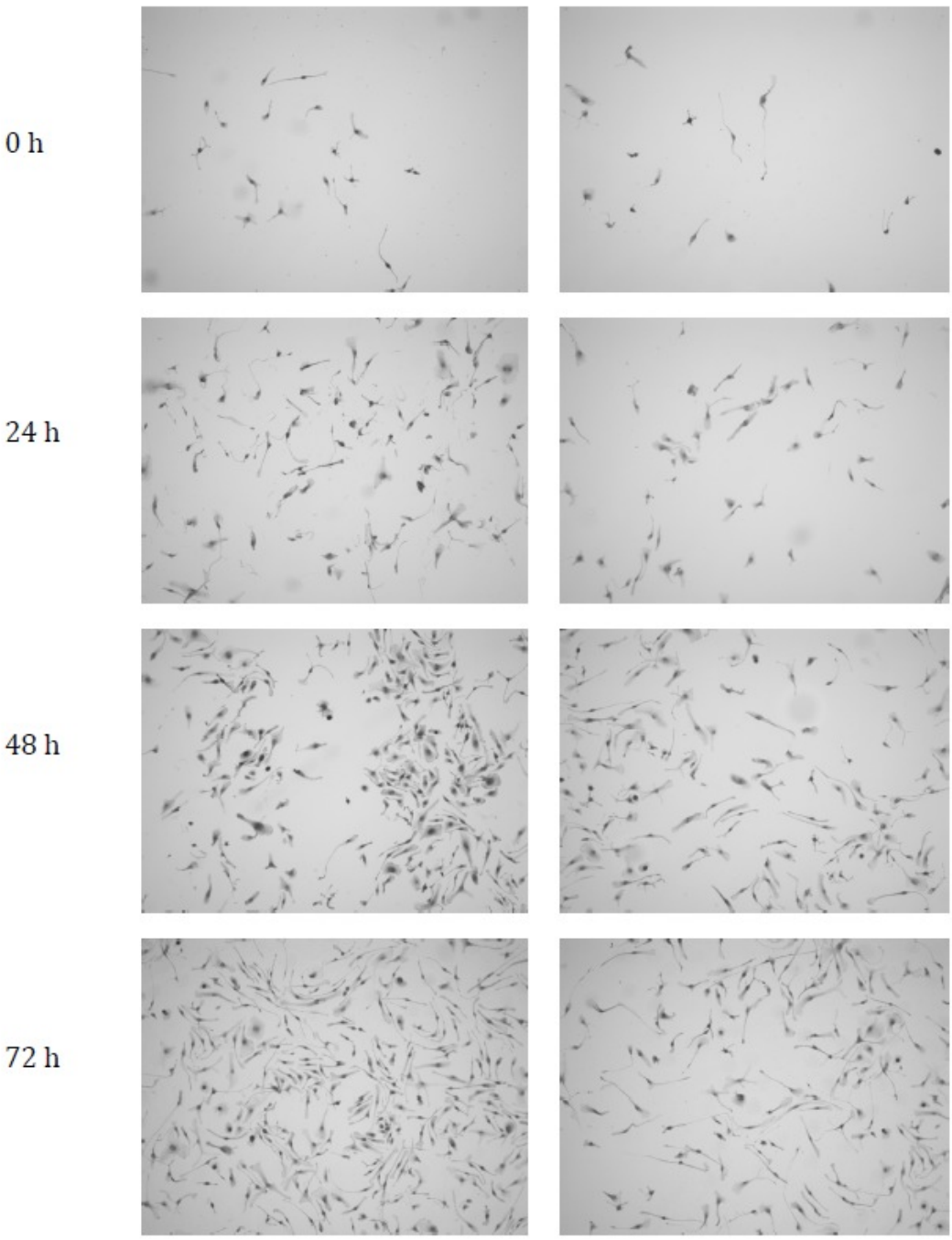


Figure 3.1: MKP-2 deletion on macrophage proliferation over 10 days. An equal number of cells ($3.5x10^6$) from the bone marrow cell suspension were seeded on the day of isolation in to petri dishes and cultured for 10 days. Culturing media was supplemented with 30 % L-929 conditioned medium (LCM) as a source for M-CSF. Macrophages were harvested from petri dishes at days 5, 7 and 10 following isolation and then counted from a diluted cell suspension in a haemocytometer as described in method section 2.2.2. Error bars represent the mean±SEM from three individual experiments. Statistical analysis was carried using the unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages ($P>0.05$, n.s).

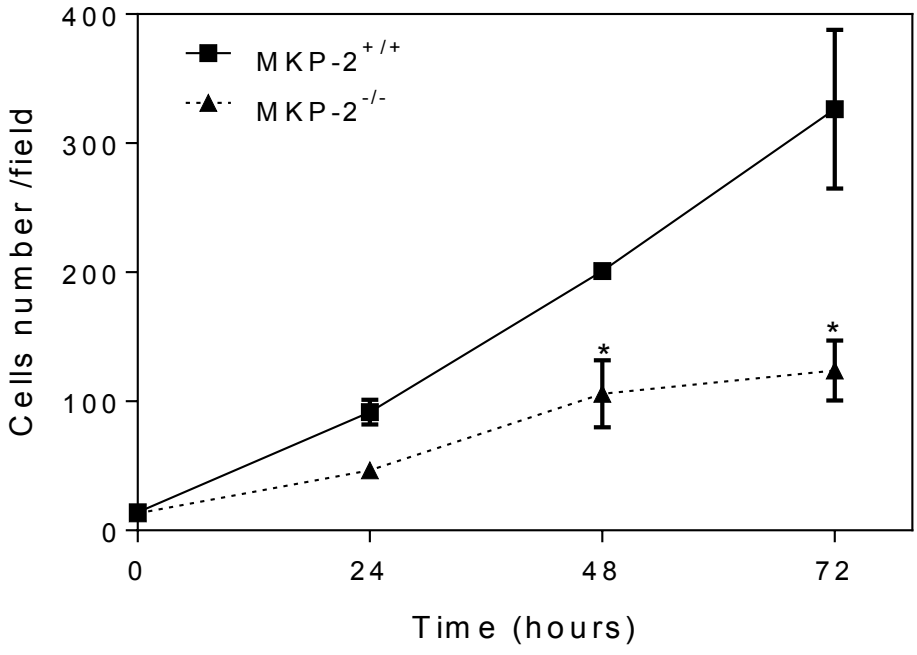
3.2.2 MKP-2 deletion negatively affects macrophage growth: Cell counting by haematoxylin staining

In previous experiments, L-cell conditioned medium was used as a cheap source of M-CSF to generate macrophages. However, the general composition of the supernatant as well as the concentration of M-CSF is not well defined. Therefore it was decided to use a specific cytokine, which is recombinant mouse M-CSF (rmM-CSF) for subsequent experiments.

Cell counting by haematoxylin staining was used for direct measurement of cell numbers (see method section). Macrophages from either MKP-$2^{+/+}$ or MKP-$2^{-/-}$ were harvested at day 3, starved of M-CSF for 24 h and then stimulated with M-CSF (10 ng/ml) for 72 hours. In MKP-$2^{+/+}$ macrophages, M-CSF was able to increase the number of MKP-$2^{+/+}$ cells 13-fold over 72 h (Figure 3.2 A and B). In contrast, in MKP-$2^{-/-}$ macrophages, M-CSF-stimulated proliferation was significantly slower at 48 and 72 h compared to wild type ($p<0.05$). In the absence of M-CSF, both cell types started to die at 72 h (Figure 3.2 C). This experiment provided additional evidence that the deletion of MKP-2 significantly impairs the growth of bone marrow derived macrophages from precursors and that MKP-2 is essential for appropriate macrophage development.


A)
MKP-2+/+
MKP-2-/-
0 h
24 h
48 h
72 h

B)


400
300
200
100
0
Cells number /field
MKP-2+/+
MKP-2-/-
*
*
0
24
48
72
Time (hours)

C)

MKP-$2^{+/+}$ MKP-$2^{-/-}$

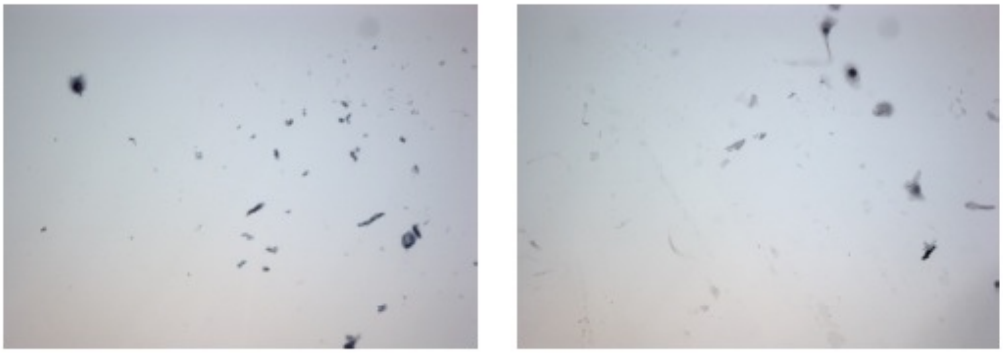


Figure 3.2: The effect of MKP-2 deletion on the proliferative capacity of bone marrow macrophages. Macrophages were harvested on day 3 of culture, and $5x10^3$ cells per well were seeded onto cover slips in 24-well plates. Cells were then allowed to attach for 24 h and subsequently stimulated with recombinant M-CSF (10 ng/ml) or left unstimulated (Controls, 0 h) (panel A and B). Cells were washed with PBS, and exposed to haematoxylin dye. The number of cells was determined by counting the cells from ten random fields per cover slip at 24 h intervals over 72 h as described in methods section 2.2.2. Control values, (0 h) MKP-$2^{+/+}$ = 20, MKP-$2^{-/-}$ = 18. Images were taken using a 20x objective, scale bar = 50 µm. In (panel C), M-CSF un-stimulated macrophages are dying at 72 h. Each value represents the mean±SEM from three individual experiments. *$P<0.05$, two-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

3.2.3 Identification of MAP kinases involved in macrophage proliferation

The prominent growth deficit observed in MKP-2$^{-/-}$ macrophages suggested the possible involvement of the MAPK pathway in macrophage development since MKP-2 is a negative regulator of this cascade. M-CSF controls macrophage differentiation, proliferation, and survival by triggering a complex of signalling pathways, including mitogen-activated protein kinases (MAPKs) (Jaworowski *et al.*, 1999, Valledor *et al.*, 2008b, Valledor *et al.*, 2000, Sanchez-Tillo *et al.*, 2007, Comalada *et al.*, 2004). The effect of M-CSF on the activation of MAPK pathway and the possible involvement of MAPK ERK, JNK, and p38 in macrophage proliferation were studied here. Firstly, a concentration curve for M-CSF-induced MAPK phosphorylation was established. Wild type macrophages were rendered quiescent for 18 h and subsequently were subjected to stimulation with increasing concentrations of M-CSF (2.5-100 ng/ml) for the duration of five minutes. Results showed that M-CSF-induced MAP kinase phosphorylation was concentration-dependent giving a maximum response at 50 ng/ml for ERK and p38 whereas 100 ng/ml was required for maximal activation of JNK (Figure 3.3). M-CSF caused a strong ERK phosphorylation at as little as 2.5 ng/ml (fold stimulation = 25.9) (Figure 3.3 A). This is in contrast to a 2.1- and 6.2-fold response for JNK and p38 phosphorylation respectively at the same concentration. Phosphorylation of JNK and p38 was potentiated clearly at 10 ng/ml (Figure 3.3 B and C). This result established that ERK is more readily phosphorylated in response to M-CSF than JNK and p38. Based on the clear activation of all three MAP kinases at 10 ng/ml, this concentration of M-CSF was used for subsequent experiments.

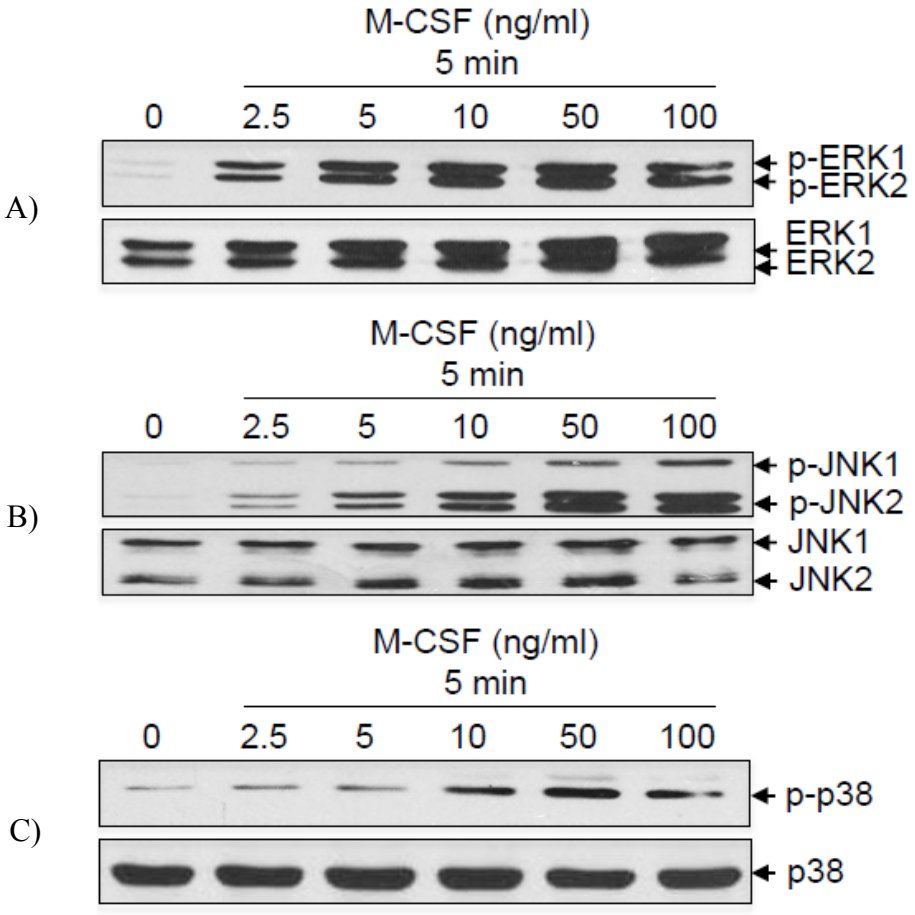


Figure 3.3: Concentration-dependent phosphorylation of MAP kinases in bone marrow derived macrophages. Cells were harvested at day 3 of isolation, deprived of L cell-conditioned medium for 18 h to render the cells quiescent and then stimulated with increasing concentrations of M-CSF for 5 min. Control cells (0) were left untreated. Whole cell lysates were prepared, and assessed for the expression of p-ERK, p-JNK and p-p38 by Western blotting as described in methods 2.2.4. Total expression for ERK (T-ERK), JNK (T-JNK) and p38 (T-p38) were used as a loading control. Data were obtained from a single experiment.

3.2.4 The effect of MKP-2 deletion on M-CSF-induced MAP kinases phosphorylation

Having established an appropriate M-CSF concentration for MAPK activation, the effect of MKP-2 deletion on a 2 h time-course of ERK, JNK and p38 phosphorylation in response to M-CSF was examined. Cells were harvested on day 3, seeded in 12-well plates and then starved of M-CSF for 18 h before the addition of 10 ng/ml of recombinant M-CSF for another 2 h. M-CSF caused a rapid phosphorylation of all three MAP kinases with a peak at 5 min (Figures 3.4, 3.5 and 3.6). Interestingly, in the absence of MKP-2, ERK phosphorylation was enhanced in MKP-$2^{-/-}$ cells from 5 to 60 min with a significant difference at 15 min (fold stimulation at 15 min: MKP-$2^{+/+}$ = 16.26 ± 8.04, MKP-$2^{-/-}$ = 29.59 ± 2.82, $p<0.05$). This response in both cell types declined at 2 h. Consistency in total ERK and β-tubulin expression suggested that differences in phosphorylated ERK bands were not due to any changes in protein levels (Figure 3.4). In contrast, there were no significant differences in the phosphorylation of JNK and p38 between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages during the same time course (Figures 3.5 and 3.6). For JNK phosphorylation, it decreased subsequently at 15 min and returned to basal levels, while p38 phosphorylation remained high until 2 h (Figures 3.5 and 3.6). These data indicate that M-CSF-induced ERK activation was enhanced in MKP-2 deficient macrophages in comparison to wild type and that MKP-2 is an important regulator of M-CSF-induced ERK signalling. In addition, the results suggest that M-CSF is capable of inducing similar phosphorylation of JNK and p38 in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages.

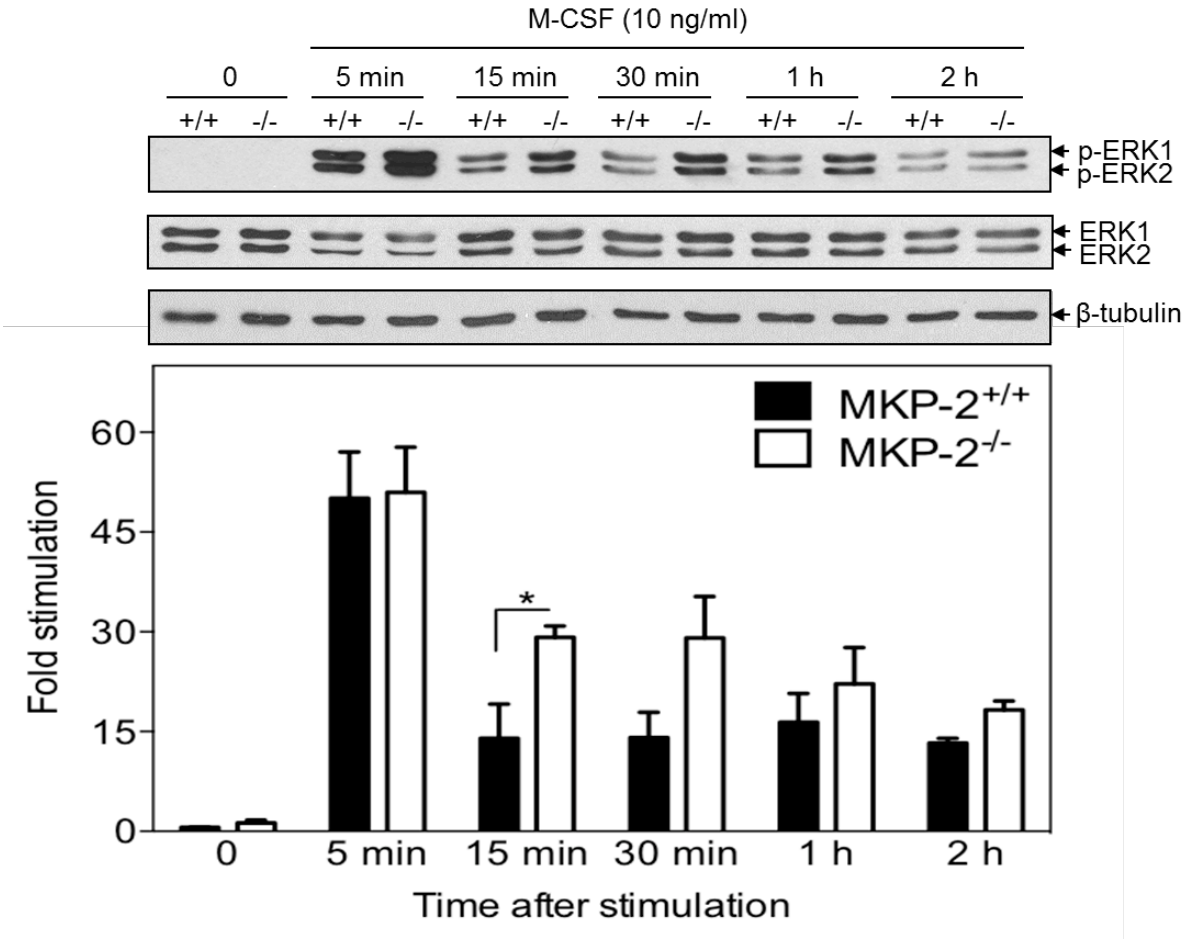


Figure 3.4: **The effect of MKP-2 deletion on M-CSF-mediated ERK phosphorylation.** Cells were harvested at day 3 of isolation, deprived of L cell-conditioned medium for 18 h to render the cells quiescent and then stimulated with M-CSF (10 ng/ml) for the indicated periods of time. Control cells (0) were left untreated. Whole cell lysates were prepared, and assessed for p-ERK (42/44 kDa) by Western blotting as described in methods 2.2.4. Expression of total-ERK (T-ERK) and β-tubulin were measured as loading control. Blots were quantified for p-ERK fold stimulation by scanning densitometry relative to the background signal. Each value represents the mean±SEM from three individual experiments. *P<0.05, two-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

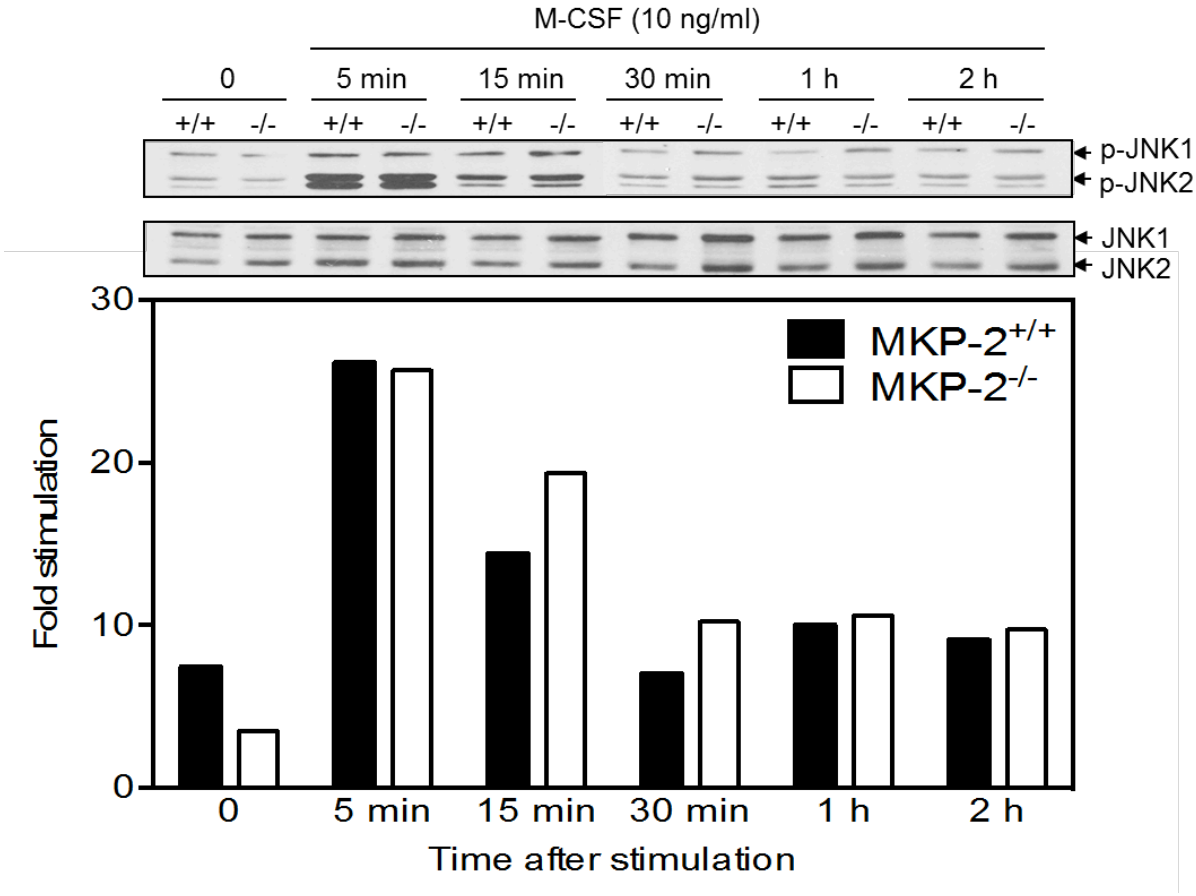


Figure 3.5: The effect of MKP-2 deletion on M-CSF-mediated JNK phosphorylation. Cells were harvested at day 3 of isolation, deprived of L cell-conditioned medium for 18 h to render the cells quiescent and then stimulated with M-CSF (10 ng/ml) for the indicated periods of time. Control cells (0) were left untreated. Whole cell lysates were prepared, and assessed for p-JNK (46/54 kDa) by Western blotting as described in methods 2.2.4. Expression of total JNK (T-JNK) was measured as a loading control. Blots were quantified for p-JNK fold stimulation by scanning densitometry relative to the background signal. The blot represents the data from two individual experiments.

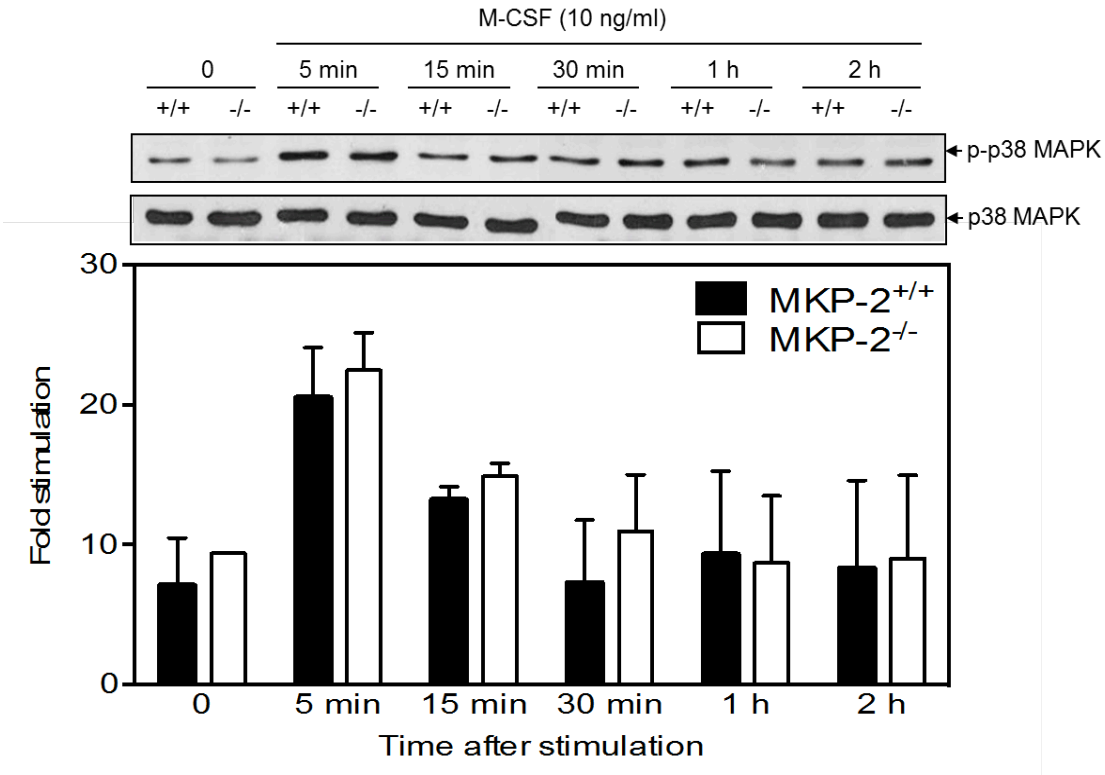


Figure 3.6: The effect of MKP-2 deletion on M-CSF-mediated p38 phosphorylation. Cells were harvested at day 3 of isolation, deprived of L cell-conditioned medium for 18 h to render the cells quiescent and then stimulated with M-CSF (10 ng/ml) for the indicated periods of time. Control cells (0) were left untreated. Whole cell lysates were prepared, and assessed for p-p38 (38 kDa) by Western blotting as described in methods 2.2.4. Expression of total p38 (T-p38) was measured as a loading control. Blots were quantified for p-p38 as fold stimulation by scanning densitometry relative to the background signal. Each value represents the mean±SEM from three individual experiments. Statistical analysis was carried using the unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages ($p>0.05$, n.s).

3.2.5 Induction of MKP-2 protein expression in response to M-CSF

Following the observation that lack of cellular MKP-2 enhanced ERK phosphorylation, the next step was to assess the expression of MKP-2 protein following M-CSF stimulation over a prolonged time course (0-24 h). Cells were harvested on day 3, seeded in 6-well plates ($2x10^6$) and then starved of M-CSF for 18 h before stimulation with recombinant M-CSF (10 ng/ml) for up to 24 hours. Detection of MKP-2 protein was analysed by Western blotting. Figure 3.7 shows that the induction of MKP-2 was already detectable at 5 min after M-CSF stimulation, increased over time and reached a maximal point at 8 h (approximately an 8.5-fold increase in expression compared with control), before decreasing again at 24 h. This suggests that MKP-2 expression coincides with ERK dephosphorylation during the macrophage response to M-CSF in Figure 3.4. The total p38 MAPK levels were unchanged, indicating equal protein loading. The current data established that M-CSF is able to induce endogenous MKP-2 expression in MKP-2 wild type cells and that the difference in ERK phosphorylation is mediated by MKP-2.

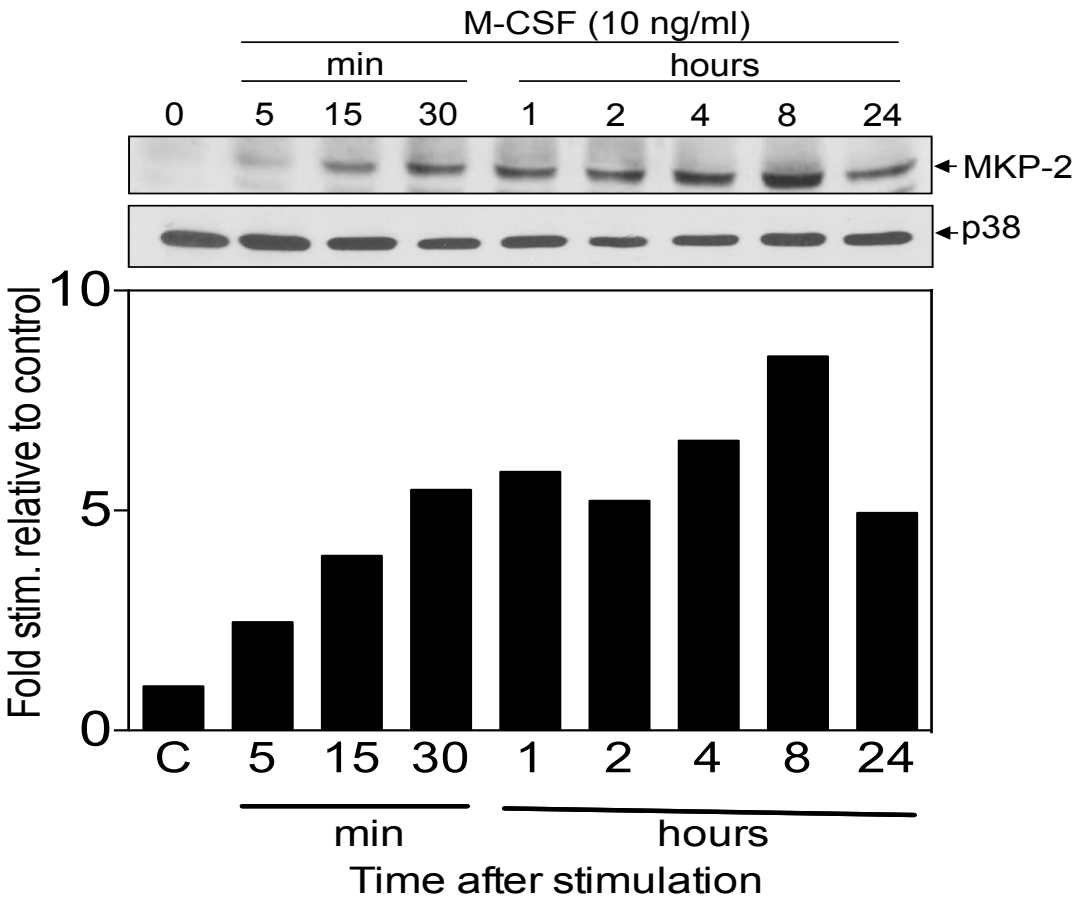


Figure 3.7: Induction of MKP-2 in wild type bone marrow macrophages in response to M-CSF. Macrophages were harvested at day 3 of culture, deprived of L cell-conditioned medium for 18 h to render the cells quiescent in 6-well plates and then stimulated with M-CSF (10 ng/ml) for the indicated periods of time. Control cells (0) were left untreated. Whole cell lysates were prepared as outlined in methods 2.2.4 and assessed for the expression of MKP-2 (43 kDa) by Western blotting. Expression of total p38 (T-p38) was measured as a loading control. Blots were quantified for MKP-2 as fold stimulation by scanning densitometry relative to the control (0). The blot represents the data from two individual experiments.

3.2.6 The role of MKP-2 in the expression of macrophage differentiation/proliferation genes

This section aimed to link differences in ERK phosphorylation obtained from section 3.2.4 to the expression of genes relevant to macrophage development. This is also to identify molecular events that are involved during monocyte/macrophage differentiation and macrophage proliferation. To obtain this, the induction of two differently expressed genes from array data generated in chapter 4 is examined in this section. These genes are: monocyte to macrophage differentiation associated (*Mmd*) and colony-stimulating factor-2 (*Csf2*). The *Mmd* gene is mainly up-regulated upon monocyte differentiation and is expressed in mature, *in vitro* differentiated macrophages but missing in monocytes (Vincent-Onabajo et al., 2012). The *Csf2* gene encodes for CSF-2 cytokine or GM-CSF that controls the production, differentiation and proliferation of granulocytes and macrophages (Morstyn and Burgess, 1988).

Macrophages from day 3 were stimulated with 10 ng/ml of M-CSF for a period up to 24 h and total RNA was extracted to examine the expression pattern of *Mmd* and *Csf2* by qRT-PCR. As shown in figure 3.8, in MKP-$2^{+/+}$ macrophages Mmd mRNA levels were increased at 2 h, reaching a peak by 4 h and decreased by 24 h (Fold stimulation= 1.9±0.23, 2.53±0.5, 3.1±0.49, 0.56±0.08 for 2, 4, 8 and 24 h respectively). Surprisingly, in MKP-$2^{-/-}$ macrophages, MMD expression was reduced at 2, 4, 8 and 24 h, reaching a significant difference at 4 h ($p<0.05$) but enhanced at 12 h.

During the same time course and as demonstrated in figure 3.9, induction of *Csf-2* in MKP-$2^{+/+}$ macrophages potentiated at 2 h, reaching a peak by 8 h and decreased again by 24 h (Fold stimulation=2.27±0.59, 3±0.51, 2.45±0.85, 1±0.03 for 2, 4, 8 and 24 h respectively). Similar to *Mmd*, in MKP-$2^{-/-}$ macrophages, the expression of *Csf2* was decreased between 2 and 8 h, reaching a significant difference at 4 and 8 h ($p<0.05$). However, level of *Csf-2* was enhanced in MKP-$2^{-/-}$ macrophages at 12 h (Figure 3.9).

These findings established that MMD and CSF-2 expression induced by M-CSF was lower in MKP-2 deficient macrophages in comparison to wild type. This demonstrates that MKP-2 is an important factor in the regulation of *Mmd* and *Csf2* gene expression. The increase of Mmd and Csf2 mRNA at 12 h in MKP-2 $^{-/-}$ mice suggests a late response from this group to induce both genes (Figures 3.8-3.9).

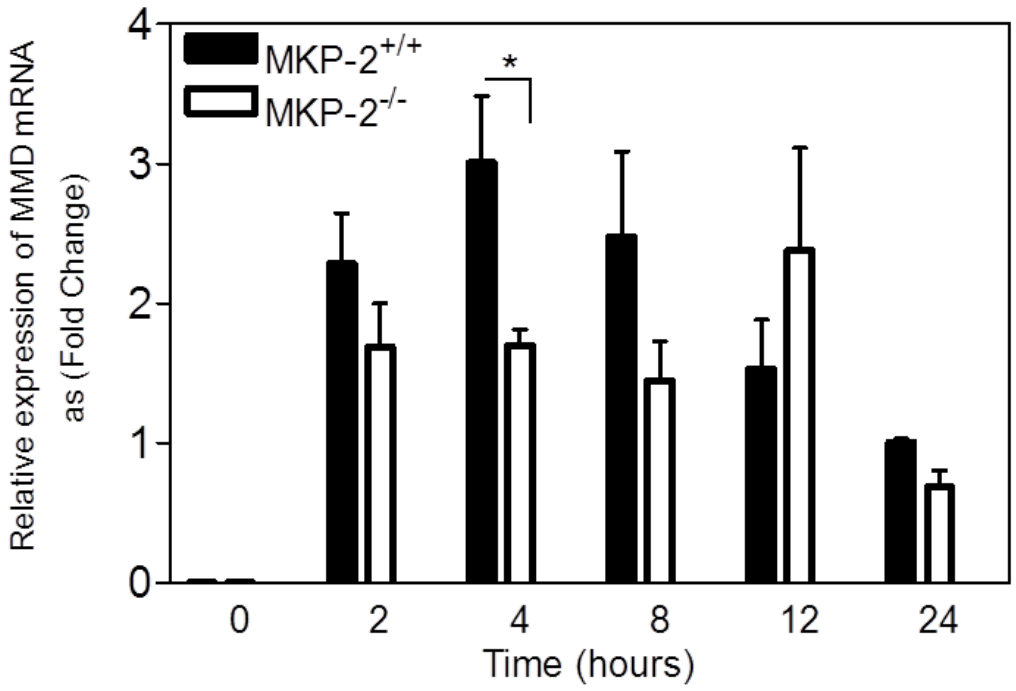


Figure 3.8: MKP-2 deletion negatively affects *Mmd* gene expression in bone marrow-derived macrophages. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h to render the cells quiescent and then stimulated with M-CSF (10 ng/ml) for the indicated periods of time. Control cells (0) were left untreated. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on cDNA using primers designed to detect *Mmd* as described in methods section 2.2.5.4. Expression levels of Mmd mRNA transcripts were normalized to the reference gene *QARS* using the delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean±SEM from three individual experiments. **P<0.05* one-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

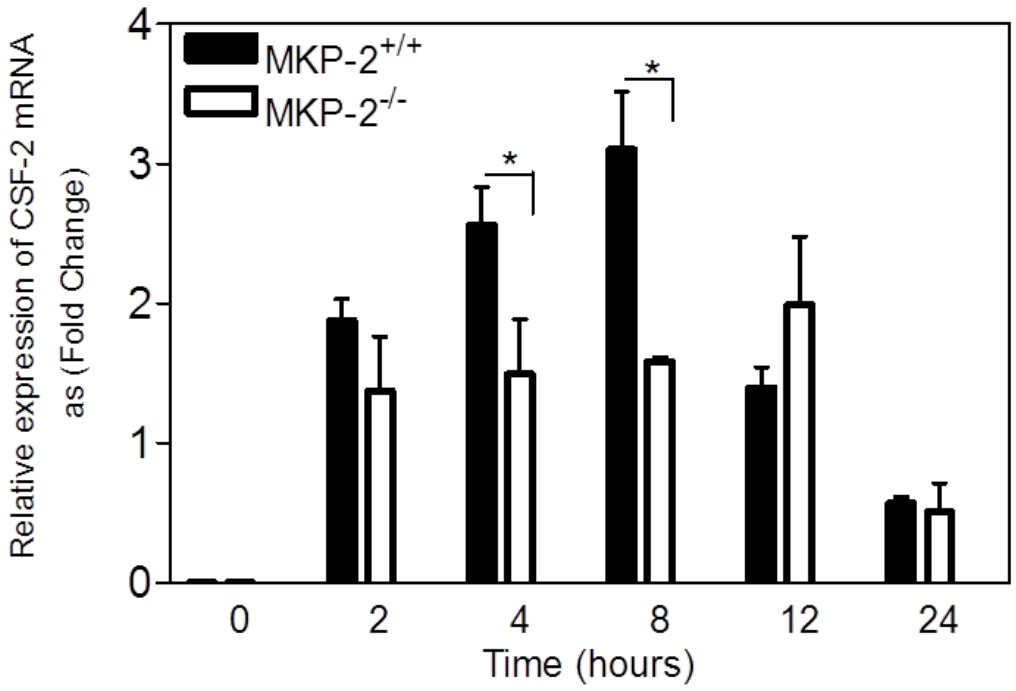


Figure 3.9: MKP-2 deletion negatively affects *Csf2* gene expression in bone marrow-derived macrophages. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h to render the cells quiescent and then stimulated with M-CSF (10 ng/ml) for the indicated periods of time. Control cells (0) were left untreated. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on cDNA using primers designed to detect *Csf2* as described in methods section 2.2.5.4. Expression levels of Csf2 mRNA transcripts were normalized to the reference gene *QARS* using the delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean±SEM from three individual experiments. **P*<0.05, one-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

3.2.7 The effect of MAPK inhibition on M-CSF-induced *Mmd* and *Csf2* expression

The previous sections 3.2.4 and 3.2.6 established that both activation of ERK and expression of *Mmd* and *Csf2* were significantly different between MKP-2 WT and MKP-2 deficient macrophages. This suggests potential involvement of ERK activation by M-CSF in the induction of *Mmd* and *Csf2* genes. To study this correlation, the effect of ERK inhibition first on M-CSF-induced ERK phosphorylation and second on *Mmd* and *Csf2* expression is studied here.

M-CSF-induced ERK phosphorylation was inhibited using U0126, a specific inhibitor for MEK the kinase upstream of ERK. MAP kinases JNK and p38 were also inhibited in this experiment with JNK (SP600125) and p38 (SB203580). These inhibitors were characterised below.

3.2.7.1 The effect of MAPK inhibition on M-CSF-induced MAPK phosphorylation

As shown in figures 3.4 to 3.6, macrophages were characterised for MAP kinase activation in response to M-CSF. MAPK kinases were all rapidly phosphorylated and peaked at 5 min after M-CSF stimulation after which this effect decreased. Therefore, macrophages were pre-incubated with the MEK inhibitor, U0126 (10 μM), JNK inhibitor, SP600125 (10 μM) or p38 MAPK inhibitor, SB203580 (10 μM) for 1 h prior to being stimulated with 10 ng/ml M-CSF for a period of 5 min. Whole cell lysates were analysed by Western blotting for ERK, JNK and p38 phosphorylation in the presence or absence of these inhibitors. A concentration of 10 μM of MAPK inhibitors was selected based on previous studies in other cell types (Gobert Gosse et al., 2005, Curry et al., 2008). The results presented in figure 3.10 shows that pre-treatment of cells with the selective ERK inhibitor U0126 completely inhibited ERK phosphorylation. (M-CSF fold stimulation relative to control, at 5 min: without U0126 WT= 54 ± 12, KO= 53.7 ± 12.7, with U0126 WT= 3.1 ± 1.9, KO= 1.15 ± 1.02). No differences in ERK phosphorylation was detected between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ cells at 5 min. This might be due to blot being

overexposed to see such a difference obtained from the blot in figure 3.4 at 5 min strong activation. In contrast, inhibiting JNK and p38 signalling using SP600125 and SB203580, respectively did not alter the phosphorylation status of either kinase (Figures 3.11 and 3.12).

The compound SB203580 blocks p38 MAPK catalytic activity by binding to the ATP-binding pocket, but does not inhibit phosphorylation of p38 MAPK by upstream kinases (Kumar et al., 1999). Similarly, SP600125 is an ATP competitive inhibitor of c-Jun N-terminal kinase (JNK) that inhibits the phosphorylation of c-Jun in a dose-dependent manner (Renlund et al., 2008, Nieminen et al., 2006). Therefore, no change the phosphorylation status of p38 and JNK was detected in figures 3.9 and 3.10

The above data proposed that the activation of the ERK pathway is required for M-CSF induced macrophage development by inducing *Mmd* and *Csf2*. As both SB203580 and SP600125 target the activity not the activation of p38 and JNK respectively, this highlights the importance of dissecting the role of both kinases on the downstream targets, particularly the expression of *Mmd* and *Csf2*.

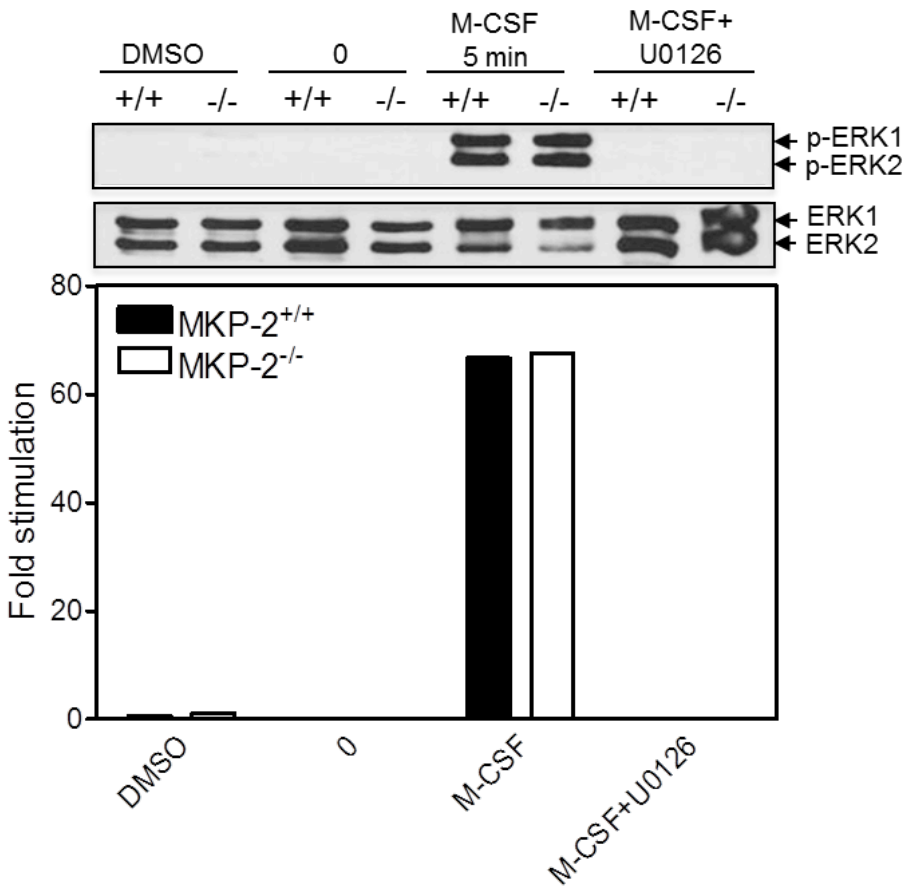


Figure 3.10: M-CSF-induced macrophage phosphorylation is blocked by ERK inhibition. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h and then pre-incubated with DMSO (vehicle control) or 10 μM of (U0126) MEK/ERK inhibitor for 1 h. Cells were either left unstimulated *(0),* or stimulated with M-CSF (10 ng/ml) for 5 min. Whole cell lysates were prepared, and assessed for the expression of p-ERK (42/44 kDa) by western blotting as stated in methods 2.2.4. Expression of the total ERK (T-ERK) was measured as a loading control. Blots were quantified for p-ERK fold stimulation by scanning densitometry relative to background signal. The blot represents the data from two individual experiments.

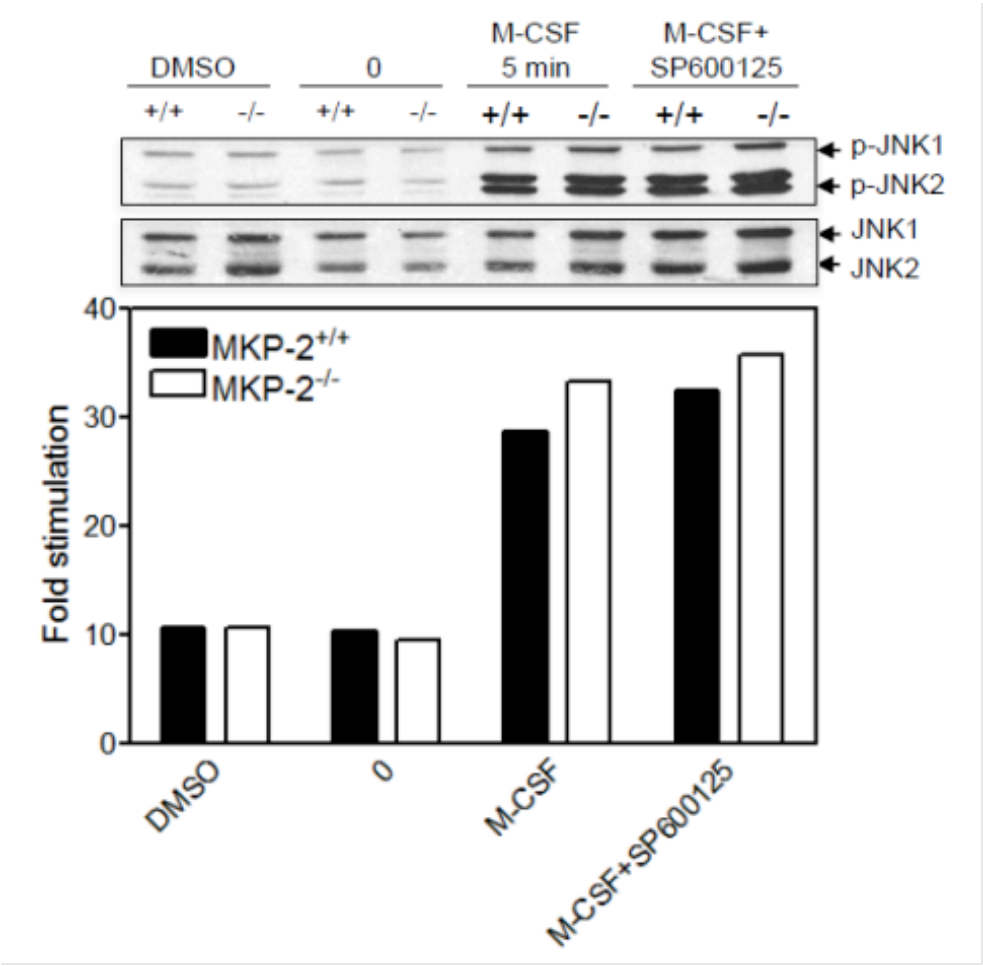


Figure 3.11: M-CSF-induced JNK phosphorylation is not inhibited by SP600125. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h and then pre-incubated with DMSO (vehicle control) or 10 μM (SP600125) JNK inhibitor for 1 h. Cells were either left unstimulated *(0),* or stimulated with M-CSF (10 ng/ml) for 5 min . Whole cell lysates were prepared, and assessed for the expression of p-JNK (46-54 kDa) by Western blotting as stated in methods 2.2.4. Expression of total JNK (T-JNK) was measured as a loading control. Blots were quantified for p-JNK fold stimulation by scanning densitometry relative to background signal. The blot represents the data from two individual experiments.

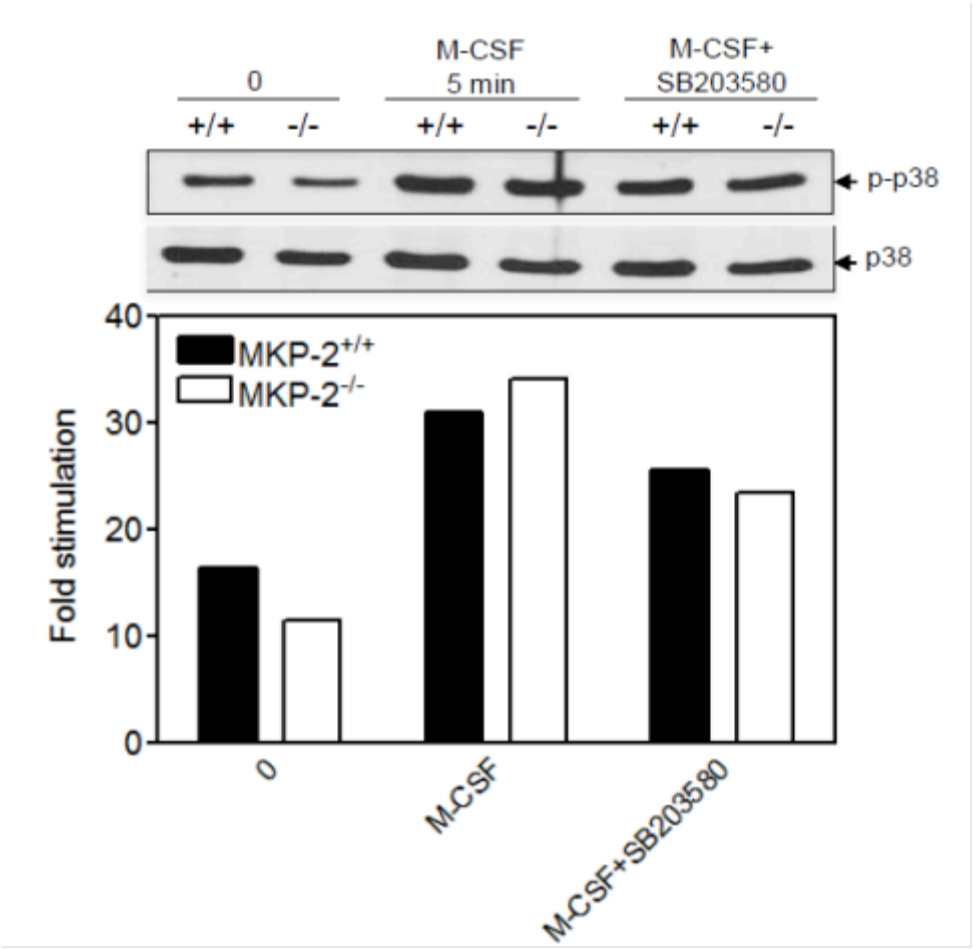


Figure 3.12: M-CSF-induced macrophage proliferation is not inhibited by SB203580. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h and then pre-incubated with DMSO (vehicle control) or 10 µM of (SB203580) p38 inhibitor for 1 h. Cells were either left non-stimulated *(0),* or stimulated with M-CSF (10 ng/ml) for 5 min . Whole cell lysates were prepared, and assessed for the expression of p-p38 (38 kDa) by western blotting as stated in methods 2.2.4. Expression of total p38 (T-p38) was measured as a loading control. Blots were quantified for p-p38 fold stimulation by scanning densitometry relative to background signal. The blot represents the data from two individual experiments.

3.2.7.2 The effect of MAPK inhibition on M-CSF-induced *Mmd* and *Csf2* expression

As obtained from the previous section, ERK inhibitor U0126 completely blocked ERK phosphorylation. To further correlate the role of ERK activation in *Mmd* and *Csf2* gene expression, the effect of ERK inhibition on the induction of both genes was studied. As shown in section 3.2.7.1, inhibiting JNK and p38 by SP600125 and SB203580 showed no significant differences in the phosphorylation of both kinases. Here, the effect of JNK and p38 inhibition using the same compounds on the expression of *Mmd* and *Csf2* was investigated. MAP kinase inhibition was performed by pre-incubating the cells for 1 h with 10 μM of the following inhibitors: U0126 (ERK), SP600125 (JNK) and SB203580 (p38). Macrophages were then stimulated with 10 ng/ml of M-CSF for 8 h and total RNA was extracted to examine the expression pattern of *Mmd* and *Csf2* by qRT-PCR.

Using this design, inhibiting ERK activity by U0126 in figure 3.13 reduced *Mmd* expression by ~52.2 % and ~33% for wild type and knock-out cells respectively to that produced from 8 h M-CSF stimulated cells. Inhibiting JNK also reduced *Mmd* induction in WT macrophages to a lesser extent when compared to ERK inhibition (JNK reduction ~39 %, p38 reduction ~17.8 %).

A similar pattern was also observed for *Csf2*, U0126 also reduced the *Csf2* mRNA levels by ~87.6 % and ~84.6 % in both wild type and knock-out cells respectively (Figure 3.14). The same figure indicated that M-CSF-induced *Csf2* expression was also reduced by both p38 or JNK inhibitors when compared to ERK inhibition (JNK reduction ~78 % and ~62 % for MKP-$2^{+/+}$ and MKP-$2^{-/-}$ respectively, p38 reduction ~52 % and ~34.4 % for MKP-$2^{+/+}$ and MKP-$2^{-/-}$ respectively).

The above data suggests the possible involvement of additional factor to regulate macrophage development which is influenced by MKP-2, since ERK inhibition downregulates both *Mmd* and *Csf2* expression. The data also indicates that induction of Mmd mRNA is not regulated by JNK or p38 as inhibitors targeting these pathways did

not affect M-CSF-induced *Mmd* expression. However, the data revealed the possible involvement of JNK and p38 activation in *Csf2* gene expression.

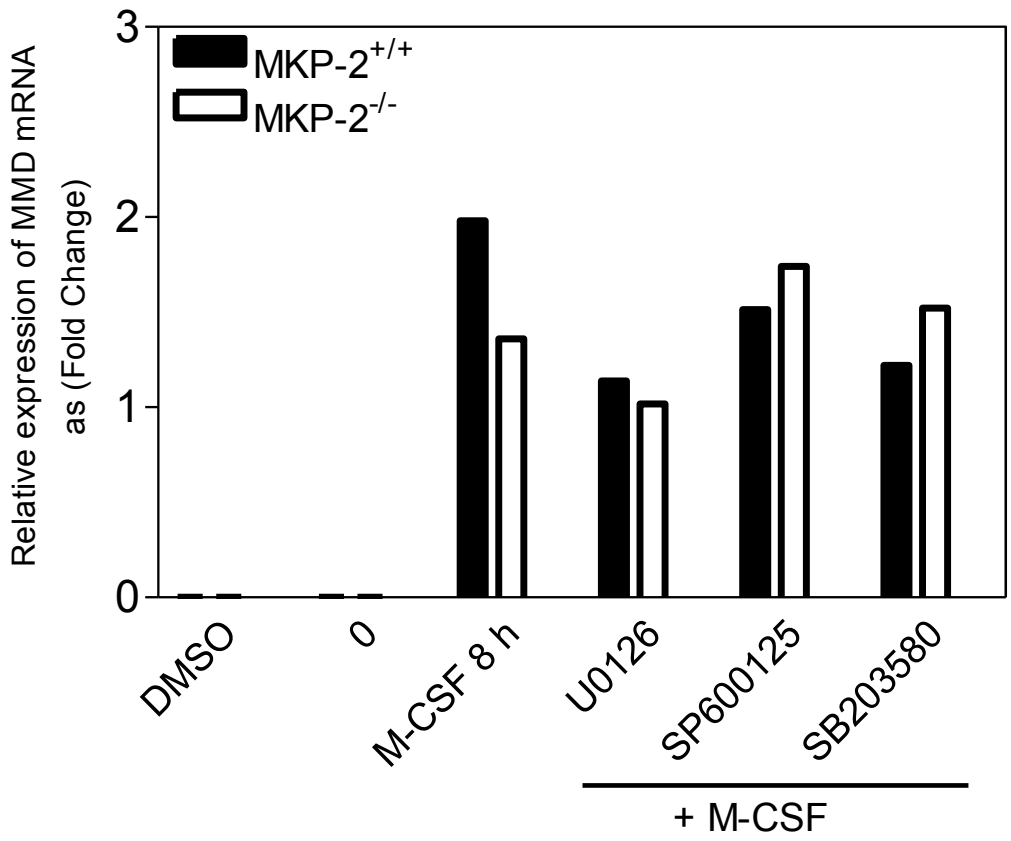


Figure 3.13: M-CSF-induced *Mmd* gene expression is affected by MAPK inhibition. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h and then pre-incubated with DMSO (vehicle control) or 10 μM of U0126, SP600125 and SB203580 inhibitors for 1 h. Cells were either left unstimulated *(0),* or stimulated with M-CSF (10 ng/ml) for 8 hours. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on cDNA using primers designed to detect *Mmd* as outlined in methods 2.2.5.4. Relative expression levels of *Mmd* mRNA transcripts were normalized to the reference gene *QARS* using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from two individual experiments.

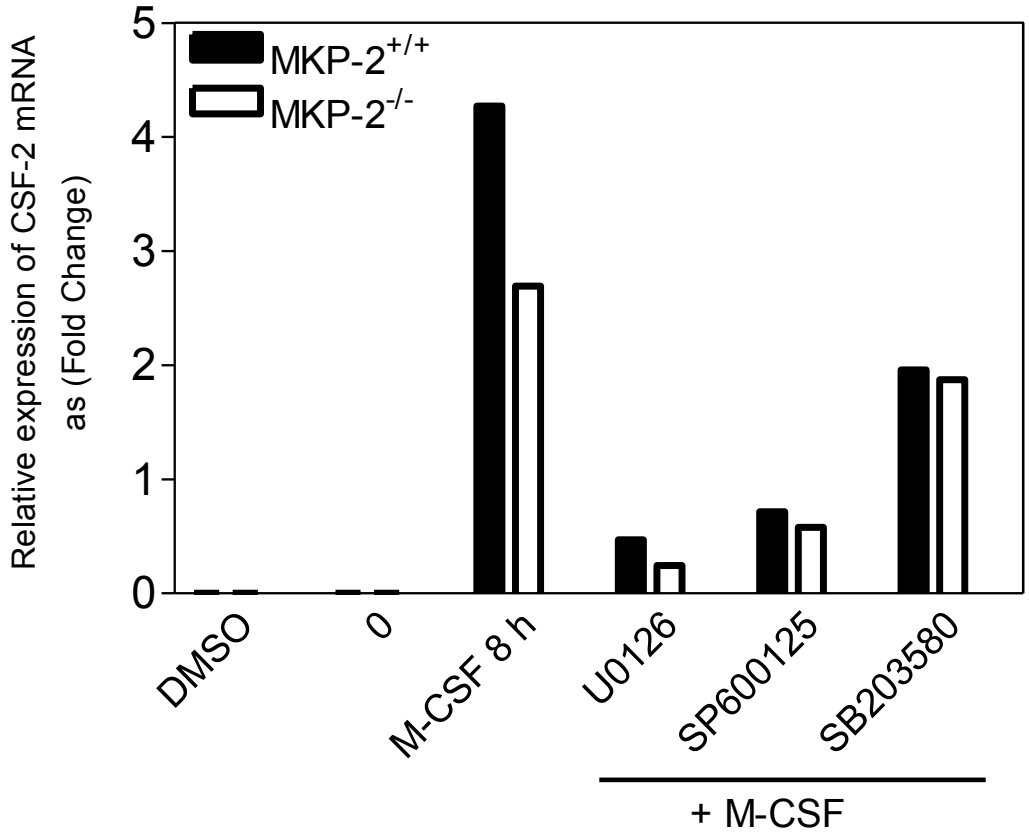


Figure 3.14: M-CSF-induced *Csf2* gene expression is affected by MAPK inhibition. Macrophages were harvested at day 3 of culturing, deprived of L cell-conditioned medium for 18 h and then pre-incubated with DMSO (vehicle control) or 10 μM of (U0126), (SP600125) and (SB203580) for 1 h. Cells were either left unstimulated *(0),* or stimulated with M-CSF (10 ng/ml) for 8 hours. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on cDNA using primers designed to detect *Csf2*, as outlined in methods 2.2.5.4. Relative expression levels of *Csf2* mRNA transcripts were normalized to the reference gene *QARS* using the delta-delta Ct method (Livak and Schmittgen, 2001). The figure represents the data from two individual experiments.

3.2.7.3 The effect of ERK inhibition on M-CSF-induced macrophage proliferation

The data in section 3.2.7.1 showed that ERK inhibition by U0126 completely blocked M-CSF-induced ERK phosphorylation. In contrast, U0126 in figures 3.13 and 3.14 reduced the induction of both *Mmd* and *Csf2* genes. An additional ERK inhibition experiment was carried out this time to investigate the definitive role of ERK in macrophage proliferation since in section 2.2.3 MKP-2 deletion was found to reduce proliferation. This was obtained by studying the effect of U0126 inhibitor in macrophage proliferation using cell counting by haematoxylin.

Macrophages from MKP-$2^{+/+}$ and MKP-$2^{-/-}$ were harvested at day 3, starved of M-CSF for 24 h and then stimulated with M-CSF (10 ng/ml) for 48 hours. ERK inhibition was performed by pre-incubating the cells for 1 h with 10 μM of U0126. In both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages, M-CSF was able to increase the number of cells at 48 h (Figure 3.15). Inhibiting ERK activity by U0126 significantly reduced the number of cells when compared to 48 h M-CSF stimulated cells (Figure 3.15). This experiment provided additional evidence that ERK significantly regulates the growth of bone marrow derived macrophages from precursors and that ERK activation by M-CSF is essential for appropriate macrophage development.

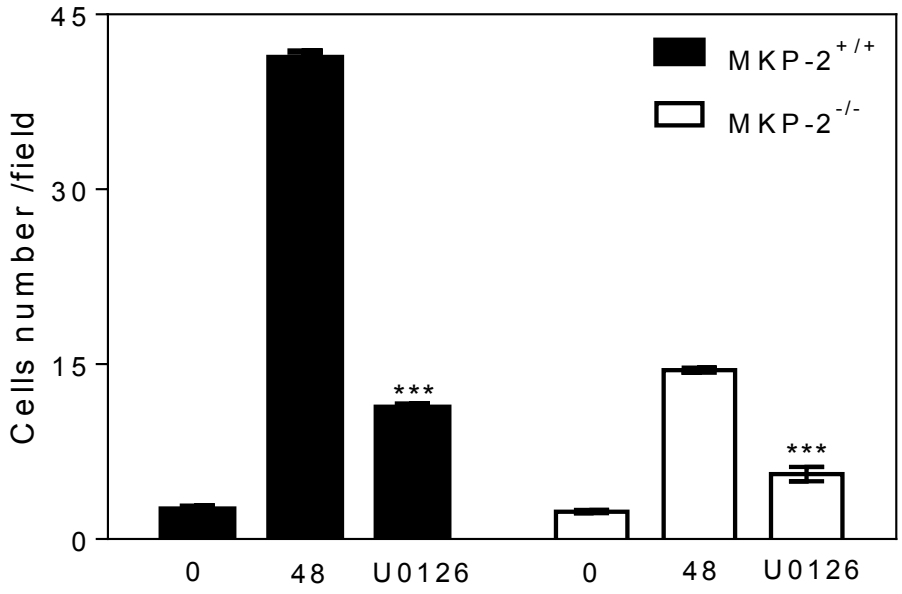


Figure 3.15: M-CSF-induced macrophage proliferation is affected by ERK inhibition. Macrophages were harvested on day 3 of culture, and $5x10^3$ cells per well were seeded onto cover slips in 24-well plates. Cells were then allowed to attach for 24 h. Cells were then pre-treated with (U0126) for 1 hour prior to stimulation with M-CSF (10 ng/ml) for 48 h, stimulated with M-CSF alone or left unstimulated (0 h). Cells were washed with PBS, and exposed to haematoxylin dye. The number of cells was determined by counting the cells from ten random fields per cover slip as described in methods 2.2.2. Each value represents the mean±SEM from three individual experiments. ***$p<0.001$, one-tailed unpaired t test relative to 48 h M-CSF treated macrophages.

3.2.8 Differential expression of CD34 and CD115 on the surface of bone marrow cells

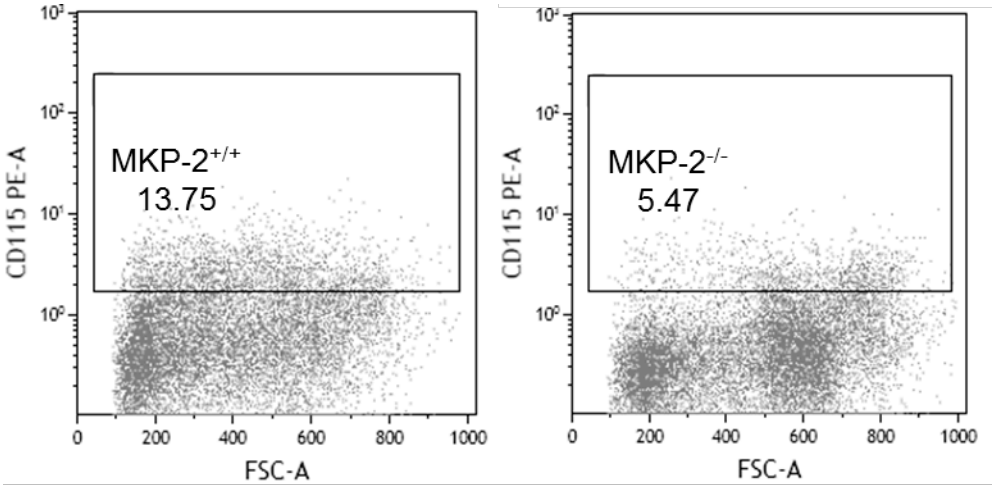
The previous section established that ERK inhibition reduced the expression of both *Mmd* and *Csf2* genes. However, results earlier in this chapter have also demonstrated that ERK activation was enhanced in MKP-2 KO macrophages but that the expression of both *Mmd* and *Csf2* was reduced. This indicates a dissociation between the cellular regulation of ERK and the effects of MKP-2. Therefore, the aim here was to examine the effect of MKP-2 loss on the expression of macrophage surface proteins CD34 and CD115.

CD34 is an important stem/progenitor cell marker (Matsuoka et al., 2001). The $CD34^+$ myeloid progenitors differentiate into monocytes and further to macrophages in the presence of M-CSF (Martinez et al., 2006). The receptor for M-CSF is a transmembrane tyrosine kinase (CD115) which has been used to identify cells that belong to the mononuclear phagocyte system including macrophages (Francke et al., 2011).

The analysis of bone marrow cells showed a significant reduction in CD115 expression on cells from MKP-$2^{-/-}$ mice in comparison to wild type; 5.6-8.2 % and 9.1-13.8 % respectively, $p<0.05$ (Figure 3.16). Based on this, the bone marrow cell suspension from MKP-$2^{-/-}$ mice contains less macrophage $CD115^+$ precursors that have the tendency to become macrophages.

Analysis of the cell suspension showed that approximately 11.3-16.5% and 2.3-7.8% of the cells were $CD34^+$ for MKP-$2^{+/+}$ and MKP-$2^{-/-}$ respectively. The percentage of CD34 positive cells was significantly lower in MKP-$2^{-/-}$ when compared to wild type counterparts ($p<0.05$) (Figure 3.17). This indicated that MKP-$2^{-/-}$ bone marrow consisted of fewer myeloid progenitors. It is noteworthy that the percentage of $CD34^+CD115^+$ cells was also reduced in MKP-$2^{-/-}$ bone marrow with 6.3%±0.9 when compared to 3.75±0.45 in MKP-$2^{+/+}$ bone marrow.

In conclusion, a significant reduction in CD34+ progenitors along with CD115 (M-CSF receptor) expression in the bone marrow of MKP-2 deficient mice might contribute to the overall reduced macrophage numbers at day 10 of culture.



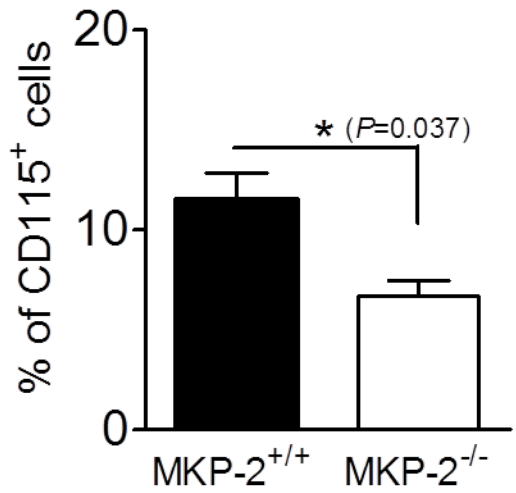


Figure 3.16: The effect of MKP-2 deletion on CD115 expression. Native bone marrow cells were isolated and subsequently analysed for CD115 the receptor for M-CSF as described in methods section 2.2.3. The gated rectangles represent the CD115 positive population as percent within the native cell suspension. Gating was performed according to negative isotype-matched controls. FACS data represent the mean ± SEM of three separate experiments. **P*<0.05, two-tailed unpaired t test comparing MKP-2$^{-/-}$ to MKP-2$^{+/+}$ macrophages.

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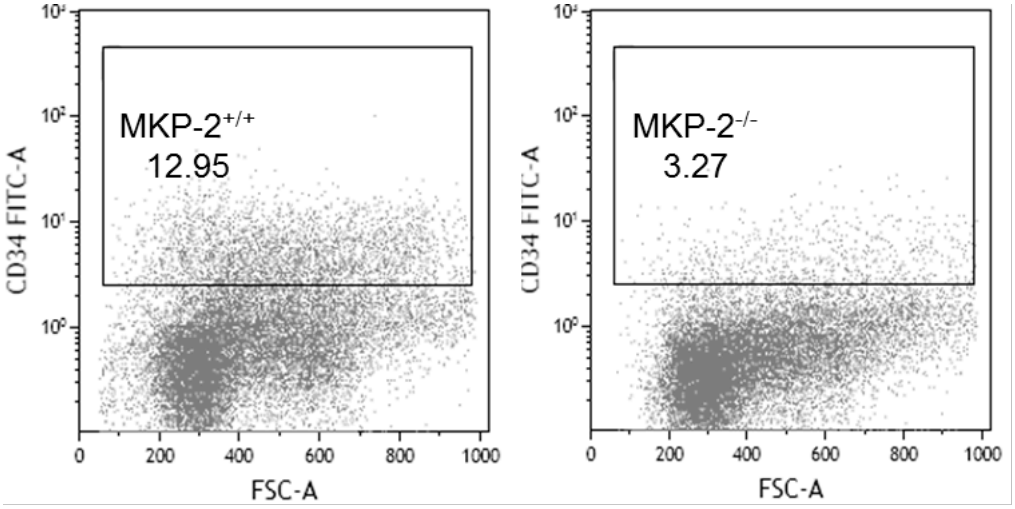


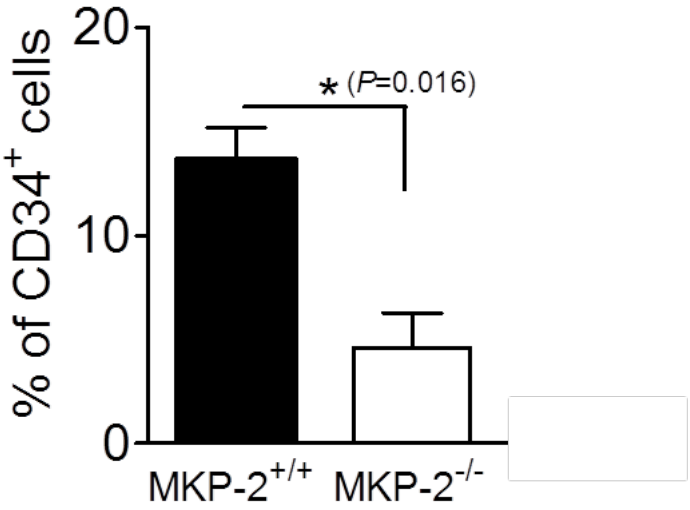


Figure 3.17: The effect of MKP-2 deletion on CD34 expression. Native bone marrow cells were isolated and subsequently analysed for CD34, a marker for progenitor cells as described in methods section 2.2.3. The gated rectangles represent the CD34 positive population as percent within the native cell suspension. Gating was performed according to negative isotype-matched controls. FACS data represent the mean ± SEM of three individual experiment experiments. **P*<0.05, two-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

3.2.9 Expression of murine mature macrophage markers

Similar to the previous experiment, flow cytometric analysis was used here to examine the expression murine macrophage markers. This was to investigate whether the developmental deficit in MKP-$2^{-/-}$ precursor cells does affect the activation/maturation to macrophages. In particular, the expression of typical macrophage markers F4/80, CD14, CD11b and MHC-II is examined here to determine if the resulting population are indeed macrophages.

Expression of macrophage markers was examined at days 5, 7 and 10 of culturing in unstimulated cells and following activation with 100 ng/ml LPS for 4 and 24 h. A similar pattern in the expression of macrophage markers was detected between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ cells. MHC-II was up-regulated with LPS treatment at 4 h (% of positive cells: MKP-$2^{+/+}$=13.55±7.65, MKP-$2^{-/-}$=12.7±6.7 vs. unstimulated MKP-$2^{+/+}$=6.6±3.3, MKP-$2^{-/-}$ =5.45±2.55 and down-regulted again at 24 h. This was considered to be a weak upregulation as usual activation by LPS at 4 h causes ~50% increase in MHC II and remains at this level for up to 48 h (Pai Kasturi et al., 2006, Casals et al., 2007, Hokuto et al., 2006). However, a non-siginificant difference in MHC II expression indicates a similar activation pattern between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages. Figure 3.18 shows the presence of surface markers on day 7. A similar data was observed from days 5 and 10. These findings indicated that lack of MKP-2 does not affect the activation/maturation status of the macrophages.

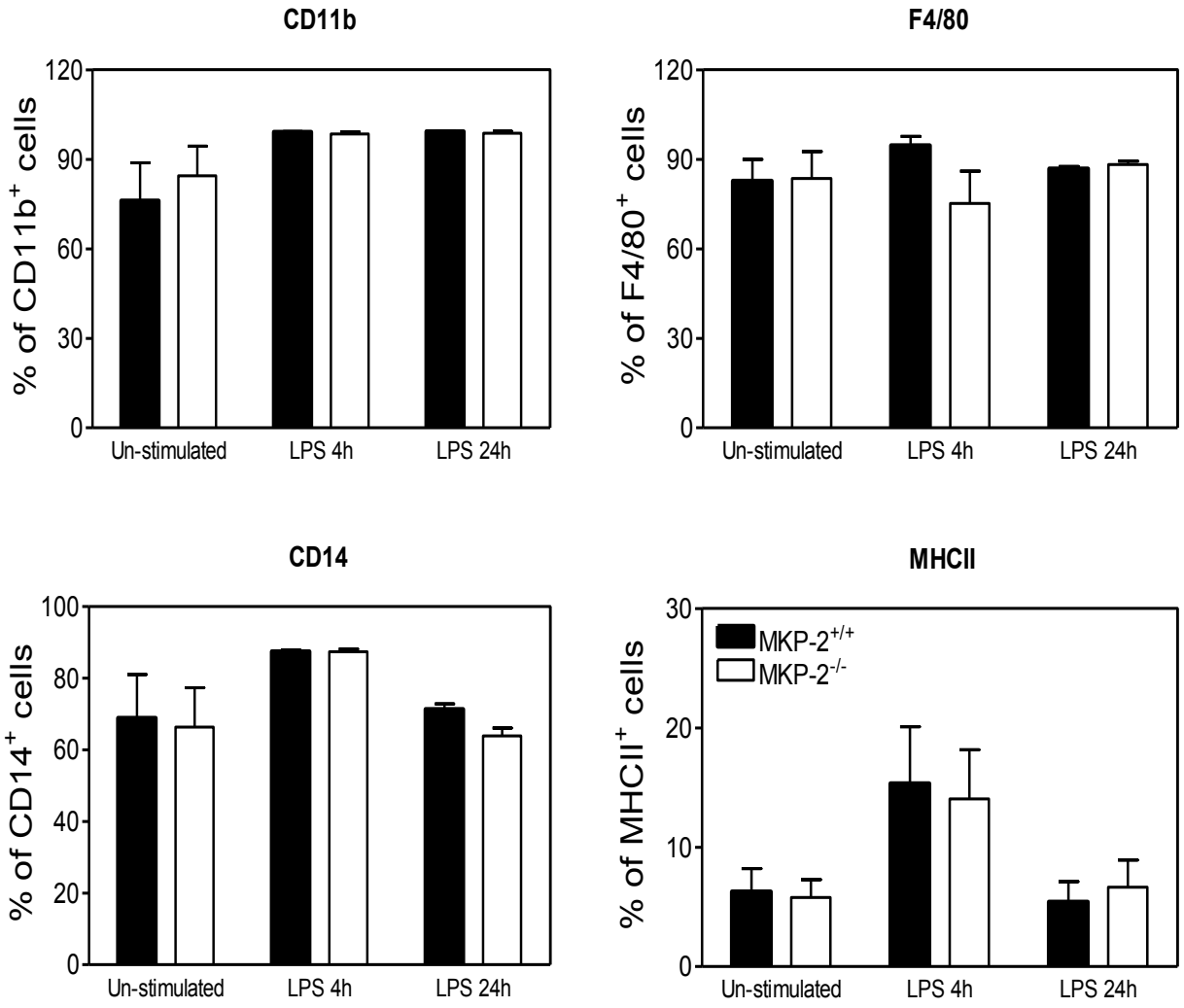


Figure 3.18: The effect of MKP-2 deletion on the expression of murine macrophage markers. Macrophages were harvested at day 7 of culturing, left untreated or were subjected to 100 ng/ml of LPS for the indicated period of time and then analysed for the presence of CD11b, CD14, F4/80 and MHC-II, as described in methods section 2.2.3. The bars represent the respective marker positive population as percent in relation to all cells. Gating was performed according to negative isotype-matched controls. Error bars represent the mean±SEM from three individual experiments. Statistical analysis was carried using the two-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages ($P>0.05$, n.s).

3.3 Discussion

MAP kinase phosphatase-2 (MKP-2) or DUSP4 is a type I member of the MKP family, which have been shown to specifically regulate the activity of ERK and JNK over p38 in vitro (Misra-Press et al., 1995b, Dickinson and Keyse, 2006, Owens and Keyse, 2007, Lawan et al., 2012). MKP-2 was found to have ubiquitous tissue distribution similar to MKP-1 (DUSP1), suggesting overlapping functions between both phosphatases. Several reports highlight the importance of MKP-1 to control ERK phosphorylation required for macrophage proliferation (Lomonaco et al., 2008, Valledor et al., 2008b, Comalada et al., 2012). However, the role of MKP-2 in macrophage development has not been thoroughly studied. This chapter has focused on the effect of MKP-2 deletion on MAPK regulation in relation to macrophage development using MKP-2 deficient mice.

The haematopoietic growth factor M-CSF is required for macrophage development and has thus been used extensively to study macrophage proliferation (Valledor et al., 1999, Valledor et al., 2000, Valledor et al., 2008b, Comalada et al., 2012). It was observed in this study that in response to M-CSF, the lack of MKP-2 protein affected the proliferation of bone marrow derived macrophages. Macrophages obtained from MKP-$2^{-/-}$ bone marrow exhibited reduced cell growth compared with MKP-$2^{+/+}$ counterparts (Figure 3.2). This is consistent with the data reported by Lawan et al. (2011), as serum-induced cellular proliferation of MKP-$2^{-/-}$ MEFs was also significantly reduced and this defect was reversed with adenoviral MKP-2 infection. In the same work, numbers of both bone marrow macrophages and cardiac fibroblasts decreased by over 50% when compared to MKP-2 wild type controls (Lawan et al., 2011). It is important to highlight that upon M-CSF removal prior to re-stimulation with FCS, macrophages failed to grow (Lawan et al., 2011). The same observation is shown in Figure 3.2 C, as cells were subsequently dying upon M-CSF removal. This is because M-CSF is the only specific growth factor for macrophage survival (Stanley et al., 1997, Pixley and Stanley, 2004). In the absence of this cytokine, macrophage growth rate declined and cells subsequently died.

M-CSF regulates macrophage development by triggering signal transduction pathways, one of which is the MAPK pathway (Comalada et al., 2012). This family includes extracellular signal-regulated protein kinase (ERK-1/2), stress-activated protein kinases (SAPK), p38, and c-Jun NH -terminal protein kinases (JNK) (Sanchez-Tillo et al., 2007). Activation of these kinases by M-CSF in macrophages further phosphorylates transcriptional regulators to induce immediate gene expression responsible for macrophage proliferation, survival and differentiation (Valledor et al., 1999, Yang et al., 2003, Comalada et al., 2012). In response to M-CSF, it was found that ERK was phosphorylated rapidly at 5 min (Figure 3.4). In agreement with our data, an early peak of ERK activation (5 min) was detected to be correlated to macrophage proliferation in response to M-CSF (Valledor et al., 1999, Valledor et al., 2000, Sanchez-Tillo et al., 2006, Comalada et al., 2012). In the same reports, a later peak (15 min) was associated with macrophage innate activation by LPS. The authors concluded that both agonists require ERK activation, and a short duration of ERK phosphorylation causes macrophage proliferation while a prolonged period activates the cells and stops proliferation. (Valledor et al., 1999, Valledor et al., 2000, Sanchez-Tillo et al., 2006, Valledor et al., 2008, Comalada et al., 2012). In addition, work by Cornell et al. showed that in LPS-stimulated bone marrow macrophages, phosphorylation of ERK was also increased in MKP-$2^{-/-}$ at 15 min (Cornell et al., 2010).

Authors also noted that ERK dephosphorylation by MKP-1 is critical to obtain either proliferation or activation. For macrophage proliferation, ERK must be dephosphorylated within the first 15 min (Valledor et al., 1999, Valledor et al., 2000, Sanchez-Tillo et al., 2006, Valledor et al., 2008b, Comalada et al., 2012). It is worth mentioning here that enhanced ERK phosphorylation at 5 min in response to M-CSF in MKP-$2^{-/-}$ macrophages was still elevated at 60 min, whilst in wild-type macrophages it returned to basal level at about 15 min (Figure 3.4). The difference in dephosphorylation kinetic may be an explanation for the defect in the proliferation of MKP-2 deficient macrophages.

To further confirm that ERK deactivation is mediated by MKP-2, the expression of this phosphatase by M-CSF was studied. The expression of MKP-2 protein was slightly detected at 5 min, peaked at 8 h and decreased again at 24 h after M-CSF stimulation in wild type macrophages (figure 3.7). Since a clear expression of MKP-2 was observed at 15-30 min, this correlates with ERK dephosphorylation during the macrophage response to M-CSF. In contrast, Western blots of M-CSF stimulated WT macrophage lysates probed for MKP-1 showed detectable protein production at 20 min, reaching the maximum between 60-75 min, and returned to basal levels within 2 h (Valledor et al., 1999). This highlights the fact that MKPs 1 and 2 have distinct patterns of activation. The current data indicates that the absence of MKP-2 critically altered the phosphorylation pattern of ERK, in response to M-CSF, and therefore identify the correlation between this regulation and macrophage proliferation. This highlights the importance of MKP-2 in deactivating ERK that is required for cell proliferation. Similarly, it has been demonstrated that M-CSF dependent macrophage proliferation was blocked when MKP-1 was inhibited using siRNA (Valledor et al., 2008, Lomonaco et al., 2008). Valledor et al., 2008 also found that the inhibition of MKP-1 by IFN-γ cytokine halts macrophage proliferation and induces activation (Valledor et al., 2008b). Another study found that overexpression of DUSP5 in FD-Fms cells that proliferate and differentiate toward macrophages in response to M-CSF, resulting in increased proliferation and decreased differentiation (Grasset et al., 2010). These studies and the data presented in this chapter indicate that deletion of some of the MKPs can lead to a decrease in cell growth.

Under similar experimental conditions, JNK and p38 activation was induced by M-CSF at 5 min to a lesser extent than that observed from ERK. Phosphorylation of both MAP kinases was not significantly altered in MKP-$2^{-/-}$ when compared to that in wild type counterparts (Figures 3.5 and 3.6). This further confirms that activation of ERK is particularly required for macrophage proliferation. Velledor et al., 2008 support the same findings, as all MAPK are activated by M-CSF whereas ERK specifically participates in the proliferation process (Valledor et al., 2008). It was found the kinetics of JNK activation by M-CSF is not involved in macrophage fate between proliferation and

activation. JNK1 activity was observed 15 min and returned to basal level at 60 min in response to both M-CSF and LPS (Valledor et al., 2008b).

An important finding in this study was the observation that the loss of MKP-2 protein caused alteration in the expression of *Mmd* and *Csf2* genes. This proposed a link between MAPK activation/deactivation by MKPs and gene expression. The Monocyte to Macrophage Differentiation (*Mmd*) gene was first identified in 1995. The gene is up-regulated during the differentiation of human monocytes to macrophages (Rehli et al., 1995). This indicates the importance of MMD in macrophage production. The gene was induced by M-CSF over 24 h time course and decreased significantly at 4 h upon MKP-2 deletion (Figure 3.8). Thus, the reduction in *Mmd* expression in MKP-2 KO cells can explain the defect in macrophage differentiation and proliferation in this group.

To date, no exact function of MMD has been identified. However, Lie et al. (2012) reported that MMD protein belongs to the progesterone and adipoQ receptor (PAQR) family and has a role in macrophage activation. It was found that LPS significantly up-regulated *Mmd* expression in macrophages and MMD over-expression enhanced LPS-stimulated production of TNF-α and NO via ERK and Akt phosphorolytaion (Liu et al., 2012). It has been suggested that MMD is a Raf kinase trapping to Golgi (RKTG) protein that blocks the Ras/Raf/MEK/ERK signalling cascade by sequestrating Raf-1 to the Golgi apparatus and thus inhibits the proliferation of tumour cells (Liu et al., 2012). Another immunological role suggesting the involvement of MMD in activated macrophage processes was provided by Sawcer et al. (2002), who reported the involvement of hMMD in Multiple Sclerosis (MS) as a susceptibility gene for the disease. The upregulation of the *Mmd* gene by LPS is confirmed in the following chapter, which coincides with the immunological role of MMD mentioned in the above literature.

A similar result was obtained from the expression of the *Csf2* gene. The protein encoded by the human CSF-2 is a cytokine that controls the production, differentiation, and function of granulocytes and macrophages (Morstyn and Burgess, 1988). An early study

showed that, total cell numbers of blastocysts from CSF-2$^{-/-}$ mice were reduced to 18 % compared with wild-type controls (Robertson et al., 2001), suggesting a role of this gene in this cell type. The reduction in CSF-2 expression in MKP-2 KO cells (Figure 3.9) can explain the deficiency in macrophage production and proliferation in this group. The late up-regulation of both genes *Mmd* and *Csf2* in the MKP-2$^{-/-}$ group at 12 h suggests a late response to M-CSF from this type of cells.

To further confirm the involvement of ERK activation in the response to M-CSF, U0126, a specific ERK kinase (MEK1/2) inhibitor was used. At 10 μM, U0126 inhibited M-CSF induced ERK phosphorylation at 5 minutes (Figure 3.10) and macrophage proliferation using haematoxylin staining (Figure 3.15). MEK inhibitor (U0126) also significantly impaired the differentiation of bone marrow mononuclear phagocytes to macrophages stimulated with M-CSF (Bourgin-Hierle et al., 2007). Pre-incubating bone marrow macrophages with PD98059 (another MEK1 inhibitor) inhibited ERK activation in a dose-dependent manner with a maximum effect at 50 μM (Valledor et al., 2000).

Inhibition of JNK and p38 had mixed effects on M-CSF. This result is difficult to interpret. Inhibition of JNK with SP600125 blocked the induction of MKP-1 mRNA, prolonged the activity of ERK and therefore controlled macrophage proliferation (Sanchez-Tillo et al., 2007). Also, the P38 inhibitor (SKB202190) did suppress CSF-1-stimulated DNA synthesis in bone marrow macrophages only at high concentrations but could not cause apoptosis following CSF-1 withdrawal (Jaworowski et al., 1999). Therefore MAPK inhibitors were used to assess the induction of the differentiation marker *Mmd* and the development gene *Csf2*. This was particularly to discover if ERK activation by M-CSF is further contributing in the expression of both genes and therefore macrophage development. However, the data in figures 3.13 and 3.14 confounds the expectations, that a reduction in ERK phosphorylation causes an increase in macrophage proliferation, which is mediated by the induction *Mmd* and *Csf2*. This suggests a possible involvement of a different factor that is affected by MKP-2 deletion, which influences macrophage proliferation. However, this does not contradict the findings that ERK

phosphorylation/dephosphorylation is important in M-CSF induced macrophage development.

Additional factors that may help to explain the growth deficit in MKP-2$^{-/-}$ macrophages is the differential expression of CD34, a progenitor cell marker and CD115, the M-CSF receptor. Profiling of haematopoietic stem cells has been extensively studied using monoclonal antibodies against surface antigens selectively expressed on primitive cells. Cell suspension from bone marrow consisted of diverse populations, including erythroid cells, B-lymphocytes, granulocytes, monocytes, and a wide range of progenitor and stem cells (Francke et al., 2011).

Antibodies against the heavily glycosylated transmembrane protein (CD34) is of particular interest because its expression is restricted to the stem/progenitor cell compartment. CD34 has been used in vitro as an important marker for primitive haematopoietic cells to identify the capacity of the cells to generate haematopoietic progenitors (Matsuoka et al., 2001). Haematopoietic stem cells (HPS) in bone marrow can differentiate to common myeloid progenitors which mediate the generation of monocytes and further to macrophages in the presence of M-CSF (Gordon and Taylor, 2005). In bone marrow from MKP-2$^{-/-}$ mice, the percentage of CD34^{+} cells was significantly lower compared to wild type mice (Figure 3.17). Therefore, a smaller number of macrophages resulted from fewer CD34^{+} progenitors able to differentiate into monocytes and then macrophages.

The analysis of CD115 expression on the surface of bone marrow cells distinguishes monocyte/macrophage population from other cells in bone marrow cell suspension (Francke et al., 2011). Detection of lower CD115/M-CSF receptors in MKP-2 KO cell suspensions (Figure 3.16) indicated less monocyte/macrophage lineage and less macrophage precursor response to M-CSF stimulation thus resulting in less numbers of macrophages. The reduction in the expression of both CD34 and CD115 could be an

additional factor to explain the defect in the growth ability of MKP-2$^{-/-}$ macrophages. Although various studies have been conducted on the importance of CD34 and CD115 to characterise bone marrow cell population (Rappold et al., 1997, Francke et al., 2011), there is no information on the potential importance of MKPs on the expression of both markers.

The later expression of macrophage markers F4/80, CD14, CD11b and MHC-II at days 5, 7 and 10 was not affected with MKP-2 deletion. Analysis for expression of cell F4/80 and CD11b is used to confirm the macrophage phenotype of the cells isolated. Membrane protein F4/80 is a specific mouse marker used to distinguished macrophages from other mononuclear phagocytes (Gonzalez-Juarrero et al., 2003, Murray and Wynn, 2011). Expression of CD11b, (α subunit of the integrin cell surface receptor) on macrophage surface has been recorded as being induced during differentiation of monocytes into macrophages, whereas, CD14 works with LPS binding protein and TLR-4 to detect and bind lipopolysaccharides (Moeenrezakhanlou et al., 2008). Major histocompatibility complex II (MHC II) is a proinflammatory cell-surface marker strongly expressed when macrophages are classically activated by LPS and IFN-γ (Gordon and Taylor, 2005b). MHC II expression was enhanced to a little extent with 4 h LPS stimulation exhibiting a similar pattern in MKP-2 wild type and knock-out cells thus excluding differences in activation status upon MKP-2 loss. Thus, similarities in the expression of the above mentioned macrophage markers exclude the earlier mentioned deficit on the developed macrophages or differentiated macrophages undergoing maturation. This verifies that development deficit is occurring at early stage during macrophage development and that cells catch up over time.

In conclusion, the findings in this chapter strongly suggest that MKP-2 is necessary for macrophage proliferation through potentially regulating the ERK pathway. It also demonstrates that MKP-2 is essential in the regulation of genes associated with macrophage development and normal progenitor surface antigen expression. Finally, it

highlights the importance to carry out further studies on the role of MKP-2 in macrophage immune response.

4. THE ROLE OF MKP-2 IN MACROPHAGE GENE EXPRESSION

4.1 Introduction

The previous chapter described the kinetics of MKP-2 induction in response to M-CSF and established the role of MKP-2 in regulating ERK MAPK signalling in bone marrow macrophages. It also demonstrated that MKP-2 was important for macrophage development through the induction of *Mmd* and *Csf2* genes in response to M-CSF. However, it was unclear if this was due to any effect on ERK signalling.

As mentioned earlier in the introduction and Chapter 3, MKP-2 has been shown to play a significant role in immune function particularly in parasite-specific immunity by influencing macrophage activity. Mice lacking MKP-2 were more susceptible to *Leishmania mexicana* infection due to enhanced macrophage arginase-1 levels and reduced iNOS activity and NO production (Al-Mutairi et al., 2010). In addition to that, MKP-2 deletion also increased LPS-stimulated induction of the inflammatory cytokines, IL-6, IL-12p40, TNF-α, and also COX-2-derived PGE_2 production (Al-Mutairi et al., 2010). This report, together with the findings in the previous chapter, highlight the importance of investigating changes in gene expression that lead to phenotypic differences following MKP-2 deletion. Since the molecular effects of MKP-2 deficiency on macrophage development and immune function are not delineated, the aim in this chapter was to clearly determine the transcriptional responses that are regulated by MKP-2 in macrophages. Microarray gene expression analysis was conducted to compare the responses of MKP-$2^{-/-}$ and MKP-$2^{+/+}$ mouse BMDMs following LPS or IL-4 stimulation.

This approach was designed to identify pro- and anti-inflammatory related gene expression in the presence and absence of MKP-2 that may play important roles in macrophage function. Based on the results of the microarray analysis, candidate genes were selected and validated using qRT-PCR assays.

4.2 Results

4.2.1 Microarray analysis of MKP-2-regulated genes in bone marrow-derived macrophages

In order to study the importance of MKP-2 in macrophage gene expression, BMDMs from MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were seeded into 6-well plates. Macrophage total RNA was extracted for microarray analysis following their activation with LPS and IL-4. Since most of the genes are induced or repressed in a temporal pattern, a 4 hour stimulation was selected to cover early and late response genes in macrophages. Transcriptional responses were examined in treated cells and compared to that of untreated macrophages using Agilent SurePrint G3 Mouse Gene Expression Arrays (8x60K). Labelling of high quality RNA, hybridization and scanning were performed using the Agilent gene expression system. Signal intensity raw data generated by Agilent Feature Extraction software v10.7 were analyzed with GeneSpring GX 12.0.1 (Agilent Technologies). Figure 4.1 shows a schematic diagram for the experimental design of the study to identify the regulation of genes with and without MKP-2. A fold change threshold of 2 or more was used for significantly regulated probes using one-way ANOVA (p-value < 0.05). For simplicity, probes for long intergenic non-coding RNAs (lincRNAs) and genes without annotations were excluded. Pathway analysis from Pathvisio software was used to set out the groups of differently expressed genes (van Iersel *et al*., 2008) available at http://www.pathvisio.org/. Hierarchical clustering was used to classify genes that were co-expressed across samples in groups according their functional relationship based on the fold change using MeV software (Saeed et al., 2003); http://www.tm4.org/mev.html) (Figure 4.2). Gene ontology (GO) analysis was carried out by GOrilla (The Gene Ontology enrichment analysis and visualization tool) (Eden *et al*., 2009) available at http://cbl-gorilla.cs.technion.ac.il/. Gene ontology (GO) is a way to associate genes with defined biological terms or phrases to help describe aspects of a gene product's biology. Such GO associations allow researchers to make inferences about groups of genes and their possible combined role rather than investigating each one individually in isolation as

single gene analysis may miss important effects on pathways. One of the most common uses of the GO is enrichment analysis which identifies GO terms that are significantly overrepresented in a given gene list. Enrichment may suggest the functional characteristics of the given genes to aid in the assessment of the mechanisms behind the biological consequences of experimental treatments. GOrilla is a web-based software for fast identification of enriched GO terms uses the hypergeometric distribution of underlining biological relevance of genes showing altered expression (>2 fold change relative to controls) to determine the possibility of whether such genes associated with a given GO term lead to its enrichment (Eden et al., 2009). The enriched GO terms are presented in hierarchical order in biological process, cellular component or molecular function GO groups.

The following sections present the results of the microarray analysis and genes regulated by MKP-2 upon LPS and IL-4 activation.

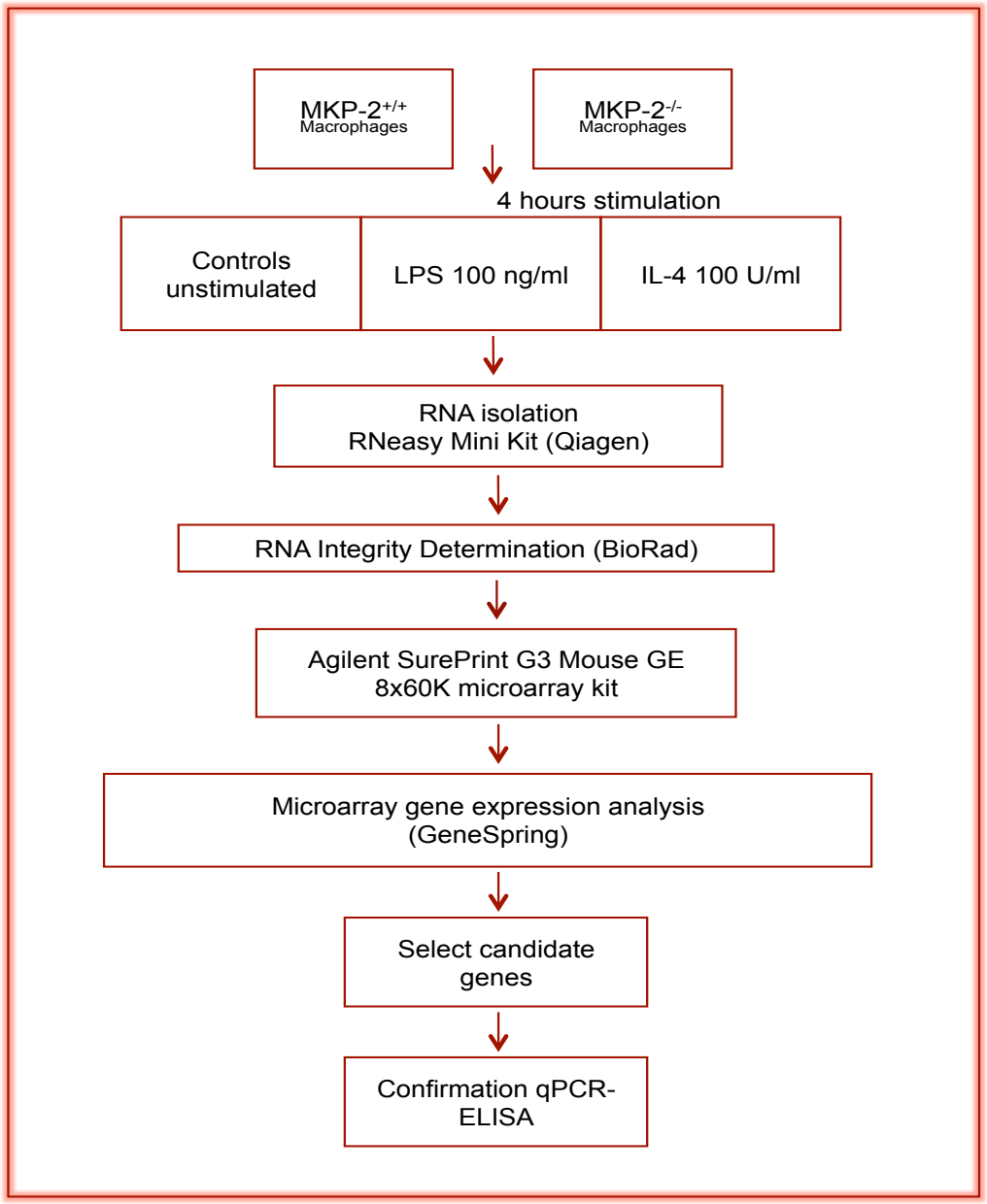


Figure 4.1: Schematic diagram showing the experimental design of the study to identify the genes regulation with/without MKP-2. Bone marrow derived macrophages ($2x10^6$) were seeded into 6-well plates. RNA was extracted from all MKP-2-WT and MKP-2-KO samples; cRNA was prepared, processed and hybridized onto the Agilent SurePrint G3 Mouse Gene Expression Arrays (8x60K) followed by the data analysis using GeneSpring software. Samples were compared to identify the differentially regulated genes. Candidate genes were selected and validated using qRT-PCR assays and ELISA.

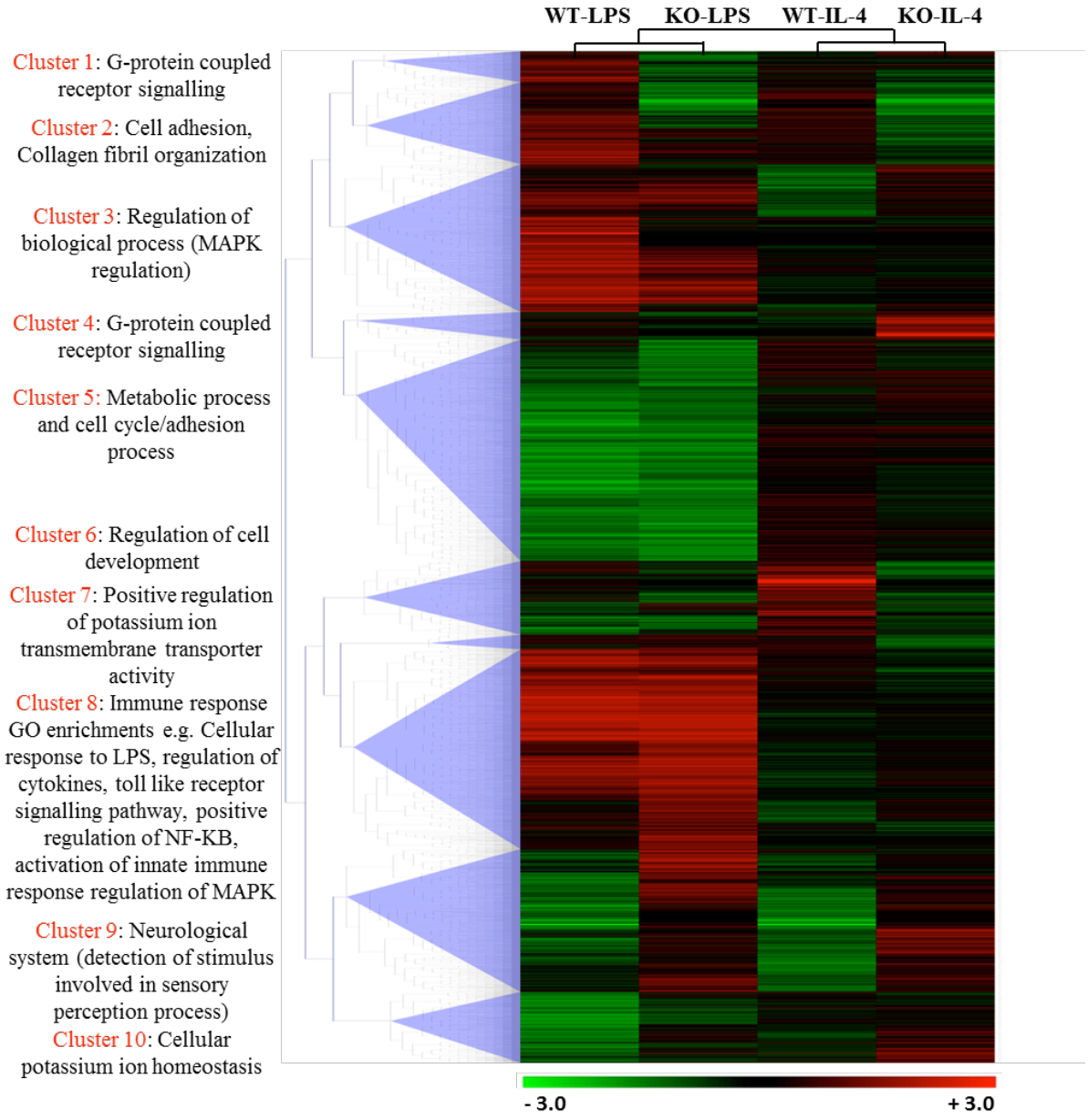


Figure 4.2: Heat maps representing hierarchical clustering of the differentially regulation, plotted using the log2 fold change values of the genes with p-value <0.05 (One-way ANOVA) between MKP-2 WT vs. MKP-2 KO. Each column represents a sample and each row in the heat map represents a gene that is differentially regulated in that particular comparison of samples. Signal intensities were normalized to the expression data of unstimulated macrophages. The colour scale represents the degree of expression of the gene, green being the downregulated (below −3.0) and red being (above +3.0) the upregulated genes in the sample sets with black as the centre of the scale at '0'.

4.2.1.1 The role of MKP-2 in the gene expression pattern of LPS-stimulated macrophages

The relative importance of MKP-2 in the macrophage response to lipopolysaccharide (LPS) was studied. LPS binds to the cell surface receptor CD14 and is then transferred to the transmembrane signalling receptor toll-like receptor 4 (TLR4) with its accessory protein MD2. This results in the induction of several intracellular signalling cascades including the MAPK pathway, which further activates a variety of transcription factors to induce genes for inflammatory mediators to support the macrophage innate immune responses. Furthermore, LPS has been shown to induce genes that are involved in cell proliferation, migration, and survival (Guha and Mackman, 2001, Aung et al., 2006).

As described in Section 4.2.1, bone marrow-derived macrophages (BMDMs) from MKP-2-WT and MKP-2-KO mice were subjected to LPS from *E. coli* (100 ng/ml) for 4 h. Expression responses were then were analysed in LPS treated cells and compared to that of untreated macrophages to assess differences in gene expression. Figure 4.2 illustrates the expression differences of significantly regulated genes in LPS-stimulated macrophages at 4 h. The LPS gene expression profiles observed in both MKP-2 WT and KO macrophages resemble previously published LPS- gene studies in mouse BMDMs (Björkbacka et al., 2004). In general, LPS-stimulated macrophages displayed changes in gene expression with thousands of genes that were induced or suppressed. A total of 3645 and 3660 entities were significantly upregulated in MKP-$2^{+/+}$ and MKP-$2^{-/-}$ respectively. An entity is a discrete feature measured by microarray analysis such as a probe for a particular transcript. LPS induced a large number cytokines, chemokines and growth factors and these were the most highly responsive gene groups in both WT and MKP-2-KO macrophages. The top 20 upregulated Agilent (8x60K) array probes in response to LPS are shown in table 4.1. It was found that the common hallmarks for LPS stimulation including *Tnf, Il12, Il6, Nos2, and Ptgs2* were all significantly induced in both MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages. This demonstrates that macrophage activation by LPS is valid in both types of cells.

The differential expression of cytokines and chemokine following to LPS stimulation was as follows: genes encoding for IL-6, IL-12β, IL-19, CSF-3, IL-1α, IL-33, CXCl1, CXCl2, CXcC3, CCL3, and CCL4 were more enhanced in MKP-$2^{+/+}$ when compared to MKP-$2^{-/-}$ counterparts. However, a slight increase was recorded for *Il1b, Ifnb1, Ifng, Cxcl10, and Ccl22* in MKP-2-deficient macrophages in relation to WT cells. There were no significant differences in *Tnf, Il10 and ll12a* between both types of cells. Fold changes are represented in tables 4.2 and 4.3 (also see appendices 6.2 and 6.3 for biological pathway analysis). The highly enriched biological processes following LPS stimulation in both MKP-2-WT and KO macrophages are related to immune function, suggesting no general difference in the immune response between both groups (Table 4.4).

Table 4.1: Top 20 up-regulated Agilent (8x60K) SurePrint G3 Mouse Gene Expression Array probe sets in by LPS

No.	Probe Name	FC-MKP-2$^{+/+}$	FC-MKP-2$^{-/-}$	Gene Symbol	Gene Name
1	A_55_P2128144	4717.09	3292.84	*Il19*	interleukin 19
2	A_51_P363187	2975.03	1604.87	*Cxcl1*	chemokine (C-X-C motif) ligand 1
3	A_51_P254855	1850.59	1746.34	*Ptgs2*	prostaglandin-endoperoxide synthase 2
4	A_52_P100926	1758.98	1369.49	*Il1α*	interleukin 1 alpha
5	A_55_P1977008	1574.15	663.96	*Gfi1*	growth factor independent 1
6	A_51_P115005	1094.3	5176.78	*Edn1*	endothelin 1
7	A_55_P1997756	769.02	742.88	*Il6*	interleukin 6
8	A_51_P217463	710.97	446.13	*Cxcl2*	chemokine (C-X-C motif) ligand 2
9	A_52_P399934	434.82	425.43	*Dusp2*	dual specificity phosphatase 2
10	A_51_P212782	423.49	494.97	*Il1β*	interleukin 1 beta
14	A_66_P109708	304.13	69.88	*Il1f6*	interleukin 1 family, member 6
11	A_55_P1971889	272.36	32.41	*F3*	coagulation factor III
12	A_52_P232813	262.54	112.79	*Cxcl3*	chemokine (C-X-C motif) ligand 3
13	A_51_P488739	233.36	150.65	*Niacr1*	niacin receptor 1
18	A_52_P409833	231.14	1.2	*Plat*	plasminogen activator, tissue
15	A_52_P550173	217.75	308.83	*Slamf1*	signaling lymphocytic activation molecule family member 1
16	A_55_P2010038	149.78	142.42	*Tnfsf9*	tumor necrosis factor (ligand) superfamily, member 9
17	A_51_P385099	133.51	135.39	*Tnf*	tumor necrosis factor
19	A_52_P482897	125.76	16.85	*Areg*	amphiregulin
20	A_51_P140710	46.82	110.86	*Ccl3*	chemokine (C-C motif) ligand 3

*A fold change of 2 or more was used for significantly regulated probes using one-way ANOVA (p-value < 0.05). Expression responses were analysed in LPS treated cells and compared to that of untreated cells. The list is sorted according to WT macrophages.

Table 4.2: List of differently expressed **cytokines** in both MKP-2$^{-/-}$ and MKP-2$^{+/+}$ bone marrow derived macrophages stimulated with LPS

Probe Name	FC-MKP-2$^{+/+}$	FC-MKP-2$^{-/-}$	Gene Symbol	Gene Name
A_55_P2017914	1.734673	2.304508	*Csf1*	colony stimulating factor 1 (macrophage)
A_51_P171075	33.78238	17.68806	*Csf2*	colony stimulating factor 2 (granulocyte-macrophage)
A_51_P317176	1260.02	248.081	*Csf3*	colony stimulating factor 3 (granulocyte)
A_51_P144180	2.298644	8.467241	*Ifnb1*	interferon beta 1, fibroblast
A_52_P68893	2.091784	6.220918	*Ifng*	interferon gamma
A_52_P100926	1758.975	1369.491	*Il1a*	interleukin 1 alpha
A_51_P212782	423.4872	494.9662	*Il1b*	interleukin 1 beta
A_51_P430766	16.03002	15.42285	*Il10*	interleukin 10
A_55_P1960436	13.91415	11.12657	*Il12a*	interleukin 12a
A_51_P385812	504.6116	370.0475	*Il12b*	interleukin 12b
A_55_P2180839	2.624529	1.061595	*Il13*	interleukin 13
A_55_P1964960	56.14	28.18	*Il33*	interleukin 33
A_52_P15461	3.279882	5.729892	*Il15*	interleukin 15
A_55_P2138386	-1.58495	-2.16077	*Il5*	interleukin 5
A_55_P1997756	769.0198	742.8751	*Il6*	interleukin 6
A_51_P385099	133.512	135.3896	*Tnf*	tumor necrosis factor

*A fold change of 2 or more was used for significantly regulated probes using one-way ANOVA (p value < 0.05). Expression responses were analysed in LPS treated cells and compared to that of untreated cells.

Table 4.3: List of differently expressed **chemokines** in both MKP-2$^{-/-}$ and MKP-2$^{+/+}$ bone marrow derived macrophages stimulated with LPS

Probe Name	FC-MKP-2$^{+/+}$	FC-MKP-2$^{-/-}$	Gene Symbol	Gene Name
A_51_P234956	5.779176	3.692485	*Xcl1*	chemokine (C motif) ligand 1
A_55_P1960386	3.816066	1.23728	*Ccl1*	chemokine (C-C motif) ligand 1
A_51_P114462	2.884584	1.910084	*Ccl17*	chemokine (C-C motif) ligand 17
A_55_P2127995	2.108601	1.58997	*Ccl19*	chemokine (C-C motif) ligand 19
A_51_P286737	2.099352	2.514401	*Ccl2*	chemokine (C-C motif) ligand 2
A_51_P327996	15.99631	20.56017	*Ccl22*	chemokine (C-C motif) ligand 22
A_51_P140710	110.865	46.82443	*Ccl3*	chemokine (C-C motif) ligand 3
A_51_P509573	25.47168	11.12756	*Ccl4*	chemokine (C-C motif) ligand 4
A_51_P436652	2.257767	2.892657	*Ccl7*	chemokine (C-C motif) ligand 7
A_51_P185660	4.17386	2.654143	*Ccl9*	chemokine (C-C motif) ligand 9
A_55_P2023542	3.462277	2.916858	*Ccr7*	chemokine (C-C motif) receptor 7
A_55_P2103097	5.068205	3.127528	*Ccr8*	chemokine (C-C motif) receptor 8
A_52_P30312	5.114318	4.798859	*Ccr9*	chemokine (C-C motif) receptor 9
A_51_P363187	2975.032	1604.875	*Cxcl1*	chemokine (C-X-C motif) ligand 1
A_55_P2016459	7.448677	37.61612	*Cxcl10*	chemokine (C-X-C motif) ligand 10
A_55_P2016462	4.049752	6.452672	*Cxcl10*	chemokine (C-X-C motif) ligand 10
A_55_P1966204	2.215787	-1.49291	*Cxcl12*	chemokine (C-X-C motif) ligand 12
A_51_P378789	5.912458	3.316522	*Cxcl13*	chemokine (C-X-C motif) ligand 13
A_51_P217463	710.9669	446.1273	*Cxcl2*	chemokine (C-X-C motif) ligand 2
A_55_P1990032	5.121231	6.725353	*Cxcl5*	chemokine (C-X-C motif) ligand 5
A_51_P319022	7.895129	1.471868	*Cxcr3*	chemokine (C-X-C motif) receptor 3
A_51_P196925	5.09714	3.691182	*Cx3cl1*	chemokine (C-X3-C motif) ligand 1

*A fold change of 2 or more was used for significantly regulated probes using one-way ANOVA (p-value < 0.05). Expression responses were analysed in LPS treated cells and compared to that of untreated cells.

Table 4.4: GO Analysis of the genes expressed between MKP-$2^{+/+}$ and MKP-$2^{+/+}$ macrophages with ≥2.0 fold change

GO Term	Description	P-value
GO:0009611	response to wounding	4.10E-14
GO:0050727	regulation of inflammatory response	1.97E-13
GO:0001817	regulation of cytokine production	2.75E-13
GO:0051239	regulation of multicellular organismal process	2.07E-12
GO:0032101	regulation of response to external stimulus	1.42E-11
GO:0048518	positive regulation of biological process	4.44E-11
GO:0023051	regulation of signalling	6.00E-11
GO:0010646	regulation of cell communication	8.51E-11
GO:0008284	positive regulation of cell proliferation	9.07E-11
GO:0050865	regulation of cell activation	1.63E-10
GO:0010941	regulation of cell death	1.32E-09
GO:0008009	chemokine activity	8.31E-08
GO:0032496	response to lipopolysaccharide	1.09E-08
GO:0001818	negative regulation of cytokine production	1.50E-08
GO:0045595	regulation of cell differentiation	7.88E-08
GO:0032680	regulation of tumor necrosis factor production	1.06E-07
GO:0042625	ATPase activity, coupled to transmembrane movement of ions	5.32E-05
GO:0017017	MAP kinase tyrosine/serine/threonine phosphatase activity	3.86E-04

***P-value** is the enrichment p-value computed according to the hypergeometric distribution model.

No obvious differences exist in GO terms obtained from Gene Ontology analysis following to LPS stimulation between the MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages. However, expression of Endothelin-1 (*Edn1*) mRNA differed significantly between LPS-stimulated MKP-2 WT and KO macrophages (1,094 vs. 5,176, respectively), with *Edn1* showing the highest fold change of any gene in the induced MKP-2 KO group. Endothelin-1 has been shown to be a potent vasoconstrictor produced from the endothelium in response to inflammation or injury (Wahl *et al.*, 2005). Furthermore, Endothelin is also produced in murine macrophages following LPS challenge and functions as an inflammatory mediator (Wahl *et al.*, 2005).

Interestingly, another gene, *Gfi1*, was also differently expressed in MKP-2 WT versus KO LPS-induced macrophages (1,574.1 vs. 663.9, respectively). Gfi-1 is a transcriptional repressor that regulates the Toll-like receptor (TLR) inflammatory response by antagonising the NF-κB pathway (Sharif-Askari *et al.*, 2010). In addition, the gene encoding CD14 was also upregulated in MKP-$2^{+/+}$ over MKP-$2^{-/-}$ macrophages (27.9 vs. 10.1, respectively). CD14 is a surface antigen that is expressed on monocytes and macrophages to mediate the innate immune response to bacterial lipopolysaccharide. Interestingly, the *Mmd* and *Csf2* genes were upregulated also following LPS stimulation by 33.7- and 24.5- fold for WT, respectively vs. 17.6 and 7.8 for KO, respectively. Up-regulation of both genes in WT over KO macrophages in response to M-CSF stimulation has been examined in chapter 3, which further confirms the data obtained here from LPS treatment.

The induction of the *Dusp4/Mkp2* gene by LPS was upregulated to 13.51 fold in MKP-$2^{+/+}$ macrophages, indicating that MKP-2 is induced under innate conditions. However in MKP-2-deficient macrophages in which signal intensities were normalized to the expression data of unstimulated macrophages, a 3.58-fold upregulation of *Dusp4/Mkp2* expression was found. This was a surprising find considering the knockout of the MKP-2 gene in these mice. This may be due to the binding of A_51_P518051 DUSP4 probe to the 3'UTR of mDUSP4 (NM_176933), which, due to the MKP-2 knockout strategy used,

might still remain and be expressed in a truncated form. Also of note is the induction of other dual-specificity phosphatases (DUSPs) upon MKP-2 loss. *Dusp2* was one of the most enhanced genes in both groups by LPS albeit showing no significant difference in mRNA expression between them (434.8 (WT) vs. 425.4 (KO)). Other DUSPs including *Dusp16, Dusp1 and Dusp5*, were more upregulated in WT macrophages; whereas *DUSP27* was the most downregulated. The rest of typical and atypical *DUSPs* did not reach a significantly altered expression in both types of macrophages when compared to the corresponding untreated cells (Table 4.5).

It is important to mention that 1652 genes were exclusively upregulated in WT macrophages by LPS stimulation. From this list, *Gata2* and *Lif* were induced in WT macrophages (FC, Gata2 = 246.7, Lif = 12.8) and are enriched for the Gene Ontology (GO) terms related to the regulation of macrophage differentiation. GO analysis of the MKP-2$^{-/-}$ macrophage upregulated gene list did not find any significant enrichment in any GO terms.

Table 4.5: List of differently expressed **DUSPs** in both MKP-2$^{-/-}$ and MKP-2$^{+/+}$ bone marrow derived macrophages stimulated with LPS

Probe Name	FC-MKP-2$^{+/+}$	FC-MKP-2$^{-/-}$	Gene Symbol
A_52_P399934	434.82	425.43	*Dusp2*
A_66_P116252	24.37	9.63	*Dusp16*
A_55_P2063216	5.77	4.37	*Dusp14*
A_55_P2063237	15.96	3.78	*Dusp5*
A_51_P518051	13.51	3.58	*Dusp4*
A_51_P288618	2.38	3.39	*Dusp11*
A_51_P430900	7.03	3.28	*Dusp1*
A_55_P1993836	2.78	1.86	*Dusp7*
A_51_P235088	-13.01	1.64	*Dusp27*
A_51_P502614	3.22	1.09	*Dusp6*
A_55_P2071092	-2.1	1.06	*Dusp22*
A_51_P259029	3.66	1.01	*Dusp26*
A_55_P2030638	-2.53	-1.53	*Dusp23*
A_51_P104418	-2	-1.96	*Dusp10*
A_55_P2063251	-2.21	-2	*Dusp3*
A_55_P2436438	-2.36	-2.06	*Dusp22*
A_55_P2028661	-1.64	-2.08	*Dusp12*
A_55_P2036171	-2.64	-2.16	*Dusp13*
A_66_P119155	-3.14	-2.67	*Dusp3*
A_52_P648601	-2.81	-3.14	*Dusp9*
A_51_P362738	-8.08	-7.86	*Dusp18*
A_52_P11618	-2.29	-7.88	*Dusp19*

*A fold change of 2 or more was used for significantly regulated probes using one-way ANOVA (p-value < 0.05). Expression responses were analysed in LPS treated cells and compared to that of untreated cells.

Since genes can be either induced or repressed by LPS treatment, the final comparison was to identify genes that were repressed by LPS treatment. There were no significant differences in GO terms obtained from the downregulated genes of both MKP-2 WT and KO macrophages (3608 and 3208 repressed probes respectively.

These above findings show that there were no major differences in the expression of cytokines and chemokines following to LPS treatment between MKP-2 WT and KO macrophages, suggesting a comparable proinflammatory response from both types of macrophages. However, the microarray data delivers candidate genes such as *Csf2*, *Mmd*, *Gfi1, Edn1* and *CD14* which may help to further explain the delayed growth properties of MKP-$2^{-/-}$ macrophages and the importance of MKP-2 in regulating the innate immune response to infections. To investigate the role of MKP-2 in the macrophage anti-inflammatory response, microarray gene expression analysis of MKP-$2^{-/-}$ and MKP-$2^{+/+}$ macrophages stimulated with IL-4 is studied in the following section.

4.2.1.2 The role of MKP-2 in the gene expression pattern of IL-4-stimulated macrophages

The previous section examined gene expression profile of MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages activated by bacterial LPS. This macrophage population is characterised by the high induction of pro-inflammatory cytokines/chemokines that are essential for innate immune response. However, this antimicrobial property can cause tissue damage (Murray and Wynn, 2011, Mills, 2012). The purpose here is to examine the role of MKP-2 in the differentiation of macrophages toward the anti-inflammatory type-2 phenotype. The activities from this type of macrophages suppress the existing inflammation and promotes matrix remodelling and tissue repair in response to the Th2 cytokine IL-4 (Murray and Wynn, 2011b, Mills, 2012). To determine genes associated with alternative activation in macrophages in the presence and absence of MKP-2, macrophages from MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were stimulated with IL-4 (100 U/ml) for 4 h. Total RNA was extracted; cRNA was prepared and hybridised onto the SurePrint G3 Mouse Gene Expression Array (8x60K, Agilent). Hierarchical clustering assessed similarities in gene expression patterns (Figure 4.2).

The IL-4 gene expression profile in mouse BMDMs was studied earlier by (Shuyi *et al.*, 2010). However, data from previously published work were also used for comparison as a source for markers of alternatively activated macrophages (Loke *et al.*, 2002, Raes *et al.,* 2002). Generally, analysis of the genes differently expressed showed that IL-4 induced a distinct activation pattern in macrophages compared to LPS. Only a small number of genes in this category were found to be significantly expressed. The intensity of 1574 and 1636 entities were increased by IL-4 in MKP-$2^{+/+}$ and MKP-$2^{-/-}$ respectively. Many of these were unclassified genes or have unknown functions. Gene ontology enrichment analysis of the expression profile of this group did not help provide a possible explanation or mechanism of this IL-4 macrophage activation. For this reason, the focus was on examining the expression pattern of commonly associated genes with IL-4-activation of MKP-2 WT and KO macrophages.

A significant increase in the expression of the well-known alternative activation markers was observed in WT macrophages including *Arg1, Chi3l3/Ym1 and Renlta/Fizz1*. This demonstrates that cell activation by IL-4 was valid in WT macrophages. However, deletion of MKP-2 caused significant alterations in the expression of these markers. One of the most abundant genes following IL-4 activation is the *Chi3l3* gene, a common alternative activation marker also known as *Ym1*. Interestingly, it was upregulated by 242.28-fold in WT macrophages vs 22.59-fold in KO macrophages. The induction of another marker, Resistin like molecule alpha *(Retnla)* (also called *Fizz1 or Relmα*), was also significantly reduced following MKP-2 gene deletion (FC, WT = 77.06 vs. KO = 11.79). Both genes have been associated with type 2 (Th2)-mediated immunity and have been proposed to participate in the wound healing process initiated by Th2-activated macrophages (Nair *et al.*, 2005). In addition, Arginase-1*(Arg1)* was significantly upregulated in MKP-$2^{-/-}$ macrophages compared with WT macrophages (FC, KO=69.58 vs WT=36.84). The upregulation of Arg1 expression has been shown to have a harmful role in parasitic infection by competing with iNOS for the same substrate L-arginine. This can prevent nitric oxide release which promotes parasite proliferation (Stempin *et al.*, 2010). The top 20 upregulated Agilent (8x60K) array probes in WT and KO IL-4-treated macrophages are shown in Table 4.6.

The expression of other genes associated with alternative activated macrophage populations was also assessed. CCL17 was found to be induced in mice primary macrophages by IL-4 and attracted macrophages and T cells (Liddiard *et al.*, 2006). IL-4 was also able to induce IL-27Rα chain (WSX-1) in bone marrow macrophages (Rückerl *et al.*, 2006). However, in the current study, both, *Ccl17* and *IL-27Rα* were not induced. Macrophage-derived insulin-like growth factor-I (IGF-I) was found to promote lung disease and pulmonary fibrosis is also induced by IL-4 (Wynes and Riches, 2003). IGF-I was only upregulated in WT macrophages up to a FC of 2.8. In addition, the expression of mannose receptor *(Mrc1)* was upregulated significantly in BMDMs at 6 and 24 h following IL-4 stimulation in a work demonstrated by Menzies et al., (2011). Here, the

induction of *Mrc1* was not significantly altered in the absence of MKP-2, although clearly induced by IL-4 with a FC of 7.7 and 9.2 for WT and MKP-$2^{-/-}$ macrophages, respectively. The suppressor of cytokine signaling-1 (*Socs1*) gene was also reported to be induced by IL-4 in macrophages {Dickensheets *et al*., 2006, Briken and Mosser 2011). The expression of this gene was different between MKP-2 WT and KO macrophages with ((FC, WT = 19.9 vs. KO = 25.9).

Table 4.6: Top 20 up-regulated Agilent (6x80K) SurePrint G3 Mouse Gene Expression Array probe sets by IL-4

No.	Probe Name	FC MKP-2$^{+/+}$	FC MKP-2$^{-/-}$	Gene Symbol	Gene Name
1	A_51_P462192	315.4	105.24	Olr1	oxidized low density lipoprotein (lectin-like) receptor 1
2	A_55_P2022743	295.36	1.15	Tenm3	teneurin transmembrane protein 3
3	A_51_P167292	242.28	22.6	Chi3l3	chitinase 3-like 3
4	A_51_P459012	146.97	1.17	Zswim2	zinc finger SWIM-type containing 2
5	A_55_P2038854	132.29	1.28	Gareml	GRB2 associated, regulator of MAPK1-like
6	A_55_P2166874	117.64	1.16	Hbb-bh2	hemoglobin beta, bh2
7	A_55_P1990116	105.4	1.15	Snrpn	small nuclear ribonucleoprotein N
8	A_52_P232802	96.48	-4.36	Tbx15	T-box 15
9	A_55_P2308239	92.66	1.15	Ccr9	chemokine (C-C motif) receptor 9
10	A_51_P452576	63.57	140.94	Zfp459	zinc finger protein 459
11	A_52_P388836	78.48	-1.76	Sec16b	SEC16 homolog B (S. cerevisiae)
12	A_51_P257951	77.07	11.79	Retnla	resistin like alpha
13	A_55_P2236291	70.98	11.79	Ppap2a	phosphatidic acid phosphatase type 2A
14	A_51_P197274	65.59	5.39	Olfr393	olfactory receptor 393
15	A_55_P2033362	56.46	27.13	Egr2	early growth response 2
16	A_55_P1986681	51.64	n/e	Olfr619	olfactory receptor 619
17	A_55_P2052563	43.58	5.4	Id1	inhibitor of DNA binding 1
18	A_55_P2187141	40.36	69.16	Pdcd1lg2	programmed cell death 1 ligand 2
19	A_51_P137336	39.80	21.25	Cdh1	cadherin 1
20	A_51_P303160	36.84	69.58	Arg1	arginase, liver

*A fold change of 2 or more was used for significantly regulated probes using one-way ANOVA (p-value < 0.05). Expression responses were analysed in IL-4 treated cells and compared to that of untreated cells. The list is sorted according to WT macrophages.

Similar to the induction of the *Dusp4/Mkp-2* gene by LPS, the expression of *MKP-2* was upregulated by 34.9 fold in MKP-$2^{+/+}$ macrophages whereas a fold change of only 1.1 (<2) was detected with MKP-2 deficient cells. This suggests the ability of IL-4 to induce MKP-2 and highlight the prominent role of MKP-2 in alternative activation. Also, it further confirms the lack of MKP-2 in MKP-$2^{-/-}$ mice. A non-significant difference in the upregulation of *DUSP27* was observed between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages (15.2 vs. 16.7, respectively). Also the induction of *DUSP18* and *DUSP9* did not reach the significant upregulation after IL-4 stimulation in both cell types (FC<2). Other typical and atypical DUSPs were not induced.

Gene ontology analysis of 2300 entities exhibiting downregulation following IL-4 activation of WT bone-marrow-derived macrophages showed significant enrichment in osteoblast differentiation GO biological process, including growth differentiation factor 2 (*Gdf2)* and bone morphogenetic proteins (*Bmp* 3, 4, and 7). In MKP-$2^{-/-}$ macrophages, IL-4 caused a downregulation of 1509 entities. Gene ontology analysis revealed downregulation in genes associated with collagen fibril organisation e.g. collagen type III alpha 1 (*Col3a1*), collagen type I alpha 2 (*Col1a2*), collagen type I alpha 1 (*Col1a1*) and cell adhesion e.g. periostin (*Postn).*

The above data suggest that MKP-2 has an important role in the differentiation of macrophages towards an alternatively activated phenotype with IL-4. Deletion of MKP-2 reduced the transcription of a number of genes associated with alternative macrophage activation, such as *Ym1* and *Fizz1*, but increased the transcription of *Arg1*. Since *Ym1* and *Fizz1* have been shown to be involved in wound healing while *Arg1* exacerbated parasitic infection (Nair *et al.*, 2005), the data obtained here are in line with previous observations that increased levels of arginase-1 expression in MKP-2 deficient mice drastically increased susceptibility to intracellular parasites *Leishmania mexicana* and *Toxoplasma gondii* (Al-Mutairi et al., 2010; Woods et al., 2013). Consequently, and based on our analysis, *ym1* and *fizz1* were selected for validation using the qRT-PCR assays.

4.2.2 Validation of microarray results by qRT-PCR

Since microarray data suggested differential expression of alternative activation markers *Ym1* and *Fizz1*, these interesting results needed to be validated by another method. Expression levels of the selected candidate genes (*Ym1* and *Fizz1*) were verified using qRT-PCR. Bone marrow macrophages from MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice were treated with 100 U/ml IL-4 over a time course of 24 h. Quantitative RT-PCR was performed to visualize any changes in gene expression. This was performed using primers specific for each gene. The samples were analysed in duplicate. The results were normalised using the reference gene *QARS* and gene expression changes determined using the ΔΔCT method. Values are expressed as relative FC of stimulated over control (untreated samples) and reference genes were evaluated as described in methods section 2.2.6.

As shown in Figure 4.3, IL-4 stimulation induced an increase in macrophage *Ym1* expression at 4 h, which subsequently increased and was still maintained at 24 h. An exciting finding is that MKP-2 deficiency resulted in a reduced expression of Ym1 mRNA during the same time course, reaching a significant difference at 8 h ($p<0.05$).

In comparison, IL-4 induced the maximum level of *Fizz1* expression after 12 h of stimulation and mRNA levels subsequently declined at 24 h (Figure 4.4). Similar to *Ym1*, in MKP-$2^{-/-}$ macrophages, the expression of *Fizz1* was diminished during the same time span to reach a significant difference at 24 h ($p<0.05$) (Figure 4.4).

The above findings demonstrated that IL-4-induced *Ym1* and *Fizz1* expression was drastically reduced in MKP-2-deficient bone marrow derived macrophages in comparison to WT macrophages, thus confirming the microarray data. The effect of MKP-2 deletion on the protein level of both markers was studied, as described in the following section.

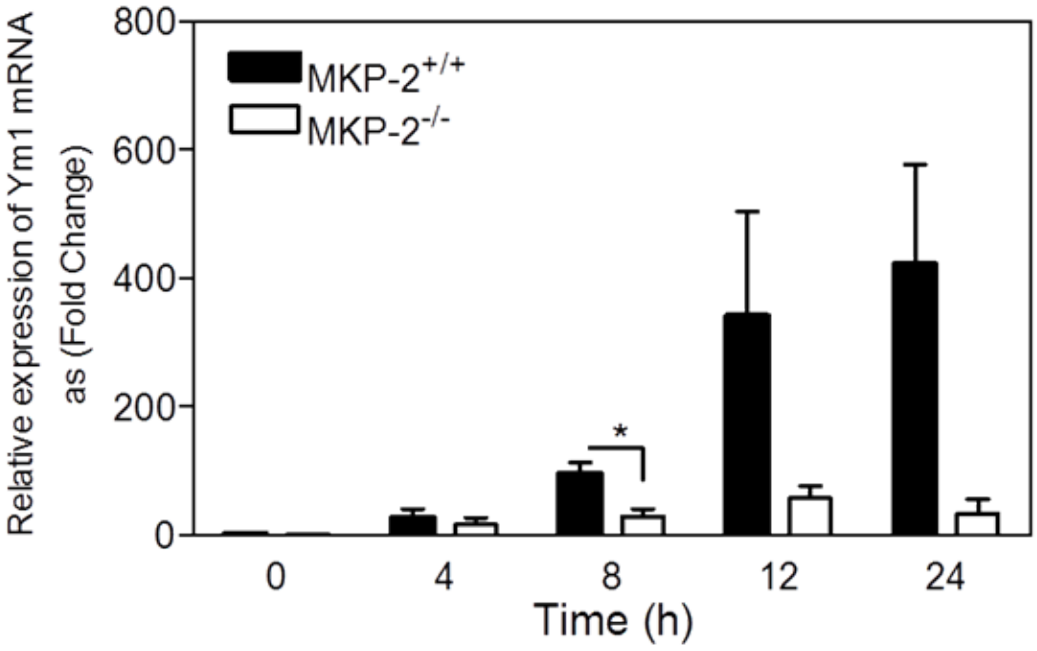


Figure 4.3: MKP-2 deletion negatively regulated the expression of alternative activation marker *Chil3l (Ym1)* in bone marrow-derived macrophages. Macrophages were harvested, seeded into 6-well plates ($2x10^6$) and then stimulated with IL-4 (100 U/ml) for the indicated periods of time. Control cells (0 h) were left untreated. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on the cDNA using primers designed to detect *Ym1* as described in methods section 2.2.5.4. Relative expression levels of *Ym1* mRNA transcripts were normalized to the reference gene *QARS* using the delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean±SEM from three individual experiments. **P*<0.05, two-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

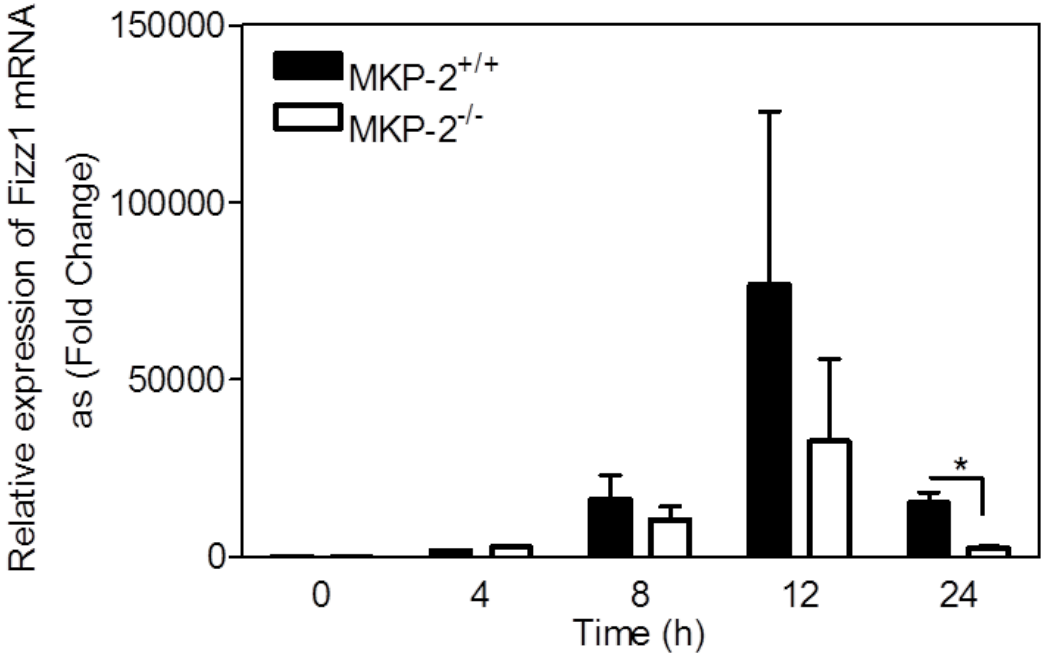


Figure 4.4: MKP-2 deletion negatively regulated the expression of alternative activation marker *Retnla (Fizz1)* in bone marrow-derived macrophages. Macrophages were harvested, seeded to 6 well plate ($2x10^6$) and then stimulated with IL-4 (100 U/ml) for the indicated periods of time. Control cells (0) were left untreated. Total RNA was prepared from the cells. After reverse transcription, quantitative PCR analysis was performed on the cDNA using primers designed to detect *Fizz1* as described in methods section 2.2.5.4. Relative expression levels of *Fizz1* mRNA transcripts were normalized to the reference gene *QARS* using the delta-delta Ct method (Livak and Schmittgen, 2001). Error bars represent the mean±SEM from three individual experiments. **P*<0.05, two-tailed unpaired t test comparing MKP-$2^{-/-}$ to MKP-$2^{+/+}$ macrophages.

4.2.3 Detection of Fizz1 and Ym1 protein expression using ELISA

Having established that mRNA levels for both *Fizz1* and *Ym1* were drastically reduced in MKP-2$^{-/-}$ compared with MKP-2$^{+/+}$ macrophages, ELISA assays were performed in order to confirm the effect on protein level. Macrophages were stimulated with IL-4 over 24 and 48 h and supernatants were collected to examine the presence of either protein. As shown in Figure 4.5, in WT macrophages, IL-4-induced Fizz1 protein was detected at 24 h and further increased at 48 h. Protein expression could not be detected before 24 h. Similar to qRT-PCR data, IL-4-induced Fizz1 expression was reduced at both 24 and 48 h (% reduction=83.4 % and 39.27 % at 24 and 48 h, respectively) in MKP-2 deficient macrophages.

The ELISA assay for Ym1 expression showed low absorbance values. As outlined in methods section 2.2.8 a standard curve was prepared from recombinant murine Ym1 in doubling dilutions from 20 ng/ml- 387.5 pg/ml. The absorbance values of the four first standards were only detected. This ELISA assay needs further optimisation that could not be performed due to time limitations. However, at 48 h after IL-4 stimulation, MKP-2$^{+/+}$ macrophages showed an absorbance value of 0.046 (optical density [OD] value between standard no. 3 and 4), whereas MKP-2$^{-/-}$ macrophages showed an undetectable absorbance value at the same time point. Regardless of the limitation of this assay, these results might indicate a reduced Ym1 protein expression in MKP-2$^{-/-}$ macrophages in relation MKP-2$^{-/-}$ counterparts.

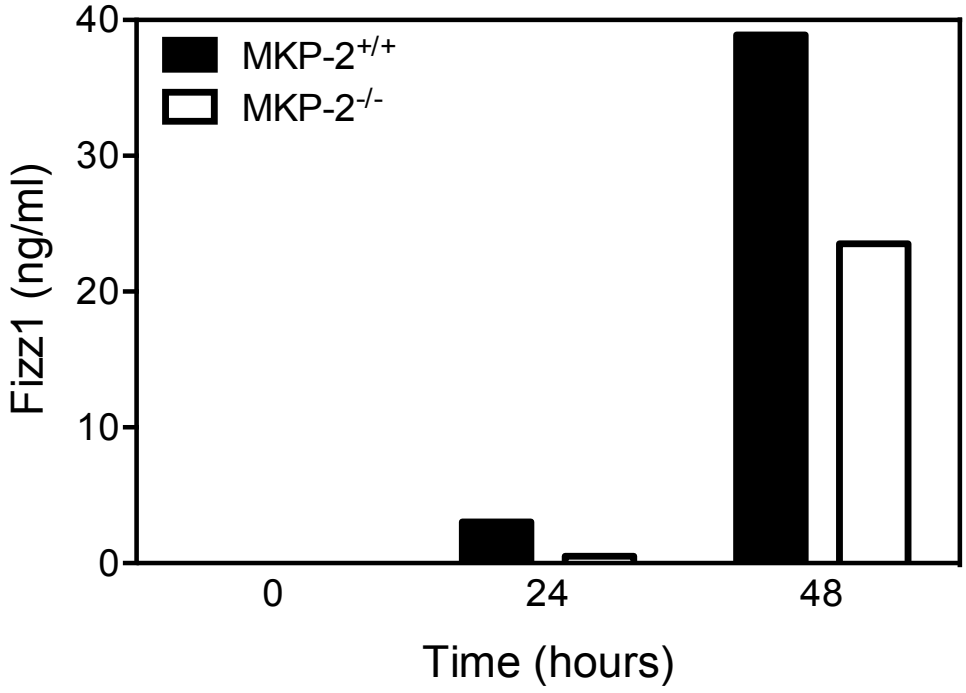


Figure 4.5: Expression level of Retnla (Fizz1) protein by ELISA. Mature macrophages were harvested, seeded into a 24-well plate to ($2x10^5$/well) and then stimulated with IL-4 (100 U/ml) for the indicated periods of time. Control cells (0) were left untreated. Supernatants were collected and Fizz1 protein level assessed by ELISA as outlined in methods section 2.2.8. The graph represents the data from two individual experiments.

4.3 Discussion

Emerging literature has established the important role of MKP-2 in macrophage inflammatory responses through the regulation of the MAPK pathway in order to influence pro- and anti-inflammatory cytokine production. MKP-2 was found to be a negative key regulator in macrophages following parasitic infections such as *Leishmania mexicana* and *Toxoplasma gondii* (Al-Mutairi *et al.*, 2010c, Woods *et al.*, 2013). On the other hand, two additional publications suggested that altering MKP-2 activity might have therapeutic potential to reduce inflammatory responses in sepsis and acute lung injury (ALI) (Cornell *et al.*, 2010c, Cornell *et al.*, 2012).

In this chapter, the importance of MKP-2 in regulating the gene expression of bone marrow macrophages was investigated. The chapter describes a transcriptome survey of genes involved in MKP-2 pathways. The focus was on dissecting the influence of MKP-2 knockout on the expression of genes in macrophages after LPS and IL-4 stimulation. This is to determine gene regulation related to pro- and anti-inflammatory immune responses in macrophages.

The initial comparison was of genes expressed in the presence and absence of MKP-2 in innate activation using LPS. It was not surprising that LPS causes a gene expression profile characterised by substantial induction of cytokines, chemokines and other inflammatory mediators (almost 8% of the genes probed in this experiment) (Tables 4.1-4.3). This is consistent with previously published LPS-induced gene programs in murine macrophages (Björkbacka *et al.*, 2004, Shuyi *et al.*, 2010). However, the number of probes showing altered intensity following induction LPS in the current study (3645 probes) was more than that identified by Björkbacka et al. (2004). Only 1,055 genes of the 13,369 genes represented on the array were differently expressed following LPS stimulation with a fold change $\geq \pm 1$; $P < 0.01$ (7.89% of the genes surveyed in this study) (Björkbacka *et al.*, 2004). An LPS concentration of 10 ng/ml was used by Björkbacka *et al*., (2004) which might explain the differences in probe list numbers. Other possible reason is LPS incubation time (4 h in this work vs. 2 h in Björkbacka's).

The predictable upregulation of cytokines by LPS was mostly reduced in MKP-2$^{-/-}$ macrophages e.g. *Il6, Il9, Il1 and Il12b* but some genes showed enhancement e.g. *Ifng, Ifnb1 and Il1b*. The attenuation in the inflammatory response was also recorded by Cornell et al., (2010), as intraperitoneal LPS injection in the MKP-2$^{-/-}$ mice caused a significant reduction in serum levels of IL-1β, IL-6, and TNF-α at 8 h. This reduction could be due to the decrease in the induction of *CD14* gene in MKP-2$^{-/-}$ macrophages (FC=10.1) in relation to MKP-2$^{+/+}$ counterparts (FC=27.9). As CD14 is critical for the recognition of LPS by macrophages to initiate the innate immunity and release of cytokines and chemokines, the decrease in *CD14* upregulation by MKP-2$^{-/-}$ macrophages could explain the attenuation in the inflammatory response in these macrophages. Levels of TNF-α and anti-inflammatory IL-10 were not significantly altered upon LPS treatment between MKP-2$^{+/+}$ and MKP-2$^{-/-}$ macrophages although both were significantly less in the MKP-2$^{-/-}$ mice after LPS injection in (Cornell *et al.*, 2010). This difference might relate to the stimulation time point selected because Cornell et al. (2010) measured late TNF-α and IL-10 production (at 24 h), while both were investigated here as early as 4 h. At the later time point, Al-Mutairi et al. (2010) reported an increased LPS-mediated induction of IL-6 at 24 h and also IL-12β and TNF-α at > 24 h in MKP-2$^{-/-}$ macrophages (Al-Mutairi *et al.*, 2010). Consistent with another study (Cornell *et al.*, 2012), TNF-α was also enhanced upon MKP-2 deletion at 24 h. The variability in cytokine production may be caused by a number of factors including the mechanism of *DUSP* transcriptional control, the complexity generated by the positive- and negative-feedback regulation of the MAP kinases and cross-regulation between different DUSPs. For standarisation, the effects of LPS require an accurate comparison among the different experimental systems. However, this does not conflict with the fact that MKP-2 is a critical component regulating macrophage inflammatory responses.

In addition, the microarray data revealed a proportional enhancement with *Dusp4* of Type I nuclear MKPs *(Dusp1, Dusp2,* and *Dusp5)* upon LPS stimulation and also the Type III MKP, *Dusp16* (Table 4.5). Deletion of DUSP4 diminished the upregulation *Dusp1,*

Dusp5, and *Dusp16,* while it has no effect on the induction of *Dusp2*. A study by Jeffrey et al. (2006) examined the expression of *Dusp* genes using Affymetrix GeneChip microarrays in LPS-activated human leukocytes including macrophages. In that study, there was a strong correlation between nuclear MKP *Dusp1, Dusp2, Dusp4, and Dusp5* with high levels of induction among other DUSPs. Furthermore, *Dusp2* was one of the most highly induced genes and was also the highest induced DUSP in our data (Table 4.5). This highlights the importance of nuclear DUSPs to act cooperatively to regulate immunological responses in leukocytes. However, similar to our data, in primary mouse macrophages stimulated with LPS, *Dusp1, Dusp2, and Dusp16* were the most strongly induced MKPs; suggesting a substantial interference of DUSP induction in activated human and mouse cells (Hammer *et al.*, 2005, Lang *et al.*, 2006b).

A recent review has also described DUSPs regulation of TLR signalling (Arthur and Ley, 2013). Following MAPK activation by TLRs, ERK1, ERK2 and p38α stimulate the expression of the *Dusp1, Dusp2* and *Dusp4* genes. DUSP4 dephosphorylates ERK1 and ERK2; DUSP2 targets JNKs and DUSP1 inactivates p38α and JNK. Also, DUSP16 limits JNK activity in macrophages infected with *Mycobacterium tuberculosis* and DUSP10 inactivates p38α in neutrophils stimulated by C5a (Arthur and Ley, 2013). As outlined in the current work, DUSP4 deficiency enhanced ERK1/2, which further augments the expression of DUSP2. Therefore, no significant differences were obtained in the expression of DUSP2 upon LPS between MKP-2 WT and KO macrophages.

The microarray data further shows that *Edn1* gene to be one the most highly induced genes in response to LPS in both WT and MKP-2 KO macrophages. However, lack of MKP-2 caused a massive upregulaton of this gene when compared to WT cells (Table 4.1). Edn-1 was initially described as a potent vasoconstrictor secreted from vascular smooth muscle cells upon inflammation to regulate vascular tone (Kedzierski and Yanagisawa, 2001, Wahl *et al.*, 2005). However, Edn-1 can also be secreted from a variety of cells, such as monocytes and macrophages, under normal and pathological conditions (Wanecek *et al.*, 2000, Kedzierski and Yanagisawa, 2001). Endothelin has

been reported to be involved in the pathogenesis of numerous diseases and particularly in sepsis (Wanecek *et al.*, 2000). Studies on transgenic mice discovered that the over expression of Edn-1 is associated with pulmonary fibrosis, chronic lung inflammation, and chronic kidney inflammation with increased recruitment of tissue lymphocytes and macrophages (Hocher *et al.*, 2000, Hocher *et al.*, 2004). These data confirms that macrophages are an important cell for the production of Edn-1 as a proinflammatory mediator in response to LPS. The production of *End1* mRNA by murine BMDMs was time and dose dependant with maximum expression at 6 h and LPS concentrations of 100 ng/ml (Wahl *et al.*, 2005). Pharmacological inhibition of (NFκB) suppressed LPS-induced Edn-1 production suggesting the importance of this signalling cascade on macrophage Edn-1 production (Wahl *et al.*, 2005). However, the abundant expression of End-1 in both MKP-2 WT and KO macrophages (Table 4.3) indicates that Edn-1 production can also be mediated through the MAPK pathway. More importantly in this chapter is the enhancement of *Edn1* expression upon MKP-2 deletion, as MKP-2 limits the activation of the MAPK pathway. As an inflammatory mediator, the massive upregulation of *Edn1* in MKP-$2^{-/-}$ macrophages could also explain the elevated inflammation challenge of MKP-$2^{-/-}$ mice and their susceptibility toward *Leishmenia mexicana* compared to WT counterparts (Al-Mutairi *et al.*, 2010c).

Furthermore, one of the highest induced genes that was affected by MKP-2 deletion is *Gfi1* (FC, WT = 1574.1 vs. KO = 663.9) (Table 4.3). Gfi1 was found to be essential to control the proliferation and maintain the functional integrity of haematopoietic stem cells (HSC) (Hock *et al.*, 2004). Studies analysing the expression of *Gfi1* in primary bone marrow-derived macrophages upon LPS suggested a Gfi1 as a negative regulator of TLR4-initiated innate immune response through antagonising the p65-RelA NF-κB subunit. *Gfi*$^{-/-}$ macrophages exhibit a high induction of p65 target genes including TNF-α (Jin *et al.*, 2006, Sharif-Askari *et al.*, 2010). Shinnakasu and others identified *Gfi1* as playing an important role in Th2 cell differentiation following Ras-ERK MAPK cascade activation (Shinnakasu *et al.*, 2008). This highlights the importance of ERK activation the subsequent induction of Gfi1 for Th2 differentiation. The differential expression of Gfi1

between MKP-2 WT and KO macrophages could be linked with the preference versus the reduction in Th2 markers, which obtained later on from IL-4 activation data.

On the other hand, the induction of both *Lif* and *Gata2* gene expression in response to LPS was abolished in the absence of MKP-2. Leukaemia inhibitory factor (*Lif*) encodes a cytokine, which was reported to induce the differentiation of a murine monocytic cell line (Hilton et al., 1988). Lif was found to facilitate monocyte differentiation and enhance the expression of tissue factor (TF) procoagulant activity in human monocyte-derived macrophages (MDMs) (Hilton *et al.*, 1988, Meisel *et al.*, 1999). Lif has also been reported to inhibit the differentiation of embryonic stem cells and promote the survival and/or proliferation of primitive haematopoietic precursors (Escary *et al.*, 1993). Agca *et al.*, (2013) showed that TNF-α was able to induce *Lif* expression in Muller cells *in vitro* and this was dependent on p38 MAPK since inhibition of its p38 abolished *Lif* expression *in vitro* and *in vivo*. Gata2 belongs to a family of transcriptional regulators that function during critical steps in haematopoietic lineages (Burch, 2005). Gata2 was also shown to be involved in macrophage differentiation. Exposure of primary macrophages to LPS significantly increased the transactivation activities of Gata2 via TLR4, MyD88, and MAPKs and further IL-1β mRNA and protein expression (Wu *et al.*, 2013). The involvement of both *Lif* and *Gata2* in the macrophage differentiation process may further explain the differences in developmental stage between MKP-2 WT and KO macrophages.

Overall, the data acquired from LPS-treated macrophages proposed novel genes regulated by MKP-2 to influence macrophage development and immune function. Validation of these genes using qRT-PCR assays is required to further discover the important role of MKP-2 in these processes.

The second comparison from microarray experiment was of genes expressed in the presence and absence of MKP-2 in alternatively activated macrophages (AAM) activated by the Th2 cytokine IL-4. The protective role of this macrophage population in Th2-mediated immunity in parasitic infections has been extensively studied (Martinez *et al.*,

2009, Gordon and Martinez, 2010, Hölscher *et al.*, 2006, Reece *et al.*, 2006). This macrophage population promotes the repair of tissue damage caused by the classical inflammatory response by inducing AAM markers, including Arg1, Fizz1, and Ym1 (Gordon and Martinez, 2010).

Arginase-1 is a classical well-known marker for alternative activation. Several reports demonstrated the participation of arginase in parasitic infections, e.g. *Trypanosoma* and *Leishmania infection* (Iniesta *et al.*, 2001, Stempin and Cerban, 2007). Alternatively activated macrophages by *Trypanosoma cruzi* (Stempin and Cerban, 2007), *Schistosoma mansoni* (Abdallahi *et al.*, 2001) and helminth or protozoan parasites (Stempin *et al.*, 2010) fail to kill these pathogens due to arginase induction. Since arginase-1 induces and turns arginine metabolism from NO production via iNOS toward production of L-ornithine, this promotes parasite survival and proliferation (Mosser and Edwards, 2008, Kreider *et al.*, 2007, Hesse *et al.*, 2001).

Ym1, or Chi3l3, is a member of the chitinase-like molecules that was suggested to degrade the chitin available on the surface of some fungi, insects, and parasites. However, Ym1 lacks chitinase activity (Bleau *et al.*, 1999, Martinez *et al.*, 2009). Alternatively, Ym1 has been identified as an animal lectin that binds to saccharides such as glycans and heparin on the cell surface and the extracellular matrix to mediate matrix organisation and tissue repair, as defence mechanisms against infections (Sun *et al.*, 2001, Chang *et al.*, 2001). Ym1 was suggested to play a role in the degradation of *Schistosoma mansoni* eggs, which express a number of oligosaccharides with amine groups (Sandler *et al.*, 2003). Also, Ym1 was found to be an eosinophil chemotactic factor (Owhashi *et al.*, 2000, Falcone *et al.*, 2001). However, due to its substantial production by macrophages and its chitin-binding ability, it has been proposed that eosinophil chemotaxis is not its primary function. Furthermore, Ym1 has been implicated in lung inflammation as a secretory lectin that forms crystals within the alveolar spaces (Feldmesser *et al.*, 2001, Guo *et al.*, 2000).

The function of Fizz1 (also called Retnla) was first reported by Holcomb et al (2000), who observed abundant secretion of Fizz1 protein in the bronchoalveolar lavage fluid of asthmatic mice. Moreover, a recent study showed that Fizz1 was involved in mediating the deposition of extracellular matrix in bleomycin-induced pulmonary fibrosis (Liu *et al.*, 2004).

Global mRNA expression analysis revealed differential expression of the above mentioned three commonly used mouse AAM indicators, *Arg1*, *Ym1*, and *Fizz1* (Table 4.6). MKP-$2^{-/-}$ macrophages displayed a significant increase in *Arg1* expression. A study in our laboratory has shown that MKP-$2^{-/-}$ mice are more susceptible to *L. mexicana* infection, as a consequence of the increased expression of arginase-I and the consequent reduction of iNOS activity. Thus, the data here contributes to the evidence that Arg1 is negatively regulated by MKP-2 and, in the absence of MKP-2, macrophages fail to eliminate parasitic risk. A key observation in IL-4-treated macrophages was the high induction of AAM signature genes *Fizz1*/*Retnla* and *Ym1*/*Chi3l3*. One of the exciting findings of this study was the significant decrease in the expression of both genes in MKP-2 deficient macrophages (Table 4.6).

It has been discovered recently that Th2 mediated immunity is associated with high levels of macrophage production of Ym1 (Welch *et al.*, 2002) and Fizz1 (Loke *et al.*, 2002b, Raes *et al.*, 2002a, Nair *et al.*, 2005, Nair *et al.*, 2003). Both genes are induced in peritoneal macrophages in an IL-4-dependent manner (Nair *et al.*, 2005). *In vitro* treatment of thioglycollate-elicited macrophages with IL-4 for 20 h induced Fizz1, with a ΔCt of ~15 between the Fizz1 levels of untreated and treated macrophages. The expression was 10-fold higher in nematode-elicited macrophages according to Nair *et al.*, (2003). This high Ct difference was also detected in our experiment, with a ΔCt of ~14-17 between the Fizz1 levels of untreated and IL-4-treated macrophages at 12 h and a ΔCt of ~12 at 24 h. Our study found that when normalised to the expression levels of the QARS reference gene using the conventional comparative Ct ($2^{-\Delta\Delta Ct}$) method for assessing relative gene expression (Livak and Schmittgen, 2001), FIZZ1 showed a mean 67,708

fold upregulation in IL-4 treated macrophages at 12 h and mean 2,432 fold upregulation at 24 h (Figure4.4). This coincides with the magnitude and direction of Fizz1 expression obtained from Nair *et al.*, (2003).

The expression *Ym1* started at 4 h and continued to increase until the end of the experiment (Figure 4.3). This is consistent with the data obtained Ruckerl et al., (2012) since significant expression of *Chi3l3/Ym1* was detectable as early as 4 h in IL-4-stimulated peritoneal macrophages and was maintained until 50 h (Ruckerl *et al.*, 2012). A similar finding was stated by another work, the mRNAs of *Ym1* and *Fizz1* were upregulated in bone marrow macrophages up to 48 h following IL-4 stimulation and remained with up to 6 days with the continuous presence of IL-4 (IL-4 stimulation every 48 h). Upon IL-4 withdrawal, a decrease in both mRNA and protein levels was observed (Ishii *et al.*, 2009). The expression of Fizz1 protein was started at 24 h and increased at 48 h. In comparison with (Ishii *et al.*, 2009), protein levels of Fizz1 assessed by Western blotting indicated an induction at 48 h post IL-4 treatment that subsequently decreased at day 4. The protein levels before 48 h were not examined by Ishii *et al.*, (2009).

The abundant expression of Fizz1 and Ym1 has been associated with Th2 immunity in nematode infection models such as *Brugia malayi* (Falcone *et al.*, 2001, Nair *et al.*, 2003), *Litimosoides sigmodontis* and *Nocardia. brasiliensis* (Nair *et al.*, 2005) and also *Trypanosoma brucei* (Raes *et al.*, 2002a). In the Th2 response elicited by infection of the filarial nematode *B. malayi*, Ym1 expression represents 10% of the transcripts in peritoneal AAM and Fizz1 is the second most abundant transcript at 2%, which is similar to the data presented in Table 4.6. The upregulation of *Ym1* and *Fizz1* mRNAs was detected in mice undergoing sham surgery. Both were downregulated again, proportionally with wound healing suggesting the strong implication of Ym1 and Fizz1 in tissue repair (Nair *et al.*, 2005). This process was associated with the migration of neutrophils to the site of damage (Falcone *et al.*, 2001).

An important finding in this chapter was the induction of MKP-2 in wild-type macrophages by IL-4. This further confirms the critical role in the regulation of type-2 macrophage markers Fizz1 and Ym1 expression. Also it raises an important question about the possible signalling pathways involved in this process. Researchers have found that the expression of *Fizz1* and *Ym1* genes induced by IL-4 was a STAT6-dependent manner (Welch *et al.*, 2002, Stutz *et al.*, 2003). The expression of Ym1/2 in primary macrophages was also shown to be STAT6-dependent as IL-4 stimulation of thioglycollate-elicited macrophages derived from STAT6-deficient mice failed to induce Ym1/2 (Welch *et al.*, 2002). In addition, the expression of *Fizz1* in allergen-challenged lungs was discovered to be STAT6-dependent. Studies have revealed that STAT-6 regulates IL-4 stimulated *Fizz1* expression at the transcriptional level by cooperation with C/EBP (Stutz *et al.*, 2003). However a study by Jin et al. (2012) demonstrated the involvement of the ERK MAPK signaling pathway and STAT6 in Dendritic Cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) differentiation by IL-4. Jin et al. suggested the activation NF-κB signaling pathways directly by IL-4, or indirectly by the ERK pathway during the same process (Jin *et al.*, 2012).

A single attempt has been done to investigate the possible targets for MKP-2 to regulate the expression of Fizz1 and Ym1. Western blotting was used to examine the expression of phosphorylated STAT6 and ERK in IL-4 stimulated BMDMs from MKP-$2^{+/+}$ and MKP-$2^{-/-}$ mice. There were no differences in ERK and STAT6 phosphorylation over 8 h after IL-4 stimulation between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages (data not shown). Due to time limitation this could not be repeated and confirmed. In the light of such limited data, further inhibition studies on major transcription factor for IL-4–mediated signaling responses are needed to confirm the mechanism by which MKP-2 is regulating Fizz1 and Ym1 expression. The possibility of STAT6 to be a physiological non-MAP kinase substrate for MKP-2 is still not clear. However, the regulatory circuits for STAT pathways can still be altered as a result of MKP-2 deletion through affecting the expression *Socs1* gene. The SOCS family consists contains eight distinct members, which regulate intracellular signaling networks. SOCS1 was reported to negatively regulate

JAK/STAT (Alexander and Hilton, 2004). It was found that *Socs1* is an IL-4-inducible gene in mice BMDMs (Dickensheets *et al*., 2006). Also, in AAM in vitro or elicited by Brugia malayi resulted in IL-4-dependent upregulation of SOCS1 and Knockdown of SOCS1 using siRNA decreased arginase I expression (Whyte *et al.*, 2011). The increase in Socs1 expression in MKP-$2^{-/-}$ macrophages in relation to WT cells might explain the enhancement of Arg1 levels in KO cells. Also, it can clarify the contradicted expression between *Arg1* and *Fizz1/Ym1*, assuming that the enhancement in Socs1 positively regulates *Arg1* while negatively regulates Ym1 and Fizz1 expression.

Finally, Il-4 stimulation showed specific downregulation of bone morphogenetic proteins (*Bmp 3,4* and *7)* in MKP-$2^{+/+}$ macrophages. These are members of the transforming growth factor (TGF)-β superfamily, which regulates bone formation (Hong *et al.*, 2009). There is no available data, which links DUSPs or MAPK pathway with the expression of these genes. Genes repressed by IL-4 in MKP-$2^{-/-}$ macrophages were collagens type I, III and *Postn*. Periostin is also a TGF-β–inducible secreted protein originally identified in mouse osteoblasts and promotes adhesion and migration (Horiuchi *et al.*, 1999). Without further investigation it is difficult to hypothesis on the role of the IL-4 repressed genes; however, it could suggest a protective role of MKP-2 in inflammation such as arthritis or a defect in bone development in MKP-2 KO macrophages.

Overall, the above-mentioned studies suggest that Fizz1 and Ym1 participate in the wound healing process of alternatively activated macrophages. The high induction of both markers by nematode-elicited macrophages is consistent with the anti-inflammatory role of alternatively activated macrophage in tissue repair. More importantly, the significant reduction of Ym1 and Fizz1 and upregulation of Arg1 in MKP-$2^{-/-}$ macrophages suggests the critical role of MKP-2 in the expression of these markers and further explains impaired ability to control parasitic infections in MKP-$2^{-/-}$ mice. Examining an *in vivo* worm model in the future would further elucidate the role of MKP-2 in macrophage alternative activation.

5. GENERAL DISCUSSION

Despite the important function of MKP-2 in regulating MAPKs, it is still one of the poorly studied particularly its role in the function of the immune cells macrophages. MKP-2 deletion gives rise to a novel phenotype associated with decreased iNOS, increased arginase-1 activity and the production of pro-inflammatory cytokines, which makes MKP-2 deficient macrophages more susceptible to infection by the intracellular parasite *Leishmania mexicana* (Al-Mutairi et al., 2010b) and, more recently, by *Toxoplasma gondii (*Woods *et al., 2013)*. The observation demonstrated by this thesis supports a key role of MKP-2 in macrophage function in terms of the basic characteristics such as macrophage development and surface protein expression as well as the gene expression of pro- and anti-inflammatory cytokines. Therefore, I studied in this thesis the effects of MKP-2 deletion on macrophage development and the expression of macrophage genes following LPS and IL-4 stimulation.

In this work, I initially demonstrated for the first time that MKP-2 is required for macrophage proliferation. Using a DUSP-4 (MKP-2) knock out mouse, I was able to show a significant reduction in macrophage proliferation in response to M-CSF following to loss of MKP-2 at the early stage of macrophage development (up to day 3 of culturing). This result supports the finding by Lawan *et al.,* (2011) who found that deletion of MKP-2 resulted in a significant reduction in proliferation rate of MEFs and this was reversed by infection with Adv.MKP-2. To further dissect the possible mechanisms underlying the development deficit in macrophages. The first area investigated was the MAPK signaling pathway which has shown to be activated by the proliferative agent M-CSF in BMDMs (Jaworowski et al., 1996, Jaworowski et al., 1999). Following gene deletion, M-CSF induced-ERK phosphorylation was observed as early as 5 min and was enhanced in MKP-2 KO cells. This is consistent with the original studies *in vitro* which suggest specificity of

MKP-2 for ERK and JNK (Misra-Press et al., 1995a, Guan and Butch, 1995, Chu et al., 1996) and more recent studies (Cornell et al., 2010a, Cornell et al., 2012). In addition, in agreement with (Valledor et al., 1999, Valledor et al., 2000, Sanchez-Tillo et al., 2006), early activation of ERK was necessary for the proliferative processes of macrophages by M-CSF and prolonged ERK activity such as that obtained by LPS stimulation blocks macrophage proliferation, and cells become activated directly. For further confirmation, inhibiting ERK significantly reduced macrophage number (Figure 3.14), which is consistent with growth arrest of macrophages at the G_1 phase of the cell cycle following ERK pathway inhibition, as stated by Valledor et al. (2000). The termination of ERK activity is correlated with the expression of MKP-2 in wild type macrophage in figure (3.7), which is consistent with the transient ERK activity as a result of MKP-1 expression demonstrated by (Valledor et al., 1999). The kinetics of MKP-1 and MKP-2 induction is different between the current work and (Valledor et al., 1999), as induction started at (clear expression at 15 min for MKP-2 vs. 20 min for MKP-1 mRNA). However, this does not conflict with ERK being dephosphorylated by MKP-2 and that differences in the activation and expression patterns are existed between both phosphatases.

Activation of p38 MAP kinase by M-CSF was not affected, which was expected since p38 is not susceptible to dephosphorylation by MKP-2 *in vitro*. Unexpectedly, JNK phosphorylation was not enhanced in MKP-$2^{-/-}$ macrophages, despite being a substrate for MKP-2 in several studies (Chu et al., 1996, Robinson et al., 2001, Cadalbert et al., 2005, Al-Mutairi et al., 2010b). This variation was stated initially in the introduction as selectivity between JNK and ERK can vary depending on the cell type (Lawan *et al.*, 2012). However, this might be due to the overlapping specificities of the MKPs and therefore redundancy may occur. A possible reason worth examining is the effect of MKP-2 deletion on the retention of phosphorylated MAP kinase within the nucleus, which has been found to be a feature of the MKPs (Keyse, 2008, Caunt and Keyse, 2013). Another possible reason is the compensation by other MKPs, which was shown later in the microarray data in chapter 4 (DUSP2 compensates for the absence of DUSP4 in MKP-$2^{-/-}$ cells, Table 4.5). Cornell et al. (2010) illustrated that MKP-1 induction was increased

in MKP-2$^{-/-}$ BMDMs in response to LPS as a result of increased ERK signaling (Cornell *et al*., 2010). Thus, further research is needed to investigate the level of endogenous MKP-1/DUSP1 or DUSP2 expression in MKP-2$^{-/-}$ BMDMs to examine to whether levels of these DUSPs are affected and can further to compensate for the loss of MKP-2 in response to M-CSF. Furthermore, it is possible that MKP-2 may have functions other than to dephosphorylate MAPKs. It has demonstrated recently that MKP-2 is able to dephosphorylate histone H3 although by suppressing vaccinia-related kinase 1 (VRK1) activity (Jeong et al., 2013). Also, other signaling pathways apart from the MAPK pathway can be in the proliferative potential of macrophages. This includes PI3K (Hamilton, 1997a, Murray et al., 2000) and STAT1/STAT3 (Novak et al., 1995), as both have shown to be target pathways for CSF-1R signaling. Studying the effect of MKP-2 deletion on these pathways following M-CSF stimulation would further confirm the discrete requirement of ERK activation for macrophage proliferation or the possible involvement of other candidate pathways for CSF-1R signaling.

The second area was investigated in examining the role of MKP-2 in macrophage development is the expression *Csf2* and *Mmd* genes. I have demonstrated for the first time that MKP-2 deletion reduced the expression of both genes. In agreement with Liu et al. (2012), *Mmd* can be used as a marker for macrophage differentiation and maturation. The expression of this gene was reduced in MKP-2$^{-/-}$ macrophages in relation to MKP-2$^{+/+}$ cells, further confirming the proliferative deficit in KO cells. Although the data here conclude that ERK plays a major role in the context of macrophage proliferation, unexpectedly, ERK inhibition also reduced the expression of *Csf2 and Mmd.* The mechanism by which MKP-2 regulates both genes can be considered another area for further investigation, since inhibiting both JNK and p38 led to no clear conclusion on their involvement in the expression of *Csf2* and *Mmd.* LPS was found to significantly up-regulate *Mmd* expression in macrophages (Liu *et al*., 2012). This suggests the possible involvement of JNK and p38 in this process and the important to further examine these pathways on the expression of *Csf2* and *Mmd* which could not be repeated due to time limitations.

A third area that was covered in chapter 3 and linked with macrophage proliferation was an examination of the effect of MKP-2 deletion on the expression of surface proteins CD115 and CD34. MKP-2 deletion reduced the expression of both markers and in the absence of data that correlate the MAPK pathway or other DUSPs with the expression of CD115 and CD34, this is suggests another important role of MKP-2 in the function macrophage progenitors.

Since, MKP-2 was found to be important in macrophage development; it was proposed that the expression of macrophage common marker (F4/80, CD14 and CD11b) would also be affected toward the maturation process of macrophages. However, the non-significant differences obtained from the expression of these markers between MKP-$2^{+/+}$ and MKP-$2^{-/-}$ cells, which suggest a late catch up from MKP-2 KO macrophages and further focus on the early role of MKP-2 in macrophage development. Clearly, the regulation of macrophage proliferation is more complicated than simply activation of ERK MAPKs. Therefore, it is reasonable to appreciate that a series of events have to take place for the final existing phenotype, which finely regulate the response to M-CSF, considering specially the defect could be at a very early stage particularly the progenitor cells.

This thesis also presents for the first time a microarray gene expression analysis for possible target genes following LPS and IL-4 stimulation in the presence and absence of MKP-2. I attempted to analyse the complex molecular network induced by both agents to fully understand phenotypic differences that follow MKP-2 deletion and susceptibility of MKP-2 KO mice to *L.mexicana* and *T.gonadii.* It is not surprising that LPS induced such a rich gene expression profile. The data presented in chapter 4 show that MKP-2 WT and KO macrophages induce almost identical gene expression profiles in terms of innate immune response represented by the induction of pro-inflammatory cytokines, chemokines and other inflammatory mediators. However, important gene expression differences appeared and proposed to play remarkable roles in immune function, which can be further confirmed qRT-PCR, including *Edn1, Gfi1*, and *Cd14*.

The analysis of macrophage responses to IL-4 stimulation revealed stark differences in genes that have been previously classified as markers of alternative macrophage activation, including *Ym1* (*Chi3l3)*, *Fizz1/Relm-alpha* (*Retnla*) and *Arg1 (*Loke *et al., 2002a)*. The reduction in the expression of *Ym1* and *Fizz1* following MKP-2 deletion, both of which are anti-inflammatory genes and found to contribute in extracellular matrix disposition (Nair et al., 2005), is considered a novel finding that may aid in understanding uncontrolled lesion growth following to *L.mexicana* infection of MKP-$2^{-/-}$ mice (Al-Mutairi et al., 2010b). This is in addition to previous findings that showed that enhanced *Arg1* activity renders MKP-$2^{-/-}$ mice more susceptible to infection by intracellular parasites (Al-Mutairi et al., 2010b, Woods et al., 2013).

Since, nematode-elicited macrophages are characterized by a high level of expression of the two novel genes, *Ym1* and *Fizz1* such as *Brugia malayi, Litomosoides sigmodontis*, and *Nippostrongylus brasiliensis (Nair et al., 2005)*, It will be important to examine the effect of MKP-2 deletion on macrophage alternative activation using an *in vivo* infection model. For example, *L.sigmodontis* can be used which showed high induction of Fizz1 and Ym1 in thoracic lavage cells and in lymph nodes BALB/c mice after subcutaneous injection with this nematode *(*Nair *et al., 2005)*. This would be a proper infection model for use in our MKP-2 KO mice to further confirm the data obtained in this thesis.

The results from chapter 4 suggest a possible scenario in which MKP-2 KO fails to fight the intracellular parasite *L.mexicana*. Lesions caused by infecting this parasite in the food pad grew more rapidly in MKP-$2^{+/+}$ mice compared to MKP-$2^{-/-}$ mice in the first 4 weeks of infection. However, parasite burdens remained higher in MKP-$2^{-/-}$ mice throughout infection (Al-Mutairi et al., 2010), which can be explained by the contribution of other inflammatory genes such as *Edn1* since it has been implicated in the pathogenesis of sepsis (Wanecek *et al.*, 2000), chronic lung and kidney inflammation (Hocher *et al.*, 2000, Hocher *et al.*, 2004). At later stages (after 15 weeks of infection), lesion size started to decrease in MKP-$2^{+/+}$ mice, whereas it remained high for MKP-$2^{-/-}$ ones (Al-Mutairi et al., 2010b). This can be explained by defect in turning on the anti-inflammatory response and

therefore macrophages to become fully alternatively activated from MKP-$2^{-/-}$ data obtained from this project. This in particular signifies the proper induction of *Ym1* and *Fizz1*, together with the fact that *Arg1* plays a major role in the high parasite burden.

The signaling pathway by which MKP-2 regulates *Fizz1* and *Ym1* expressions is unknown as of yet. *Stat6* would be a first target, since several studies have shown that the induction of *Fizz1* and *Ym1* is STAT6 dependent (Welch et al., 2002, Stutz et al., 2003). However, the involvement of ERK in IL-4 signaling has also been stated by Jin et al. (2012). This is the last area that remains to be established. From a single Western blotting experiment to check ERK and STAT6 proteins in BMDMs, I found that there were no changes between MLP-$2^{+/+}$ and MKP-$2^{-/-}$ macrophages in ERK and STAT6 phosphorylation levels over 8 h of IL-4 stimulation. Due to time limitation this could not be repeated. However, it is possible that MKP-2 is not negatively regulating immune responses by directly dephosphorylating ERK or STAT6. Instead, MKP-2 might compete with the upstream or downstream substrates for binding to ERK or STAT6. Thus, MKP-2 can further regulate signal perpetuation indirectly and further alter the expression of Fizz1 and Ym1. Also of note is the possible insolvent of *Socs1* the expression of *Arg1* as well as *Fizz1* and *Ym1*.

Due to time restriction and all the optimisation work which has been done during microarray experiment, selecting/validating reference and target genes and PCR efficiency, very interesting candidate genes obtained from microarray data could not be confirmed. However, the novel findings obtained from this thesis highlights the significant role of MKP-2 in the immune responses by regulating the function of the immune cells macrophages.

6. APPENDICES

6.1 MIQE check list (The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines)

ITEM TO CHECK	IMPORTANCE	CHECK LIST
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	**E**	✓
Number within each group	**E**	✓
Assay carried out by core lab or investigator's lab?	D	✓
Acknowledgement of authors' contributions	D	-
SAMPLE		
Description	**E**	✓
Volume/mass of sample processed	D	✓
Microdissection or macrodissection	**E**	-
Processing procedure	**E**	✓
If frozen - how and how quickly?	**E**	samples were used
If fixed - with what, how quickly?	**E**	directly
Sample storage conditions and duration (especially for FFPE samples)	**E**	-
NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	**E**	✓
Name of kit and details of any modifications	**E**	✓
Source of additional reagents used	D	✓
Details of DNase or RNAse treatment	**E**	✓

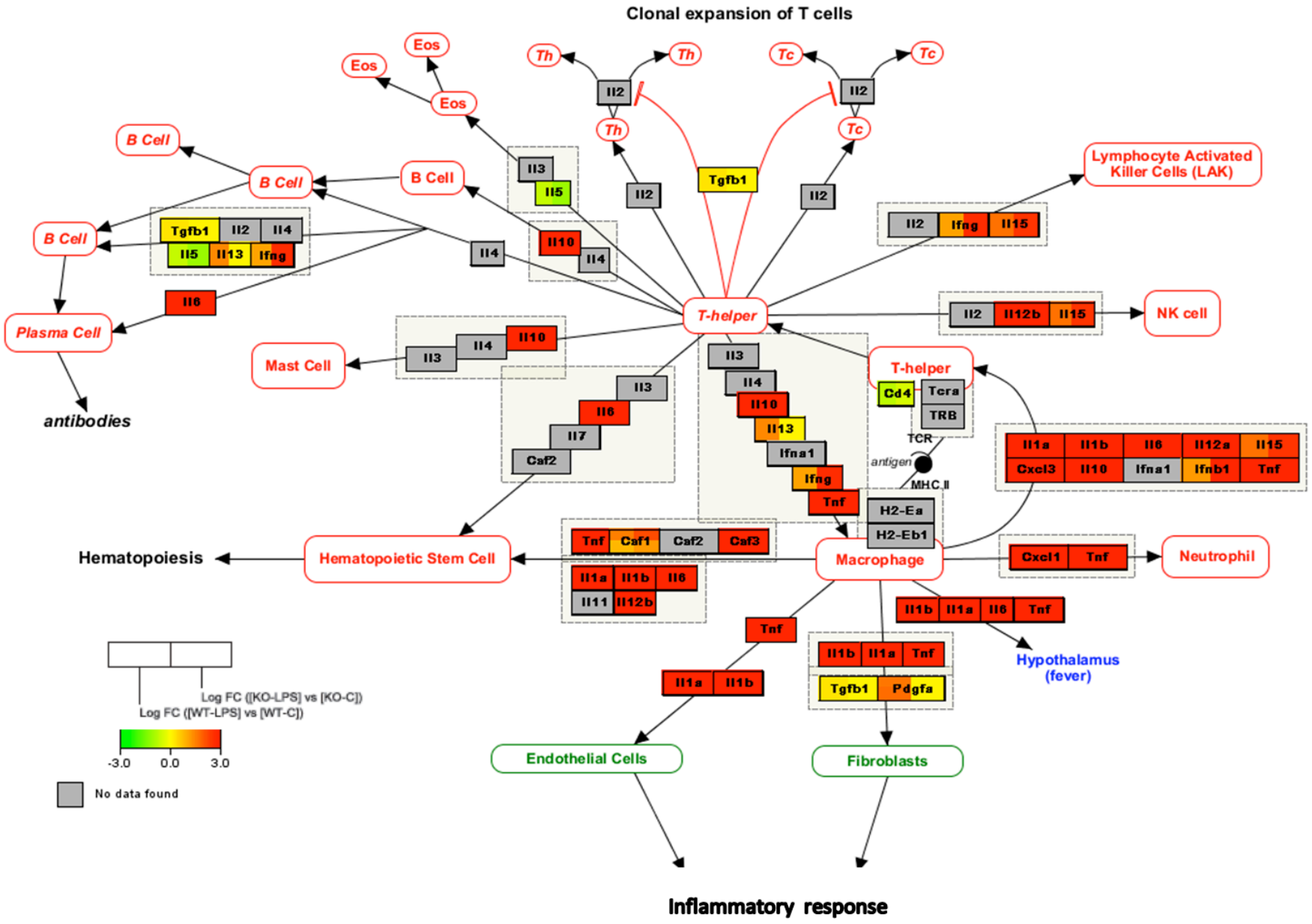
Contamination assessment (DNA or RNA)	**E**	✓
Nucleic acid quantification	**E**	✓
Instrument and method	**E**	✓
Purity (A260/A280)	D	✓
Yield	D	✓
RNA integrity method/instrument	**E**	✓
RIN/RQI or Cq of 3' and 5' transcripts	**E**	✓
Electrophoresis traces	D	RQI
Inhibition testing (Cq dilutions, spike or other)	**E**	-
REVERSE TRANSCRIPTION		
Complete reaction conditions	**E**	✓
Amount of RNA and reaction volume	**E**	✓
Priming oligonucleotide (if using GSP) and concentration	**E**	✓
Reverse transcriptase and concentration	**E**	✓
Temperature and time	**E**	✓
Manufacturer of reagents and catalogue numbers	D	✓
Cqs with and without RT	D*	-
Storage conditions of cDNA	D	✓
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay.	**E**	Efficiency
Sequence accession number	**E**	✓
Location of amplicon	D	✓
Amplicon length	**E**	✓
In silico specificity screen (BLAST, etc)	**E**	✓
Pseudogenes, retropseudogenes or other homologs?	D	✓
Sequence alignment	D	-
Secondary structure analysis of amplicon	D	-

Location of each primer by exon or intron (if applicable)	**E**	-
What splice variants are targeted?	**E**	-
qPCR OLIGONUCLEOTIDES		
Primer sequences	**E**	✓
RTPrimerDB Identification Number	D	-
Probe sequences	D**	-
Location and identity of any modifications	**E**	-
Manufacturer of oligonucleotides	D	✓
Purification method	D	✓
qPCR PROTOCOL		
Complete reaction conditions	**E**	✓
Reaction volume and amount of cDNA/DNA	**E**	✓
Primer, (probe), Mg++ and dNTP concentrations	**E**	✓
Polymerase identity and concentration	**E**	-
Buffer/kit identity and manufacturer	**E**	✓
Exact chemical constitution of the buffer	D	-
Additives (SYBR Green I, DMSO, etc.)	**E**	✓
Manufacturer of plates/tubes and catalog number	D	✓
Complete thermocycling parameters	**E**	✓
Reaction setup (manual/robotic)	D	✓
Manufacturer of qPCR instrument	**E**	✓
qPCR VALIDATION		
Evidence of optimisation (from gradients)	D	✓
Specificity (gel, sequence, melt, or digest)	**E**	✓
For SYBR Green I, Cq of the NTC	**E**	✓
Standard curves with slope and y-intercept	**E**	-
PCR efficiency calculated from slope	**E**	✓
Confidence interval for PCR efficiency or standard error	D	✓

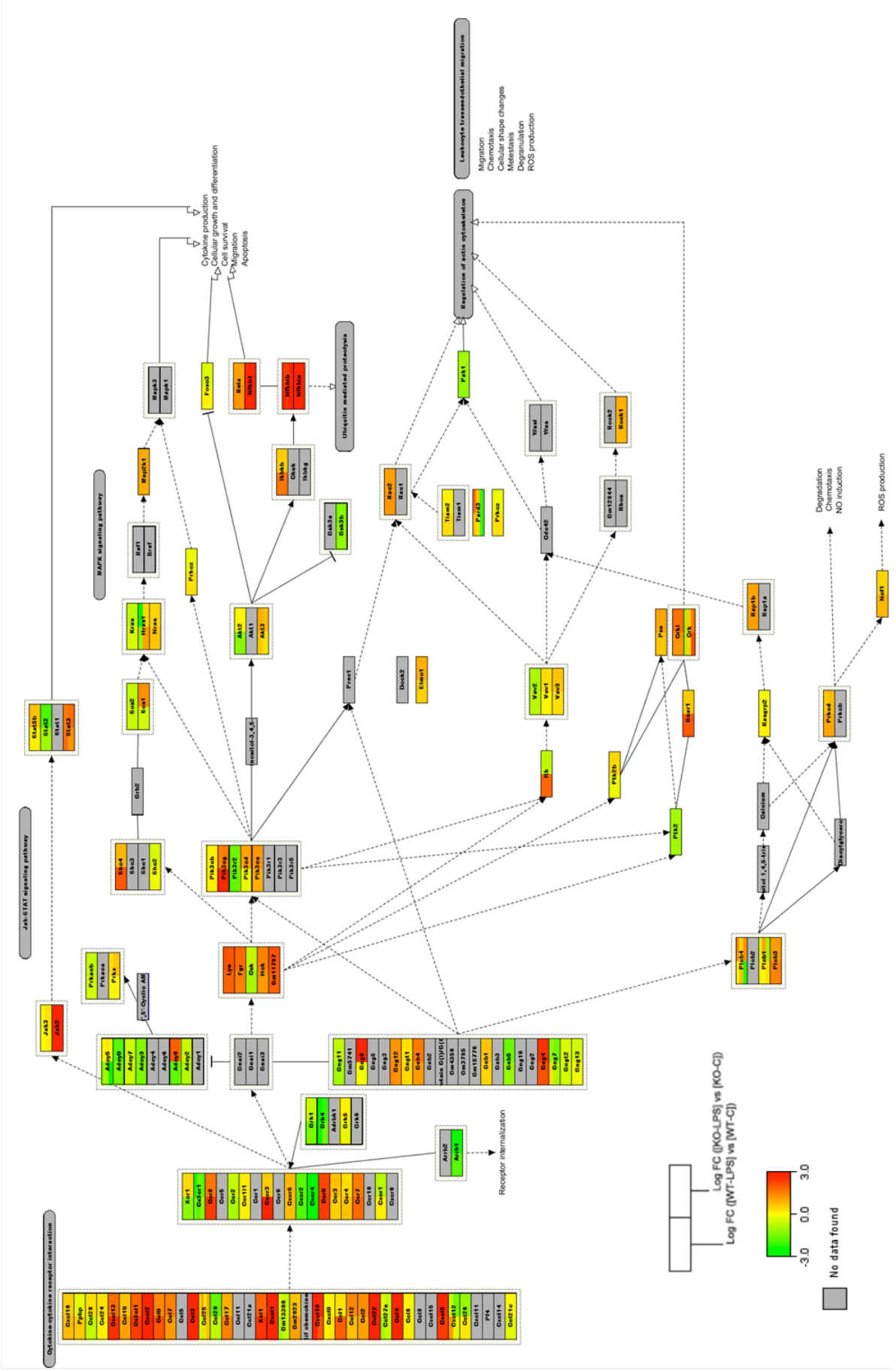
r2 of standard curve	**E**	✓
Linear dynamic range	**E**	-
Cq variation at lower limit	**E**	-
Confidence intervals throughout range	D	-
Evidence for limit of detection	**E**	-
If multiplex, efficiency and LOD of each assay.	**E**	✓
DATA ANALYSIS		
qPCR analysis program (source, version)	**E**	✓
Cq method determination	**E**	✓
Outlier identification and disposition	**E**	✓
Results of NTCs	**E**	✓
Justification of number and choice of reference genes	**E**	✓
Description of normalisation method	**E**	-
Number and concordance of biological replicates	D	✓
Number and stage (RT or qPCR) of technical replicates	**E**	✓
Repeatability (intra-assay variation)	E	✓
Reproducibility (inter-assay variation, %CV)	D	✓
Power analysis	D	-
Statistical methods for result significance	**E**	✓
Software (source, version)	E	✓
Cq or raw data submission using RDML	**D**	-

Table 6.1: MIQE checklist for authors, reviewers and editors. All essential information (E) must be submitted with the manuscript. Desirable information (D) should be submitted if available.

6.2 Cytokines pathway analysis



6.3 Chemokines pathway analysis



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