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Regulation of cytokine and chemokine production in macrophages

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Declarations

I hereby declare that the following thesis is based on the results of investigations conducted by myself, and that this thesis is of my own composition. Work other than my own is clearly indicated in the text by reference to the researchers or their publications. This dissertation has not in whole or in part been previously presented for a higher degree.

Michael Pattison

I certify that Michael Pattison has spent the equivalent of at least nine terms in research work in the Division of Cell Signalling and Immunology, College of Life Sciences, University of Dundee and that he has fulfilled the conditions of the Ordinance General No. 14 of the University of Dundee and is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Dr J. Simon C. Arthur

University of Dundee

Summary

Macrophages are an important part of the innate immune response. They are capable of sensing pathogens via pattern recognition receptors (PRRs) and then initiating an appropriate inflammatory response. Dysregulation of the inflammatory response can result in chronic inflammation and tissue damage. This thesis aimed to investigate signaling pathways regulating production of cytokines and chemokines in response to PRR agonists.

Interleukin (IL)-10 is a potent anti-inflammatory cytokine which suppresses the production of pro-inflammatory cytokines such as TNFα, IL-6 and IL-12. In this thesis, a requirement for an interferon (IFN) β-mediated feedback loop was identified for sustained transcription of IL-10 in response to lipopolysaccharide (LPS), a Toll-like receptor (TLR)4 agonist. Ruxolitinib treatment of macrophages led to an increase in pro-inflammatory cytokine production in response to LPS. Another JAK inhibitor, Tofacitinib, also blocked the sustained transcription of IL-10 and led to elevated secretion of pro-inflammatory cytokines in response to LPS.

The IFNβ-mediated feedback loop also regulated the transcription of another mediator in the inflammatory response, monocyte chemotactic protein-1 (MCP-1). Blocking a type I IFN-mediated feedback loop prevented maximal transcription and secretion of MCP-1 in response to LPS or poly(I:C). IFNβ directly activated MCP-1 transcription and was shown to promote binding of STAT1 to a STAT binding site in the MCP-1 promoter.

Numerous transcription factors such as CREB, Sp1 and NFκB play a role in regulating cytokine production in response to LPS. Other transcription factors may be important in mediating TLR signaling. MEF2D was identified as being phosphorylated at Ser121 in response to LPS. Interestingly, phosphorylation of STAT3 was increased in MEF2D-deficient macrophages compared to wildtype macrophages. MEF2D KO macrophages have elevated levels of IL-10 mRNA and protein. Elevated secretion of IL-10 resulted in decreased levels of pro-inflammatory cytokines TNFα, IL-6 and IL-12. IL-10 mRNA levels were increased in response to a range of TLR agonists. Expression of the regulatory

macrophage markers (Arg1, LIGHT and SphK1) was enhanced in MEF2D-deficient cells.

Regulatory macrophages are characterised by high expression of IL-10 amongst other markers. Interestingly, Zymosan stimulation of macrophages leads to a regulatory-like phenotype. Expression of SphK1 mRNA is strongly induced by Zymosan but not by LPS. Three SphK inhibitors were used to investigate the role of SphK1 in the Zymosan-stimulated macrophages. Interestingly, SphK inhibitors blocked the transcription of IL-10 in response to Zymosan. Treatment of Zymosan-stimulated macrophages with SphK inhibitors also reduced phosphorylation of Akt and STAT3. LPS-stimulated macrophages had lower IL-10 secretion and reduced activation of Akt and STAT3 when treated with a SphK inhibitor. Addition of exogenous DHS1P or sphingosine 1-phosphate (S1P) did not restore phosphorylation of STAT3 in the presence of a SphK inhibitor. This suggests the SphK inhibitors had off-target effects that reduced IL-10 transcription.

The work presented in this thesis demonstrates novel pathways that regulate the transcription of the important mediators, IL-10 and MCP-1.

Publications

Work I have carried out during my PhD studies has contributed to the following publications:

Inhibition of JAKs in macrophages increases lipopolysaccharide-induced cytokine production by blocking IL-10-mediated feedback. Pattison MJ, MacKenzie KF, Arthur JS. J Immunol. 2012 Sep 15;189(6):2784-92.

IFNβ autocrine feedback is required to sustain TLR induced production of MCP-1 in macrophages. Pattison MJ, MacKenzie KF, Elcombe SE, Arthur JS. FEBS Lett. 2013 May 21;587(10):1496-503

X-ray crystal structure of ERK5 (MAPK7) in complex with a specific inhibitor. Elkins JM, Wang J, Deng X, Pattison MJ, Arthur JS, Erazo T, Gomez N, Lizcano JM, Gray NS, Knapp S. J Med Chem. 2013 Jun 13;56(11):4413-21

PGE(2) induces macrophage IL-10 production and a regulatory-like phenotype via a protein kinase A-SIK-CRTC3 pathway. MacKenzie KF, Clark K, Naqvi S, McGuire VA, Nöehren G, Kristariyanto Y, van den Bosch M, Mudaliar M, McCarthy PC, Pattison MJ, Pedrioli PG, Barton GJ, Toth R, Prescott A, Arthur JS. J Immunol. 2013 Jan 15;190(2):565-77

Transcriptional regulation of IL-10 and its cell-specific role in vivo. MacKenzie KF, Pattison MJ, Arthur JS. Crit Rev Immunol. 2014 (In Press)

Abbreviations

15-PDGH	15-hydroxyprostaglandin dehydrogenase
ABIN2	A20-binding inhibitor of NF-kappa-B activation 2
Acy1	aminoacylase 1
AhR	aryl hydrocarbon receptor
AIR	anti-inflammatory response
AP-1	activator protein 1
APC	antigen-presenting cell
ARE	AU-rich element
Arg1	arginase-1
Ash2L	ash2 (absent, small, or homeotic)-like
ASK1	apoptosis signal-regulating kinase 1
ATF	activating transcription factor
ATP	adenosine triphosphate
B10	IL-10-producing B-cell
BATF	basic leucine zipper transcription factor, ATF-like
Bcl10	B-cell lymphoma/leukemia 10
BM	bone marrow
BMDMs	bone marrow-derived macrophages
BMMCs	bone marrow-derived mast cells
BSA	bovine serum albumin
Btk	Bruton's tyrosine kinase
bZip	basic leucine zipper
c-Fos	FBJ osteosarcoma oncogene
c-Myc	myelocytomatosis oncogene
C-terminal	carboxyl-terminal
C1P	ceramide 1-phosphate
Cabin1	calcineurin binding protein 1

cAMP	cyclic adenosine monophosphate
CARD9	caspase recruitment domain-containing protein 9
CBP	CREB binding protein
CD	cluster of differentiation
CDK5	cyclin-dependent kinase 5
CerS	ceramide synthase
ChIP	chromatin immunoprecipitation
CIB1	calcium and integrin-binding protein 1
CLP	cecal ligation and puncture
CLR	C-type lectin receptor
CMV	cytomegalovirus
CNS	central nervous system
COX2	cyclooxygenase 2
CRE	cAMP response element
CREB	cAMP response element-binding protein
CRTC	CREB regulated transcription coactivator
CTLs	cytotoxic T-cells
DAMPs	damage-associated molecular patterns
DBD	DNA binding domain
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DCs	dendritic cells
DD	death domain
DHS	dihydrosphingosine
DHS1P	dihydrosphingosine 1-phosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNCB	2,4-dinitrochlorobenzene
DNFB	1-fluoro-2,4-dinitrobenzene
dsRNA	double-stranded ribonucleic acid
DUSP	dual-specificity phosphatase
EAE	experimental autoimmune encephalomyelitis
EBI3	Epstein-Barr virus induced gene 3
ECM	extracellular matrix
eEF2	eukaryotic Elongation Factor 2

EGF	epidermal growth factor
Egr	early growth response protein
Elk1	ETS-like gene 1
EMSA	electrophoretic mobility shift assay
EP	prostaglandin E receptor
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FADD	FAS-associated death domain-containing protein
FcγR	Fc gamma receptor
FERM	band 4.1 ezrin, radixin and moesin
G-CSF	granulocyte colony-stimulating factor
GAS	gamma-activated site
GPBAR1	G protein-coupled bile acid receptor 1
GPCRs	G protein-coupled receptors
GSK3	glycogen synthase kinase 3
HDACs	histone deacetylases
HMGN1	high mobility group nucleosome binding domain 1
HP1	heterochromatin protein 1
HuR	human antigen R
i.p.	intraperitoneal
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
IBD	inflammatory bowel disease
IC50	half maximal inhibitory concentration
IEGs	immediate early genes
IFNAR	interferon (alpha and beta) receptor
IFNs	interferons
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IRAK	interleukin 1 receptor-associated kinase
IRF	interferon regulatory factor
ISGF3	IFN-stimualted gene factor 3
ISGs	interferon-stimulated genes

JAK	janus kinase
JH	janus homology domain
JunB	Jun B proto-oncogene
K48	Lysine48
KO	knockout
LPS	lipopolysaccharide
LRRs	leucine rich repeats
MADS	MCM1, AGAMOUS, DEFICIENS and SRF box
MAF	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog
MAP2Ks	MAPK kinase
MAP3Ks	MAPK kinase kinase
MAPK	mitogen activated protein kinase
MCP-1	monocyte chemotactic protein-1
MEF2	myocyte enhancer factor 2
MGL	macrophage galactose lectin
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary response 88
N-terminal	amino-terminal
NECA	5'-N-ethylcarboxamidoadenosine
NEMO	NF$\varkappa$B essential modulator
NES	nuclear export signal
NF$\varkappa$B	nuclear factor kappa-light-chain-enhancer of activated B cells
NFIL3	nuclear factor, interleukin 3 regulated
NIK	NF-kappa-B-inducing kinase
NK	natural killer
NLS	nuclear localisation signal
NO	nitric oxide
NOD	nucleotide-binding oligomerization domain
NOS2	nitric oxide synthase 2
Nur77	nuclear receptor subfamily 4, group A, member 1
OH	hydroxyl
PAMPs	pathogen-associated molecular patterns
PAR1	protease activated receptor 1
PASI	psoriasis area severity index

PBMCs	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
Pbx1	Pre-B-cell leukemia homeobox 1
PCR	polymerase chain reaction
PECAM-1	platelet/endothelial cell adhesion molecule 1
PGE	prostaglandin E
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PKD1	protein kinase D1
PMA	phorbol myristate acetate
PP2A	serine/threonine protein phosphatase 2A
PRRs	pattern recognition receptors
PTMs	post-translational modifications
qPCR	quantitative PCR
RA	rheumatoid arthritis
RHD	Rel homology domain
RHIM	RIP homotypic interaction motif
RIP	receptor-interacting protein
RNA	ribonucleic acid
ROCK	Rho-associated protein kinase
RPK118	ribosomal protein S6 kinase, 52kDa, polypeptide 1
RSKs	ribosomal s6 kinases
S1P	sphingosine 1-phosphate
S1P1-5	S1P receptor 1-5
SH2	Src-homology-2
shRNA	short hairpin ribonucleic acid
SIK2	salt-inducible kinase 2
SILAC	stable isotope labeling by amino acids in cell culture
siRNA	small interfering ribonucleic acid
SLE	systemic lupus erythematosus
SphK	sphingosine kinase
SPNS2	spinster homolog 2
SPP	S1P phosphatase

ssRNA	single-stranded ribonucleic acid
STAT	signal transducer and activator of transcription
STIM	stromal interaction molecule
SUMO	small ubiquitin-like modifier
Syk	spleen tyrosine kinase
TAB	TAK1-binding protein
TAD	transcriptional activation domain
TAK1	TGFβ-activated kinase 1
TALE	three amino acid loop extension
TAO	thousand and one amino acid protein kinase
TCR	T-cell receptor
TGFβ	transforming growth factor beta
Th	T helper
TIR	Toll/IL-1R homology
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFAIP3	tumor necrosis factor, alpha-induced protein 3
Tpl2	tumour progression locus 2
Tr1	type I regulatory T cell
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor-associated factor
TRAM	toll-like receptor 4 adaptor protein
Treg	regulatory T-cell
TRIF	TIR-domain-containing adapter-inducing interferon-β
TTP	tristetraprolin
UBAN	ubiquitin binding in ABIN and NEMO
VEGF	vascular endothelial growth factor
WT	wildtype

Chapter 1

Introduction

1.1 Innate immune response

Inflammatory responses have evolved to protect a host from infection and other insults such as cardiac infarction. Infections induce an innate immune response that leads to inflammation. The macroscopic symptoms of the inflammatory response are redness, swelling, heat, pain and loss of tissue function. At the cellular and molecular level, the inflammatory response is characterized by the production of pro-inflammatory cytokines, interferons and chemokines leading to the recruitment of other immune cells (Kawai and Akira, 2010). The inflammatory response will promote the removal of detrimental stimuli and help promote the healing process. It is then rapidly terminated and damaged tissues healed. Failure to terminate the inflammatory response leads to overproduction of cytokines, termed a "cytokine storm", which can cause tissue damage, organ failure and death.

The innate immune response triggers an inflammatory response after recognition of microbial infection or tissue damage. Macrophages, dendritic cells (DCs) and neutrophils are involved in the innate immune system and therefore must be capable of sensing and responding to various pathogens (Hespel and Moser, 2012; Savina and Amigorena, 2007; Basset et al., 2003; Medzhitov and Janeway, 2000). Macrophages, DCs and neutrophils are also able to phagocytose infectious particles and in the case of macrophages and DCs present them to other immune cells to drive the adaptive immune response (Basset et al.,

2003; Medzhitov and Janeway, 2000). In order to generate and then terminate the inflammatory response to prevent the development of cytokine storms, innate immune cells must initiate signaling to drive the inflammatory response and then appropriately signal to promote resolution of inflammation (Liew et al., 2005).

1.1.1 Innate immune signaling

Signaling is the process of transmitting a signal from one place to another such as from the cell membrane to the nucleus. Signaling often works in a cascade or pathway and requires the sequential activation of signaling molecules, although signaling pathways often crosstalk and form networks exhibiting both classical negative feedback and positive feed forward signaling. Small post-translational protein modifications including phosphorylation, ubiquitination, SUMOylation, acetylation and glycosylation are often used within signaling pathways (Liu et al., 2013; Karve and Cheema, 2011; Prabakaran et al., 2012). Phosphorylation is the reversible addition of a phosphate group to OH groups located on tyrosine, threonine or serine amino acid side chains. Two classes of enzyme mediate this phosphorylation; kinases which catalyse the addition of phosphate and phosphatases, which catalyse the removal of a phosphate group. Phosphorylation works in many ways and can lead to activation, inhibition, translocation, sequestration, altered binding properties or degradation of the modified protein (Ubersax and Ferrell, 2007; Cohen, 2000). In macrophages, signaling is triggered by receptors located at the plasma membrane, endosome or within the cytoplasm responding to a microbial infection and the signaling pathways activated produce the desired immune response.

1.2 Macrophage polarisation

Recent work in the field has been focused on macrophage polarization. Initially, the model proposed for T cell polarization was adopted to explain macrophage polarization (Gordon, 2003). Thus, classically activated macrophages which demonstrated a pro-inflammatory phenotype and were induced by interferon (IFN)γ and tumour necrosis factor (TNFα) were classed as M1 macrophages. The alternatively activated macrophages, which produce high levels of IL-10 and thus have an anti-inflammatory phenotype that can be in-

duced with IL-4 treatment, were termed M2 macrophages. Subsequent studies identified the need for further, more precise definitions and led to the M2 macrophage subtypes, M2a and M2b macrophages (Martinez et al., 2008). An alternative classification replaced the M2a and M2b terms with regulatory and wound healing macrophages respectively (Edwards et al., 2006; Mosser and Edwards, 2008), with the suggestion of increased plasticity allowing macrophages to possess characteristics of host-defense, immune regulation and wound healing, such as the macrophages found in obese patients which demonstrate both wound healing and classically activated characteristics. It remains unclear whether macrophage polarisation is a stable phenotype or rather a context-dependent response based upon the incoming signals.

1.2.1 Classically-activated macrophages

A combination of IFNγ and TNFα led to macrophages with a high microbicidal activity, which produced high levels of pro-inflammatory cytokines (Mackaness, 1977; O'Shea and Murray, 2008). IFNγ can be produced by both innate and adaptive immune cells but a likely source in the innate immune response to infection is from natural killer (NK) cells. This will then induce a macrophage population secreting pro-inflammatory cytokines, oxygen and nitrogen radicals, thus increasing killing ability and promoting better resistance to infection (Dale et al., 2008). Due to the rapid and transient nature of the NK cell response, an adaptive immune cell, such as Th1 cells, would be required to maintain the IFNγ levels for the classically activated macrophages.

Classically activated macrophages are vital to host defense. Mice lacking IFNγ or humans with mutations in those signaling pathways are more susceptible to infections by viruses, bacteria and protozoa. Classically activated macrophages may be insufficiently activated in these situations, although other immune cells also respond to IFNγ (Mackaness, 1977; Dale et al., 2008; Gordon and Taylor, 2005). The high levels of pro-inflammatory cytokines secreted by classically activated macrophages can, if not properly regulated, lead to immunopathology such as that seen in inflammatory bowel disease or rheumatoid arthritis (RA) (Zhang and Mosser, 2008; Szekanecz and Koch, 2007).

1.2.2 Wound-healing macrophages

Wound-healing macrophages develop in response to innate or adaptive signals and one of the first innate signals caused by tissue injury is release of interleukin (IL)-4 (Loke et al., 2007). IL-4 can be released from basophils and mast cells in response to injury but also in response to chitin which is found in some fungi and parasites (Brandt et al., 2000; Reese et al., 2007). One key effect of IL-4 on macrophages is to induce arginase, an enzyme that catalyses the production of ornithine from arginine (Kreider et al., 2007). Ornithine is a precursor to collagens and polyamines and thus the macrophage is ready to produce extracellular matrix (ECM). Increased arginase activity, not only promotes ECM deposition, it also reduces the levels of nitrogen radicals by competing with nitric oxide synthase 2 (NOS2/iNOS) for arginine, thus promoting wound healing and limiting classical activation of the macrophage (Munder, 2009).

In addition to IL-4 release from granulocytes, adaptive immune cells such as Th2 cells also secrete IL-4. Th2 immune responses can be induced at mucosal surfaces, such as the lungs or intestines, and at non-mucosal surfaces, often in response to helminth infection (Reese et al., 2007; Wilson et al., 2007). A Th2 immune response is characterised by IL-4 and IL-13 secretion. IL-4 or IL-13 treatment of macrophages *in vitro* leads to macrophages that secrete ECM components and produce much lower levels of pro-inflammatory cytokines, oxygen and nitrogen radicals compared to classically activated macrophages (Edwards et al., 2006). Alternatively activated macrophages are important to helminth and nematode clearance, although precisely how this occurs is unclear (Anthony et al., 2006; Zhao et al., 2008). Wound-healing macrophages express YM1,YM2 and acidic mammalian chitinase (Raes et al., 2002; Zhu et al., 2004). These proteins do not seem to affect chitins found on pathogens but appear to play a role in matrix reorganisation (Bleau et al., 1999; Fusetti et al., 2002). Similarly to classically activated macrophages, control of wound-healing macrophage responses are important to prevent immunopathology. Chronic schistosomiasis leads to tissue fibrosis and this is attributed to deregulated wound-healing macrophage activity (Hesse et al., 2001).

1.2.3 Regulatory macrophages

Both innate and adaptive immune responses can induce a regulatory macrophage population. Interestingly, stress responses can also affect macrophages through glucocorticoid release from adrenal cells (Sternberg, 2006; Mosser and Edwards, 2008). Glucocorticoids, such as dexamethasone, inhibit transcription of pro-inflammatory cytokine genes and decrease mRNA stability (Sternberg, 2006). For example, dexamethasone represses TNFα, IL-1β and COX2 in a DUSP1-dependent manner (Abraham et al., 2006). Glucocorticoids can also induce expression of DUSP1, which can target p38. It has been shown that iNOS, an important characteristic of classically activated macrophages, is induced by LPS stimulation in a p38-dependent manner (Turpeinen et al., 2011). Furthermore, this study demonstrated that DUSP1-deficient cells have enhanced iNOS expression compared to wildtype cells.

Regulatory macrophages can develop during later stages of adaptive immune responses to dampen the response and thereby limit inflammation. Activation of extracellular signal-regulated kinase (ERK)1/2 is important in the development of regulatory macrophages as it allows hyper-secretion of IL-10 by modifying the IL-10 locus (Lucas et al., 2005). *In vitro* stimulation of macrophages with a Toll-like receptor (TLR) agonist and IgG immune complexes induces a regulatory macrophage population secreting high levels of IL-10 (Gerber and Mosser, 2001). Other stimuli also act as a signal for differentiation to regulatory macrophages, including prostaglandins, IL-10, adenosine and sphingosine 1-phosphate (Strassmann et al., 1994; Haskó et al., 2007; Weigert et al., 2007).

Regulatory macrophage subpopulations differ in some characteristics due to the different stimuli. However, most require two stimuli to induce the regulatory phenotype. The key characteristic of regulatory macrophages is high levels of IL-10 secretion coupled with down-regulation of IL-12 production leading to a high IL-10:IL-12 ratio (Edwards et al., 2006; Gerber and Mosser, 2001). IL-10 is a potent anti-inflammatory cytokine and therefore it is the hyper-secretion of IL-10 that is responsible for regulatory macrophages limiting inflammation. In contrast with wound-healing macrophages, regulatory macrophages do not affect ECM composition, although they continue to function

as antigen-presenting cells (APCs) due to high levels of CD80 and CD86, which are both important co-stimulatory molecules (Edwards et al., 2006).

Regulatory macrophages are important for limiting inflammation and thereby preventing immunopathology, however this ability can be utilised by pathogens. At one stage in the life cycle of the intracellular protozoan *Leishmania spp*., the protozoan is capable of binding to host IgG which activates the FcγR, the macrophage Fc receptor for IgG. This stimulation initiates signaling that induces a regulatory macrophage phenotype and allows intracellular growth (Miles et al., 2005). A second example is that of African trypanosomes that escape the humoral immune response by altering their main surface antigen. This leads to the formation of immune complexes that will bind FcγR, inducing a regulatory phenotype (Baetselier et al., 2001).

1.2.4 Macrophage phenotype markers

Expression of specific markers has been used to identify the polarisation state of macrophages within an experimental system (Mosser and Edwards, 2008). Many different markers have been suggested, however some markers may be shared between phenotypes and others do not translate into human systems. This may be as result of transient expression and the short stimulation times used to induce polarisation. However, they still function as useful markers to identify changes in macrophages phenotype (figure 1.1). Recent work has shown that prostaglandin E_2 (PGE_2) can alter the phenotype of macrophages leading to elevated levels of IL-10 and the expression of regulatory macrophage markers such as LIGHT and SphK1 (MacKenzie et al., 2013a).

1.3 Pattern recognition receptors

In macrophages, the recognition of pathogens is mediated by a group of receptors known as Pattern recognition receptors (PRRs) (table 1.1). These germline-encoded receptors are capable of recognizing pathogen associated molecular patterns (PAMPs), which are groups of molecules with conserved patterns that are associated with pathogens and are recognised by the host (Kawai and Akira, 2011). PRRs recognise structures that are

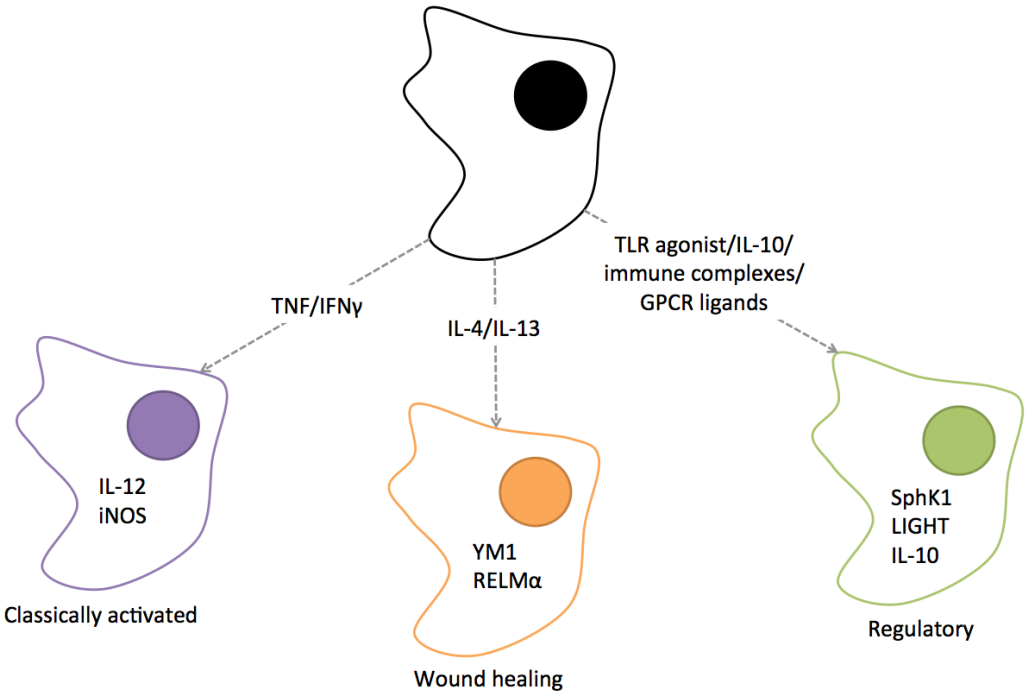

Figure 1.1: Macrophage phenotypes
Macrophages can be polarised into three phenotypes; classically activated, wound healing and regulatory. Macrophages display sufficient plasticity to switch between phenotypes or share certain characteristics. Classically activated macrophages are generated in response to TNFα or IFNγ and are characterised by production of IL-12 and iNOS. Wound healing macrophages are induced in response to IL-4 or IL-13 and are characterised by expression of proteins associated with extracellular matrix remodelling. Regulatory macrophages are generated often in response to TLR agonist and a second stimuli such as immune complexes. Regulatory macrophages are characterised by a high IL-10:IL12 ratio and expression of SphK1 and LIGHT.

conserved across microbial species and, more recently, have been identified as being responsible for recognising endogenous molecules from damaged cells, damage-associated molecular patterns (DAMPs). One family of PRRs is the TLRs. These type I membrane proteins recognize a variety of PAMPs such as lipids, nucleic acids and carbohydrates from viruses, bacteria, fungi and protozoa. In addition to TLRs, other PRRs are found in innate immune cells. C-type lectin receptors, Nod-like receptors, RigI-like receptors and DNA receptors all function to recognize pathogens. Recognition of PAMPs results in the initiation of transcription of genes involved in the inflammatory response. The pattern of genes induced varies between the activated PRRs.

1.3.1 Toll-like Receptors

TLRs can be found upon the surface of many cells including macrophages and also within cells on endosomes and lysosomes. TLRs have N-terminal leucine-rich repeats (LRRs) followed by a transmembrane region and a cytoplasmic Toll/IL-1R homology

(TIR) domain (Akira and Takeda, 2004b). Different TLRs recognise different conserved structures (table 1.1). TLR2 in complex with TLR1 or TLR6 recognises di- and tri-acylated lipids respectively from bacteria, mycoplasma, fungi and viruses. The TLR1/2 or TLR2/6 heterodimers form M shaped structures and their ligands bind to internal pockets within the complexes (Jin et al., 2007). TLR10 possesses a similar sequence identity to TLR1 and TLR6, although the TLR10 ligand has not yet been identified. TLR4 will bind lipopolysaccharide (LPS), a major component of Gram negative bacterial cell walls. TLR4 binds LPS in conjunction with CD14 and MD2 (Kim et al., 2007a; Park et al., 2009). TLR4 can also sense viral envelope proteins (Kurt-Jones et al., 2000).

TLR5 is highly expressed by dendritic cells and recognises flagellin from flagellated bacteria. Flagellin-activated DCs can then trigger B and T cell responses (Uematsu et al., 2008). Mice deficient for TLR5 had elevated pro-inflammatory gene expression in the intestine along with colitis with incomplete penetrance (Carvalho et al., 2012). Interestingly, TLR11 which is present in mice, but not humans, is broadly homologous to TLR5. TLR11 will recognise uropathogenic bacteria and also a profilin like molecule from protozoa (Yarovinsky et al., 2005). More recently, it has been identified as being protective in response to *Salmonella* infection (Shi et al., 2012).

TLRs 3, 7, 8 and 9 bind nucleic acids derived from bacteria and viruses. These nucleic acid-sensing TLRs are found at the endosome and can also sense endogenous nucleic acids (Akira and Takeda, 2004b). TLR3 will sense viral dsRNA in the endolysosome. The structure of TLR3 bound to dsRNA shows dsRNA bound to the N- and C-terminal regions of the LRRs and promotes dimerisation of two TLR3 molecules (Choe et al., 2005; Liu et al., 2008b). Mouse TLR7 and human TLR7/8 bind ssRNA from RNA viruses and from bacteria such as Group B Streptococcus (Mancuso et al., 2009). TLR9 will bind and recognize unmethylated CpG sequences in DNA from bacteria and viruses. A key feature of signaling from TLR3 is the induction of type I interferons as well as pro-inflammatory cytokines. The pattern of cytokine production downstream of TLR7, 8 and 9 is cell type-dependent.

1.3.2 C-type lectin receptors

C-type lectin receptors (CLRs) recognize carbohydrates from fungal pathogens and initiate downstream signaling (reviewed in Kerrigan and Brown, 2011; Hardison and Brown, 2012). The CLR family includes Dectin-1, Dectin-2 and Mincle. Signaling downstream of Dectin-1 involves recruitment of Src and Syk and activates a complex containing CARD9, Multi and Bcl10 (Rogers et al., 2005). Dectin-1 signaling can activate MAPK and NFκB signaling and leads to the production of cytokines and chemokines (Gross et al., 2006; Goodridge et al., 2007). Dectin-1 specifically recognizes β1-3 glucans (Taylor et al., 2007) and can be activated by Zymosan; a crude preparation from fungal cell wells, which also results in TLR2/6 activation. Curdlan, a purified β-glucan, will also activate Dectin-1 signaling.

Dectin-1 signaling is important in the response to fungal infections (Gross et al., 2006; Taylor et al., 2007; Werner et al., 2009; Saijo et al., 2007; Ferwerda et al., 2009), as well as in triggering adaptive immune responses such as Th1, Th17 and cytotoxic T-cell responses (Reid et al., 2009; Kerrigan and Brown, 2010). In particular, Dectin-1 dependent triggering of Th17 responses has been shown to contribute to fungal clearance (LeibundGut-Landmann et al., 2007). Several factors caused by Dectin-1 stimulation of dendritic cells are thought to enhance Th17 responses. Firstly, increased ratio of IL-23 compared to IL-12 production thus limiting Th1 differentiation and promoting Th17 cells (Smeekens et al., 2010). Secondly, β-glucans cause the secretion of prostaglandin E_2 (PGE_2), which can induce IL-6 and IL-23, both important in Th17 polarisation (LeibundGut-Landmann et al., 2007). Zymosan stimulation of macrophages results in a significant secretion of IL-10, surprsingly, Zymosan-stimulated macrophages are resistant to the IL-10 mediated repression of pro-inflammatory cytokines (Elcombe et al., 2013). Zymosan stimulation also induces regulatory macrophage markers such as SphK1 and LIGHT (Elcombe et al., 2013).

1.4 TLR signaling

Once TLRs have bound their agonists, signaling is initiated via adaptor proteins and leads

Table 1.1: Pattern recognition receptors

Name	Ligand	Pathogens detected	Signaling Adaptor
TLR1	Triacyl lipopeptide	Bacteria	MyD88
TLR2	Lipoprotein	Bacteria, Viruses, Parasites	MyD88
TLR3	dsRNA	Viruses	TRIF
TLR4	Lipopolysaccharide	Bacteria, Viruses	MyD88/TRIF
TLR5	Flagellin	Bacteria	MyD88
TLR6	Diacyl lipopeptide	Bacteria	MyD88
TLR7/8	ssRNA	Viruses, Bacteria	MyD88
TLR9	CpG-DNA	Viruses, Bacteria, Protozoa	MyD88
TLR10	Unknown	Unknown	Unknown
TLR11	Profilin-like molecule	Protozoa	MyD88
TLR12	Profilin-like molecule	Protozoa	MyD88
TLR13	23S rRNA	Bacteria	MyD88
Dectin-1	β-glucan	Fungi	Syk
Dectin-2	β-glucan	Fungi	Syk
MINCLE	SAP130	Fungi	Syk
NOD1	iE-DAP	Bacteria	RIP2
NOD2	MDP	Bacteria	RIP2
RIG-I	short dsRNA	Viruses	IPS-1
MDA-5	long dsRNA	RNA viruses	IPS-1
LGP2	Unknown	RNA viruses	Unknown
cGAS	dsDNA	DNA viruses	STING
DAI	dsDNA	DNA viruses	STING
AIM2	dsDNA	DNA viruses	ASC
DHX9	dsDNA	DNA viruses	MyD88
DHX36	dsDNA	DNA viruses	MyD88

to the transcription of distinct genes depending on the TLR activated (Akira and Takeda, 2004b). These distinct transcriptional programmes are partly explained by the signaling molecules used. TLRs can be broadly split into two groups, which use either the adaptors TRIF or MyD88. All TLRs except TLR3 signal via MyD88. TLRs 1, 2, 4 and 6 are found at the plasma membrane. TLR3, 7/8 and 9 are located on the endosome. In addition to MyD88, TLR4 is capable of signaling via TRIF and its partner adaptor TRAM.

1.4.1 MyD88 signaling

MyD88 contains a death domain (DD) and a TIR domain, which mediates recruitment to the TIR domain of the receptor. In the case of TLR4 and TLR2, MyD88 recruitment occurs via the adpator protein Mal. MyD88 signaling results in the recruitment of IL-1R-associated kinase (IRAK)4 (Lin et al., 2010; Warner and Núñez, 2013). IRAK4 interacts

with MyD88 via its own DD and is a serine/threonine kinase (figure 1.2). IRAK4 will activate IRAKs 1 and 2 (Kawagoe et al., 2008). The activated IRAKs can then interact with TRAF6. TRAF6 is an E3 ubiquitin ligase and in conjunction with Ubc13 and Uev1A, the formation of a Lys-63 linked polyubiquitin chain on TRAF6 and IRAK1 is catalysed.

Following the ubiquitination of TRAF6, a complex of TGFβ-activated kinase 1 (TAK1), TAK1-binding protein 1(TAB1), TAB2 and TAB3 is activated as TAB2/3 bind to polyubiquitin chains through zinc finger-type ubiquitin-binding domains (Mendoza et al., 2008; Wang et al., 2001; Kanayama et al., 2004; Sato et al., 2005). In addition, NEMO is also capable of binding polyubiquitin chains, bringing TAK1 into proximity with the NEMO/IKKα/IKKβ complex (Wu et al., 2006). TAK1 will phosphorylate and activate the NEMO/IKKα/IKKβ complex (Sato et al., 2005). Active IKKα/IKKβ will in turn phosphorylate IκBα. Once phosphorylated, IκBα becomes ubiquitinated and degraded by the proteasome (Chen et al., 1996). This allows NFκB to translocate to the nucleus and drive transcription (Vallabhapurapu and Karin, 2009).

In addition to recognition of lysine-linked polyubiquitin chains, NEMO can bind to linear ubiquitin chains. It has been shown that the linear ubiquitin chain assembly complex, which consists of HOIP, HOIL-1 and sharpin, generates polyubiquitin chains which are linked C-terminal to N-terminal (Kirisako et al., 2006; Stieglitz et al., 2012; Tokunaga et al., 2011). Several studies have demonstrated the importance of linear ubiquitin chains in NFκB activation. NEMO binds linear ubiquitin chains via its UBAN motif and binding to linear chains is required for maximal NFκB activation (Rahighi et al., 2009; Kensche et al., 2012; Tokunaga et al., 2009; Emmerich et al., 2013). Many of these studies used mouse embryonic fibroblasts stimulated with IL-1 to investigate the role of ubiquitin chains, although evidence for these chains downstream of TLRs in macrophages is more controversial (see (Clark et al., 2013)).

TAK1 and IKKs can also activate mitogen-activated protein kinase (MAPK) signaling, which activate Sp1, CREB, AP-1 and other transcription factors that promote gene transcription. A series of studies has shown that for activation of ERK1/2 in macrophages,

IKK activation is required to release inhibition of Tpl2 by p105 and ABIN2 (Beinke et al., 2004; Roget et al., 2012; Robinson et al., 2007; Lang et al., 2004; Gantke et al., 2012). TAK1 is thought to be the MAP3K for the JNK pathway, as myeloid-specific deletion of TAK1 prevented activation of JNK as well as NFκB (Eftychi et al., 2012) and, in line with this, TAK1 is required for JNK activation in response to TLR agonists in B cells (Sato et al., 2005).

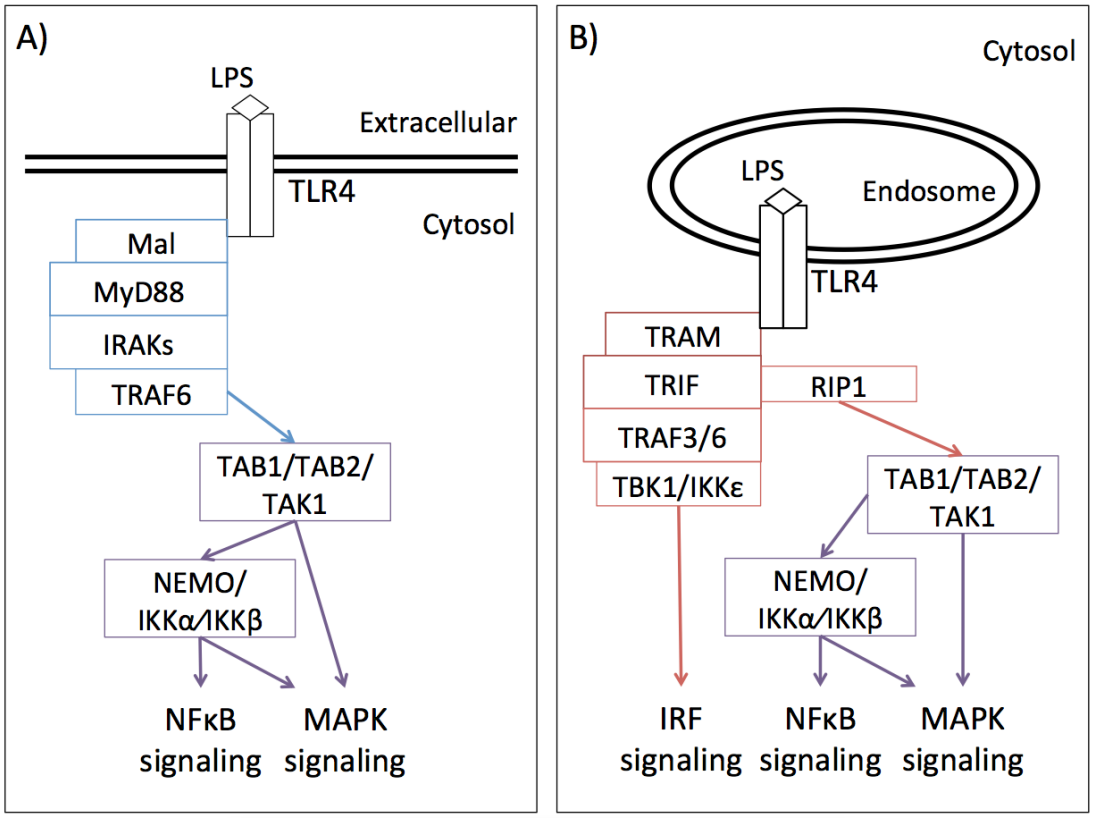

Figure 1.2: TLR4 signaling
A) LPS can bind to TLR4 in complex with MD2 and CD14 at the plasma membrane. This leads to the recruitment of Mal and MyD88. IRAK1, 2 and 4 are recruited and this leads to the activation of TRAF6. TRAF6 can then form K63-linked polyubiquitin chains that promote activation of TAK1. TAK1 is responsible for activation of the IKKs which can activate NFκB signaling and ERK signaling via Tpl2. TAK1 may also activate p38 and JNK signaling. Other MAP3Ks are expected to be activated in response to TLR4 signaling. Additionally, IRF5 is activated downstream of TRAF6, although the precise mechanism is unclear. B) TLR4 can also be internalised to the endosome where it recruits TRAM and TRIF to initiate downstream signaling. TRAF3 and TRAF6 are recruited and activate TBK1/IKKε, which in turn activate IRF3/7. TRIF can also recruit RIP1, which leads to the activation of NFκB and MAPK signaling.

In plasmacytoid DCs, TLR7 and TLR9 also induce the production of type I interferons via a complex of IRAK1, TRAF6, TRAF3 and IKKα. In plasmacytoid DCs, this complex phosphorylates IRF7, which translocates to the nucleus to drive type I interferon expression (Honda et al., 2006). In conventional DCs, IRF3 is activated and is responsible for

type I interferon gene transcription (Negishi et al., 2006; Schmitz et al., 2007; Honda et al., 2006).

1.4.2 TRIF signaling

TRIF will interact with TRAF3 and TRAF6 via TRAF-binding motifs (figure 1.2) (Kawai and Akira, 2010). TRIF also contains a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) that allows interactions with RIP1 and RIP3. RIP1 becomes polyubiquitinated in response to TLR4 activation and this is dependent on interaction with TRADD, FADD and RIP1. Polyubiquitinated RIP1 can then activate the TAK1 complex, which results in NFκB and MAPK activation (Cusson-Hermance et al., 2005). TRIF signaling also results in the activation of TBK1/IKKe which will phosphorylate IRF3, active IRF3 can then induce transcription (Yamamoto et al., 2002; Oshiumi et al., 2003; Hoebe et al., 2003; Fitzgerald et al., 2003). Importantly, IRF3 mediates the transcription of type I interferons (Honda et al., 2006).

1.5 MAPK signaling

Mitogen activated protein kinases (MAPKs) are Ser/Thr kinases of which 14 have been identified in mammals (reviewed in Cargnello and Roux, 2011). MAPK signaling classically involves a three-tiered kinase cascade (see figure 1.3). Stimulation results in the activation of MAPK kinase kinases (MAP3Ks). MAP3Ks will then phosphorylate MAPK kinases (MAP2Ks). MAP2Ks phosphorylate the conserved Thr and Tyr of the TXY motif in the activation loop of the kinase domain of MAPK. Active MAPKs phosphorylate many targets including transcription factors which can then initiate transcription .

1.5.1 TLR-induced MAPK signaling

The identity of all the MAP3Ks responsible for activation downstream of TLR signaling is still being elucidated. Tpl2 is responsible for the activation of MEK1/2-ERK1/2 in macrophages (Beinke et al., 2004). Recent studies have suggested that TAK1 is required for JNK activation in macrophages, although other kinases have been suggested including ASK1 (Eftychi et al., 2012). The MAP3K responsible for p38 activation is still unclear

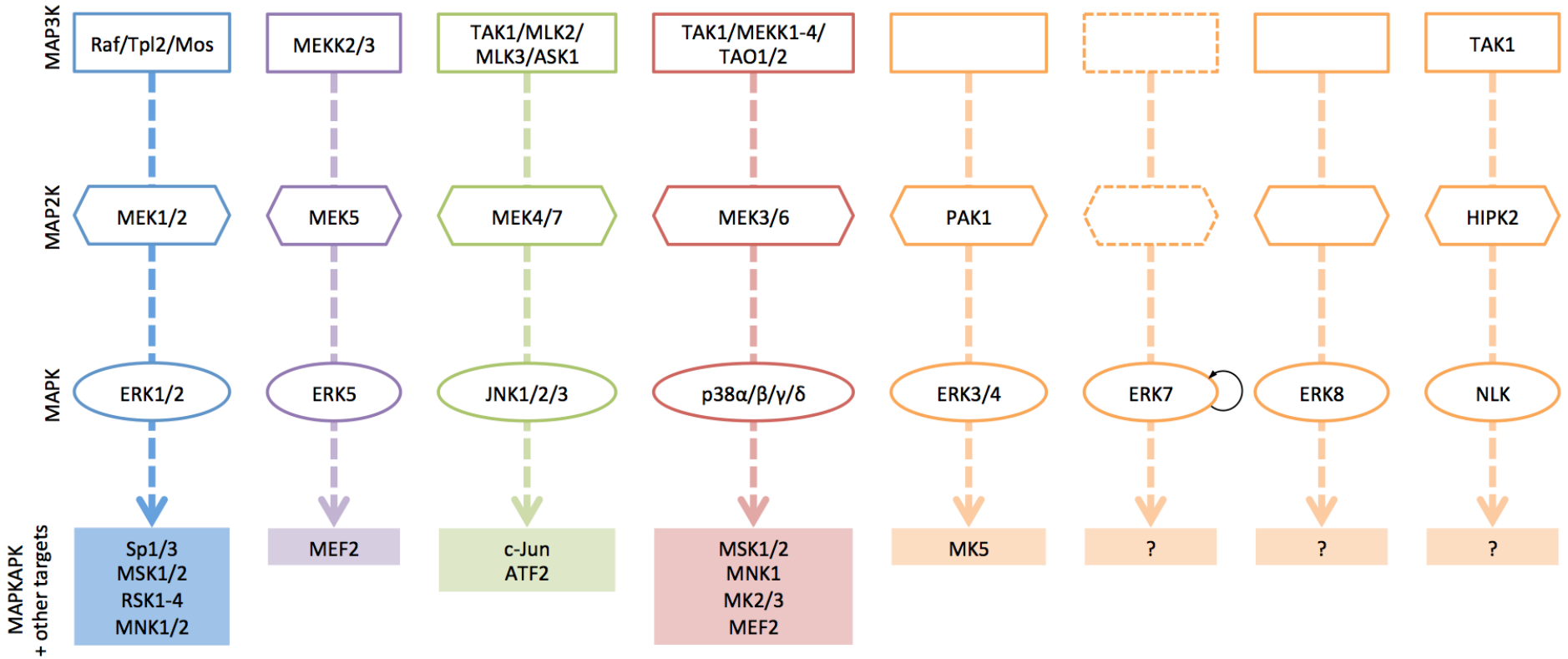

Figure 1.3: Mitogen-activated protein kinases
MAP3Ks can be activated by a variety of stimuli. They then phosphorylate and activate downstream MAP2Ks. Activated MAP2Ks will phosphorylate and activate MAPKs. MAPKs have numerous substrates including transcription factors and a group of kinases, known as MAPKAPKs. Raf/Tpl2/Mos have been shown to activate MEK1/2, which in turn activate ERK1/2. The targets of ERK1/2 include Sp1/3, MSK1/2, RSK1-4 and MNK1/2. MEKK2/3 target MEK5 which can then phosphorylate ERK5. ERK5 is known to activate the transcription factor, MEF2. Several MAP3Ks have been proposed to activate both p38 and JNK signaling. MEK4/7 phosphorylate the three JNK isoforms, whilst MEK3/6 can activate the four p38 isoforms. JNKs are known to target c-Jun and ATF2. The targets of p38 include MSK1/2, MNK1, MK2/3 and MEF2. The atypical MAPKs have less clearly defined signaling pathways. NLK is thought to be activated downstream of TAK1-HIPK2. ERK7 has been shown to autophosphorylate residues important for activation. ERK3/4 are activated by PAK1 and phosphorylate the kinase, MK5. Little is known about the upstream signaling that activates ERK8, nor what the substrates of ERK8 are.

with TAK1, ASK1 and MEKK3 all being suggested (Ajibade et al., 2012; Matsuzawa et al., 2005; Huang et al., 2004; Kim et al., 2007b). Cell type specific deletion of potential MAP3Ks will be required to identify the responsible kinase in the context of TLR signaling in macrophages.

1.5.2 ERK1/2

ERK1 was the first identified mammalian MAPK and was phosphorylated in response to growth factors (Cooper et al., 1982; Kazlauskas and Cooper, 1988). ERK1 and 2 share 83% amino acid identity and are widely expressed. In most cell types, ERK1/2 are activated downstream of Raf-MKK1/2. However, in macrophages, Tpl2/Cot is utilised in response to TLR agonists (reviewed in Raman et al., 2007; Shaul and Seger, 2007). MEK1/2 are two MAP2Ks that become phosphorylated by Raf or Tpl2, depending on cell type, and then activate ERK1/2.

The ERK1/2 pathway is important in cytokine production from macrophages via effects on transcription and post-transcriptionally. Through use of MEK1 and MEK2 inhibitors and the analysis of Tpl2-/- mice, it has been shown that ERK1/2 induce TNFα, IL-1β and IL-10 production and negatively regulate IL-12, IFNβ and NOS2 (Kaiser et al., 2009; Mielke et al., 2009; Dumitru et al., 2000). ERK1/2 is also important for the induction of chemokines such as MCP-1 (Bandow et al., 2012). ERK1/2 also induce the transcription of DUSP1, DUSP2 and DUSP4 resulting in negative feedback loops (Brondello et al., 1997; Cagnol and Rivard, 2013). DUSP4 will dephosphorylate and inhibit ERK1/2 while DUSP1 targets p38 and JNKs and DUSP2 inactivates JNKs (Lang et al., 2003; Huang et al., 2000). ERK1/2 phosphorylates and activates MSKs and RSKs (Deak et al., 1998; McCoy et al., 2007; Dalby et al., 1998).

1.5.3 p38

p38α was identified in 1994 and, subsequently, 3 other isoforms have been found to make up the p38 family, which are responsive to stress stimuli (Han et al., 1994; Lee et al., 1994; Rouse et al., 1994). p38α and β are widely expressed, whereas p38γ and p38δ have more restricted expression profiles (Cuadrado and Nebreda, 2010; Jiang et al., 1996). p38

isoforms are activated by environmental stresses and inflammatory cytokines and MKK3 and MKK6 are responsible for their activation through phosphorylation of a TGY motif (Dérijard et al., 1995; Han et al., 1996; Stein et al., 1996; Cuadrado and Nebreda, 2010). Many MAP3Ks can activate MKK3/6 including MEKK1-4, ASK1, Tpl2, TAK1 and TAO1/2 (Cuadrado and Nebreda, 2010). p38α can also activate multiple kinases downstream including MNK1/2, MSK1/2 and MK2/3 (Ananieva et al., 2008; Cargnello and Roux, 2011).

The four p38 isoforms have been well studied through genetic and chemical approaches. p38α and β are sensitive to most common p38 inhibitors and these inhibitors are protective in autoimmune and inflammatory models (Cohen, 2009). The role of p38β in cytokine production is minimal, as p38β deficiency has little effect on their production from myeloid cells (Beardmore et al., 2005). Deletion of p38α from macrophages leads to reduced TNFα after TLR stimulation and confers greater resistance to endotoxic shock compared to wild-type mice (Kang et al., 2008). Using a chemical genetic approach to render either p38α or p38β insensitive to common p38 inhibitors has shown that the inhibition of p38α is crucial to the inhibitors' effects on TNFα production and in arthritis models (O'Keefe et al., 2007). Recent work has shown that p38α has cell specific roles. A study using myeloid-specific deletion of p38α demonstrated the importance of p38α in rheumatoid arthritis. Unexpectedly, mice lacking p38α in macrophages showed increased disease severity and delayed resolution as well as elevated synovial levels of IL-6 and IL-1 (Guma et al., 2012). This is in contrast to the protective effect of p38 inhibitors, suggesting p38 may have either pro- or anti-inflammatory roles depending on cell type.

One anti-inflammatory role of p38 is to drive transcription of IL-10 via MSK1/2 thus initiating negative feedback pathways to limit TLR signaling (Kim et al., 2008a; Ananieva et al., 2008). Another feedback pathway regulated by p38 involves the phosphorylation of TAB1 and TAB3 and then results in inhibition of TAK1. As a result, p38 inhibitors cause JNK1/2 and ERK1/2 hyperactivation (Cheung et al., 2003; Mendoza et al., 2008). p38 also drives transcription of dusp1, which encodes a phosphatase that dephosphorylates p38α and JNKs and thus sets up a negative feedback pathway (Hammer et al., 2006; Zhao

et al., 2006; Kim et al., 2008a; Ananieva et al., 2008).

p38γ and p38δ are required for maintaining Tpl2 stability as myeloid cells lacking p38γ and p38δ have decreased Tpl2 levels (Risco et al., 2012). Reduced TNFα levels are evident in LPS-stimulated liver macrophages from mice lacking p38γ and p38δ. p38γ and p38δ phosphorylate and inhibit eukaryotic elongation factor 2 (eEF2) kinase, promoting TNF elongation as eEF2 becomes dephosphorylated and activated (González-Terán et al., 2013). However, the changes in Tpl2 protein levels may partly explain the changes seen in TNFα levels in myeloid cells lacking p38γ and p38δ (Risco et al., 2012).

1.5.4 JNK

Stress stimuli promote the phosphorylation of JNKs and there are 3 JNK isoforms; JNK1 and JNK2 are widely expressed, while JNK3 has a more restricted distribution (Rincón and Davis, 2009; Kyriakis and Avruch, 1996; Kyriakis et al., 1991, 1994; Dérijard et al., 1994; Bode and Dong, 2007). JNKs are phosphorylated by MKK4 and MKK7 which can be activated by several MAP3Ks including MKK1-4, Tpl2, TAO1/2, TAK1 and ASK1/2 (Bogoyevitch et al., 2010; Kyriakis and Avruch, 2001; Wagner and Nebreda, 2009; Lawler et al., 1998). JNK1/2 are activated in response to multiple stimuli in macrophages (Rincón and Davis, 2009). A well known target of JNK is c-Jun but other transcription factors such as ATF2, Elk1 and JunB are also phosphorylated (Weston and Davis, 2002; Raman et al., 2007; Bogoyevitch et al., 2010).

Jnk1 and Jnk2 deficiency leads to embyronic lethality but conditional deletion of JNK1 and JNK2 in myeloid cells has demonstrated some of the physiological roles of these kinases (Kuan et al., 1999; Sabapathy et al., 1999). Mice with myeloid cells lacking JNK1 and 2 become obese when on a high-fat diet but do not develop insulin resistance like wild-type mice (Han et al., 2013). Also, macrophages from these mice fail to polarise to the M1 classically activated phenotype (Martinez et al., 2009). This pro-inflammatory role for JNKs is further illustrated by reduced expression of classical M1 macrophage-specific genes in bone marrow-derived macrophages (BMDMs) from mice lacking JNK1 and JNK2 in myeloid cells stimulated with IFNγ or LPS (Martinez et al., 2009).

1.5.5 ERK5

ERK5 was identified in 1995 and is a 98 kDa protein and therefore is also called Big MAPK (BMK) (Zhou et al., 1995; Lee et al., 1995). ERK5 has homology to the ERK1/2 kinase domain and shares the TEY motif in the activation loop but has a large unique C-terminal domain (Drew et al., 2012; Zhou et al., 1995; Lee et al., 1995). ERK5 is activated by MEK5, which is thought to be activated by MEKK2/3 (Chao et al., 1999; Zhou et al., 1995; Sun et al., 2001; Nakamura and Johnson, 2003). ERK5 has a range of substrates including c-Myc, c-Fos and MEF2 family members (English et al., 1998; Kato et al., 1997, 2000; Terasawa et al., 2003). ERK5 is widely expressed and activated in response to oxidative stress and growth factors (Lee et al., 1995; Abe et al., 1996; Kato et al., 1998; Kamakura et al., 1999). Deletion of *erk5* is embryonic lethal with defective vasculature evident at embryonic days 9.5-11.5 (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003; Pi et al., 2004).

1.6 MAPK activated protein kinases

MAPKs have a number of downstream targets including further kinases. There are a group of kinases activated by MAPKs known as MAPK activated protein kinases (MAPKAPKs or MKs) (reviewed in Cargnello and Roux, 2011; Arthur and Ley, 2013). These are RSK1-4, MSK1/2, MNK1/2, MK2/3 and MK5. These kinases are activated by their upstream MAPK and thus contain an activation loop sequence that these kinases can recognise. ERK1/2 can activate RSKs, MSKs or MNKs. p38 isoforms will phosphorylate the MSKs and MNK1 similarly to ERK1/2. They also activate MK2 and MK3. Lastly, MK5 is phosphorylated by the atypical MAPKs, ERK3 and ERK4.

1.6.1 MSK

ERK and p38 MAPKs target two nuclear protein kinases – MSK1 and MSK2. MSK1/2 were identified through genome-wide homology searches to RSK or through yeast two-hybrid screens using p38α bait (Deak et al., 1998; New et al., 1999; Pierrat et al., 1998). MSK1 and MSK2 are 63% identical and interact with ERK1/2 and p38 isoforms through the MAPK binding domain near their C-terminus (Tomás-Zuber et al., 2001). MSKs

have two autonomous kinase domains; the N-terminal kinase domain is related to the AGC family and the C-terminal kinase domain has a CAMK-like sequence. Both isoforms are widely expressed and are located within the nucleus due to a functional nuclear localisation signal (Deak et al., 1998; Pierrat et al., 1998).

Phosphorylation of MSK1/2 by ERK or p38 MAPK leads to their activation and they have a number of key targets in the immune response (Deak et al., 1998; Pierrat et al., 1998; McCoy et al., 2007; Tomás-Zuber et al., 2001; Wiggin et al., 2002). MSK interacts with ERK or p38 via its MAPK binding domain leading to phosphorylation of three Pro-directed sites on MSK; Ser360, Thr581 and Thr700. Phosphorylation of Thr700 is thought to relieve auto-inhibition of the C-terminal kinase domain and along with the phosphorylation of Thr581 within the activation domain of the C-terminal kinase domain results in an active C-terminal kinase domain that phosphorylates two sites within the linker region of MSK and one site (Ser212) within the activation loop of the N-terminal kinase domain (McCoy et al., 2007). The active N-terminal kinase domain can then phosphorylate target substrates.

MSK1/2 can phosphorylate the transcription factor CREB on Ser133 (Deak et al., 1998; Pierrat et al., 1998; Hamm et al., 2002; Lee et al., 2003; Wiggin et al., 2002). CREB recognizes and binds CRE sites in promoter regions of many genes including c-Fos, JunB and Egr1 (Lonze and Ginty, 2002). Phosphorylation of Ser133 was thought to promote recruitment of p300/CBP and lead to the initiation of transcription, however recent work has suggested that downstream of MSK, Ser133 phosphorylation does not lead to strong recruitment of these co-activators (Naqvi et al., 2014). MSKs also phosphorylate the closely related transcription factor ATF1 at Ser63 (Wiggin et al., 2002). MSKs are also crucial in driving the transcription of the important anti-inflammatory mediators, DUSP1, IL-1ra and IL-10 (Ananieva et al., 2008; Darragh et al., 2010). MSK1 and 2 deficient mice are hypersensitive to endotoxic shock and demonstrate prolonged inflammation after toxic contact eczema (Ananieva et al., 2008). This hyper-inflammation is probably due to the loss of the anti-inflammatory mediators under the control of MSKs.

MSKs also target two proteins involved in shaping the chromatin environment; histone H3 and HMGN1 (Soloaga et al., 2003). Histone H3 is phosphorylated at Ser10 by MSKs. The C-terminal tail of histone H3 undergoes numerous post-translational modifications (PTMs) and embryonic fibroblasts from mice lacking MSK1 and MSK2 lacked phosphorylation of histone H3 in response to stress or mitogens (Soloaga et al., 2003). Histone H3 is additionally phosphorylated at Ser28 by MSKs (Dyson et al., 2005). These two serine residues lie in a putative 14-3-3 binding site, which may alter binding of repressors to promoter regions (Macdonald et al., 2005). Interestingly, phosphorylation of histone H3 was shown to block the binding of HP1, a transcriptional repressor (Zeng et al., 2010). HMGN1 regulates chromatin compacting and studies using HMGN1-null cells suggest it suppresses histone H3 phosphorylation (Hock et al., 2007; Lim et al., 2004; Vermeulen et al., 2009). Therefore the role of MSK-mediated phosphorylation of HMGN1 at Ser6 may be to allow MSK-mediated phosphorylation of core histones by affecting the interaction between HMGN1 and nucleosomes.

1.6.2 MK2/3

MAPK-activated protein kinase 2 (MK2) and 3 were identified as p38 substrates and share 75% amino acid identity (Rouse et al., 1994; McLaughlin et al., 1996; Zu et al., 1994; Stokoe et al., 1993). Both MK2 and 3 contain a NLS found at their C terminus (Smith et al., 2000). The NLS is found within the D domain that is important for interactions with p38α and β. MK2 and 3 also possess a NES that promotes nuclear export following stimulation (Ben-Levy et al., 1998; Engel et al., 1998). MK2 and MK3 are widely expressed, particularly in muscle and kidney (Engel et al., 1993; Stokoe et al., 1992).

In quiescent cells, MK2 and MK3 are predominantly found within the nucleus (Engel et al., 1998; Neufeld et al., 2000; Ben-Levy et al., 1998). Stimulation results in an exportin-1-dependent export of these proteins. Unmasking of the NES appears to be regulated by phosphorylation and thus stress stimulation and kinase activation results in MK2/3 export to the cytoplasm (Engel et al., 1998). Stimuli that activate p38 result in activation of MK2/3 and this activation can be blocked by p38 inhibitors and is absent in p38α-deficient cells (Freshney et al., 1994; Rouse et al., 1994; Clifton et al., 1996; Adams

et al., 2000). Activated p38α phosphorylates MK2 at a threonine residue within its activation loop and at second threonine within a hinge region which regulates auto-inhibition. Phosphorylation of the hinge region leads to conformational change, releasing a helix from the kinase domain and exposing the NES (Ben-Levy et al., 1995; White et al., 2007; Meng et al., 2002; Engel et al., 1998). p38α and MK2 mutually stabilise each other and deletion of one leads to reduced expression of the other (Gaestel, 2006; Kotlyarov et al., 2002; Sudo et al., 2005).

p38 is important in many processes, including cytokine production and gene expression, and MK2 appears to be important in many of these (Gaestel, 2006; Ronkina et al., 2008). Notably, following LPS stimulation, MK2 is important in regulating the cytokine response through post-transcriptional mechanisms. MK2 promotes stability and/or translation of IL-6 and TNFα (Kotlyarov et al., 1999; Neininger et al., 2002). MK2-deficient mice are more resistant to endotoxic shock but more susceptible to bacterial infection (Lehner et al., 2002). MK2 can bind or phosphorylate numerous proteins, including TTP and HuR, which can bind AU-rich elements (AREs) (Neininger et al., 2002; Winzen et al., 1999; Mahtani et al., 2001; Tran et al., 2003). MK2 can therefore regulate stability of mRNAs containing AREs such as TNFα and IL-6. MK2 has been shown to phosphorylate TTP at Ser52 and Ser178. p38 inhibitors promote localisation of TTP to the nucleus, proteasomal degradation and loss of phosphorylation at Ser52 and Ser178 (Brook et al., 2006). The control of TTP stability and expression by MK2 was shown to influence TNFα mRNA stability as TTP binds an ARE and destabilises TNFα mRNA. MK2-deficient macrophages produce significantly less TNFα mRNA in response to LPS, while TTP knockout macrophages produce large amounts of TNFα (Hitti et al., 2006). Recently, it has been shown that phosphorylation of TTP by MK2 decreases its binding to AREs. It also reduces displacement of HuR and therefore allows HuR-mediated translation of TNFα mRNA (Tiedje et al., 2012).

1.7 NFκB

NFκB is an important transcription factor in many cellular processes including cancer and the innate immune response (reviewed in Ben-Neriah and Karin, 2011; Hayden and

Ghosh, 2011). NFκB plays an important role in driving the transcription of pro-inflammatory cytokines and anti-inflammatory cytokines. NFκB signaling is activated in response to multiple stimuli including pathogens, cytokines and other stressful conditions. Due to the number of important processes that are controlled by NFκB, the activation of NFκB is regulated to prevent inappropriate activation that can be linked to autoimmune diseases and chronic inflammation (Toubi and Shoenfeld, 2004).

1.7.1 Canonical NFκB signaling

In resting cells under normal conditions, NFκB is found bound to the inhibitory protein, IκBα. IκBα masks the nuclear localisation signal (NLS) found on NFκB. Thus, the NFκB-IκBα complex remains in the cytoplasm and NFκB cannot bind to DNA and drive transcription. Downstream of TLR activation, TRAF3/6 activate IκB kinases (IKKs) . The IKK complex will phosphorylate IκB on two serine residues in the cytoplasm, leading to K48-coupled ubiquitination which marks IκB for proteasomal degradation (Mercurio et al., 1997; Zandi et al., 1997; Rothwarf et al., 1998; Ghosh and Baltimore, 1990; Jacobs and Harrison, 1998; Sen and Baltimore, 1986). This frees NFκB from the repressive function of IκB leading to translocation of NFκB to the nucleus where it binds to target sequences within the promoter of NFκB-responsive genes (Huang and Miyamoto, 2001; Huang et al., 2000).

1.7.2 Non-canonical NFκB signaling

In addition to the canonical pathway, NFκB can be activated by another mechanism. In resting conditions, TRAF3 promotes the degradation of NIK via the proteasome. Following stimulation, TRAF3 is degraded and NIK is stabilised. NIK then activates IKKα which in turn phosphorylates p100. Phosphorylated p100 becomes ubiquitinated and undergoes partial processing. p52 forms dimers with RelB and is able to drive transcription of NFκB-dependent genes (Zarnegar et al., 2008; Vallabhapurapu et al., 2008).

1.7.3 NFκB family

The NFκB family consists of RelA, RelB, c-Rel, p50 and p52. These family members share the Rel homology domain (RHD) that contains a highly conserved DNA-binding

domain (DBD). NFκB family members exist as hetero- or homodimers and the RHD facilitates dimerisation (Liu et al., 1994a,b; Nolan and Baltimore, 1992; Dobrzanski et al., 1993; Basith et al., 2013). RelA, RelB and c-Rel contain a C-terminal transcriptional activation domain (TAD) that will stimulate transcription of target genes. p50, p52 and Relish possess a long ankyrin repeat-containing domain at their C-terminal and thus cannot activate transcription as homodimers. These dimers will bind to a 10 base pair sequence of DNA using the RHD domains which form β-sandwich structures to make contacts to the phosphate backbone as well as specific interactions with DNA bases (Müller et al., 1995; Dobrzanski et al., 1993; Liu et al., 1994a,b).

1.7.4 NFκB dependent genes

TLR signaling activates NFκB in macrophages and NFκB is required for the transcription of many genes crucial to the innate immune response. NFκB plays a role in controlling the induction of four important pro-inflammatory cytokines, IL-1β, IL-6, IL-12(p40) and TNFα, as well as many other pro-inflammatory cytokines, chemokines, immunoreceptors, transcription factors and important pro-resolution factors such as IL-10 (reviewed in Pahl (1999); Blackwell and Christman (1997); Saraiva and O'Garra (2010)).

1.8 Immune response mediators

The signaling processes downstream of TLRs are required to induce a transcriptional programme that allows the generation of an immune response (Medzhitov and Horng, 2009). The immune response involves the production of pro-inflammatory cytokines, anti-inflammatory cytokines, chemokines, interferons and prostaglandins. These effector molecules are secreted by macrophages and then can act in a number of ways. Some molecules will act in an autocrine or paracrine manner creating feedforward or feedback signaling loops. Chemokines will establish a chemo-attractant gradient leading to the recruitment of other immune cells, which is important in the formation of an adaptive immune response (Ransohoff, 2009). Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-12 and tumour necrosis factor-α (TNFα) are secreted by macrophages in response to TLR stimulation. These cytokines act to maintain a pro-inflammatory

environment to promote clearance of the pathogen. However, prolonged inflammatory responses can lead to chronic inflammation and tissue damage. Therefore the production of anti-inflammatory cytokines such as IL-10 is critical (Goldszmid and Trinchieri, 2012). As monocyte chemotactic protein-1, prostaglandin E_2 and interleukin-10 are relevant to this thesis, they will be discussed in more detail.

1.8.1 Chemokines

Chemokines can be broadly split into four groups based on the arrangements of key cysteine residues within their structure (Ransohoff, 2009). Chemokines are responsible for creating chemotactic gradients to attract immune cells (Zlotnik and Yoshie, 2012). Inflammatory chemokines are released during infection and recruit immune cells such as macrophages, neutrophils and T cells to sites of inflammation. Homeostatic chemokines are important in tissue maintenance and development. Homeostatic chemokines can guide cells to certain locations for appropriate maturation during development or to sites where they are required for screening for pathogens. Chemokines mediate their effects by binding to their cognate receptors which are members of the G-protein coupled receptor (GPCR) family.

1.8.1.1 Monocyte chemotactic proteins

Monocyte chemotactic proteins (MCPs) form a subfamily of 4 proteins (MCP-1, 2, 3 and 4). MCP-1 displays over 60% homology with other members of the MCP family (Melgarejo et al., 2009). MCP-1/CCL2 is encoded by the ccl2 gene and can be produced by several cell types including macrophages in response to different stimuli (Yadav et al., 2010). In response to LPS, MCP-1 is produced by macrophages in a Tpl2/ERK1/2-dependent manner (Bandow et al., 2012). Other transcription factors have also been implicated in controlling transcription of MCP-1. Sp-1 is thought to promote the assembly of complexes at the promoter to initiate MCP-1 gene expression in response to TNFα (Ping et al., 2000). Using EMSA and small molecule inhibitors, it has been shown that NFκB, AP-1 and CREB may regulate hydrogen peroxide-induced MCP-1 transcription in BR10 cells, a macrophage cell line (Jaramillo and Olivier, 2002). Several studies have proposed regulation of MCP-1 transcription by STAT transcription factors. In an osteoblast cell

line, STAT1 and STAT3 were required for maximal MCP-1 expression in response to oncostatin M, which activates JAK through two cell surface receptors (Kok et al., 2009). Furthermore, MCP-1 expression in macrophages was dependent on IFNβ signaling via STAT4 (Iida et al., 2011).

MCP-1 triggers chemotaxis in monocytes, memory T lymphocytes and natural killer cells through CCR2 (Deshmane et al., 2009; Yadav et al., 2010; Handel and Domaille, 1996). Knockout of MCP-1 negatively affects monocyte recruitment in a number of contexts including intraperitoneal thioglycollate administration, induction of delayed-type hypersensitivity models and in response to *Schistosoma mansoni* eggs (Lu et al., 1998). MCP-1 has been shown to be detrimental in certain diseases. MCP-1 will induce immune cell infiltration and stimulate histamine release from mast cells and basophils and thereby promote allergic inflammation (Luster and Rothenberg, 1997). Interestingly, MCP-1 protein levels are elevated in the plasma of patients with RA and diabetes (Rantapää-Dahlqvist et al., 2007; Kamei et al., 2006). Nucleotide polymorphisms that result in elevated levels of MCP-1 have been identified in several pathologies. An A-2518G promoter polymorphism is linked to infection with *Mycobacterium tuberculosis*. Individuals with the AG or GG genotypes were more susceptible to tuberculosis. These individuals also had altered expression of inflammatory mediators, with increased levels of MCP-1 and decreased levels of IL-12(p40) (Flores-Villanueva et al., 2005). The A-2518G polymorphism has also been linked to increased susceptibility to psoriasis (Wang et al., 2008). Two other polymorphisms, G-928C and G-362C, have also been implicated in increased disease risk. Individuals with the polymorphisms were identified in being more at risk for carotid atherosclerosis and these polymorphisms also correlated to elevated expression of MCP-1. Interestingly, G-362C is situated within a STAT binding site in the MCP-1 promoter (Nyquist et al., 2010).

1.8.2 Prostaglandin E_2

Prostaglandins are synthesized from arachidonic acid by cyclooxgenases and PG synthases (Phipps et al., 1991; Kalinski, 2012). PGE_2 is synthesized in a multi-step process, although the rate-limiting step is controlled by the inducible enzyme, cyclooxgenase 2

(COX2). PGE_2 is rapidly degraded *in vivo* by 15-hydroxyprostaglandin dehydrogenase (15-PDGH) (Tai et al., 2002). Myeloid and stromal cells produce PGE_2, which is secreted and then can activate four GPCRs, EP1-4. EP2 and EP4 activate the cAMP-PKA-CREB pathway by activating adenylate cyclase (Fujino et al., 2005; Hata and Breyer, 2004). EP1 signaling results in calcium release, whilst signaling from EP3 is through G_i and inhibits adenylate cyclase (Hata and Breyer, 2004; Sugimoto et al., 1992). EP1-4 are expressed on multiple cells and can influence a range of processes including haematopoesis, vascular permeability and smooth muscle function (Kalinski, 2012). PGE_2 can also modulate the inflammatory response. It has been shown to promote the attraction and activation of macrophages and mast cells, but also has anti-inflammatory roles. PGE_2 induces expression of IL-10, the potent anti-inflammatory cytokine (Strassmann et al., 1994). It has been shown that PGE_2 boosts TLR-stimulated IL-10 production by activating the CREB transcription co-factor, CRTC3. Notably, PGE_2 also repressed pro-inflammatory cytokine production independently of IL-10 (MacKenzie et al., 2013a). Interestingly, COX2 induction in macrophages is MSK-dependent in response to LPS (MacKenzie et al., 2013b). In macrophages, PGE_2 reduces the phagocytic capabilities of alveolar macrophages and limits pathogen killing (Aronoff et al., 2004; Serezani et al., 2007).

1.8.3 Pro-resolution factors

Unrestricted inflammation will result in tissue damage and potentially septic shock, therefore the fine tuning of the inflammatory response is crucial (Goldszmid and Trinchieri, 2012). The resolution of inflammation is mediated by several cytokines including IL-10 and IL-1ra. IL-1ra is an antagonist of the IL-1 receptor and will therefore block the pro-inflammatory signals of IL-1β (Garlanda et al., 2013). IL-10 is a potent anti-inflammatory cytokine and through JAK/STAT signaling drives a transcriptional programme known as the anti-inflammatory response (AIR) (Hutchins et al., 2013; Ouyang et al., 2011).

1.9 JAK/STAT signaling

JAK/STAT signaling is a key mediator of cytokine responses (O'Shea and Plenge, 2012). There are 4 janus kinases (JAK)s, JAK1, 2, 3 and Tyk2 and seven signal transducers and

activators of transcription (STAT) isoforms (STAT1, 2, 3, 4, 5a, 5b and 6), which are activated by different cytokines and other growth factors (table 1.2). JAK/STAT signaling is important in immune responses and other cellular processes (O'Shea and Plenge, 2012; O'Shea et al., 2013a).

Table 1.2: JAK/STAT signaling combinations

Cytokine	Receptor	JAKs	STATs	Response
IFNγ	IFNγR1+2	JAK1/JAK2	STAT1	Antiviral and microbial responses
IFNα/β	IFNαR1+2	JAK1/Tyk2	STAT1/2	Viral response
IL-4	IL-4Rα+γc	JAK1/JAK3	STAT6	Th2 differentiation
IL-6	IL-6Rα+gp130	JAK1/JAK2	STAT3	Inflammatory response
IL-10	IL-10Rα+β	JAK1/Tyk2	STAT3	Anti-inflammatory response
IL-12	IL-12Rβ1+2	JAK2/Tyk2	STAT4	Th1 differentiation

1.9.1 JAKs

The 4 JAK isoforms are required to transduce the signals from multiple cytokines which are important in immune cell differentiation and function as well as being associated with other diseases including myelofibrosis. JAKs are large intracellular tyrosine kinases which possess seven regions of conserved homology. Janus homology domain (JH) 1 is the kinase domain and contains two conserved tyrosine residues required for activation (Feng et al., 1997; Zhou et al., 1997). Phosphorylation of these tyrosine residues results in a conformational change which improves substrate binding. JH2 is a pseudokinase domain and is thought to regulate activity of the kinase domain (Chen et al., 2000; Saharinen et al., 2000). JH3-4 resemble Src-homology-2 (SH2) domains and are therefore important in binding to phosphorylated tyrosine residues (Kampa and Burnside, 2000). JH5-7 are found at the amino terminal end of JAKs and form a FERM (band 4.1 ezrin, radixin and moesin) domain, which aids association of JAKs to cytokine receptors (Zhou et al., 2001; Hilkens et al., 2001; Chen et al., 1997). The primary substrates of JAKs are their receptors and STATs, which are a group of transcription factors responsible for driving the cellular response to the cytokine signal.

1.9.2 STATs

STAT proteins are approximately 800 amino acids in length and consist of a N-terminal domain, coiled-coil domain, DNA binding domain, linker region, SH2 domain and a

C-terminal transactivation domain. Each STAT has a conserved tyrosine residue phosphorylated by JAKs and STAT1, 3, 4 and 5 have a conserved serine residue (Decker and Kovarik, 2000). A conserved arginine residue is found within the N-terminal domain. Arginine 31 of STAT1 is methylated and prevents binding of the protein inhibitor of activated STATs (PIAS1) and this results in increased transcriptional activity (Mowen et al., 2001). The coiled-coil domain promotes interactions with other proteins, for example STAT1 with IRF9 and STAT3 with c-Jun (Horvath et al., 1996; Zhang et al., 1999). The DNA-binding domains within a STAT dimer recognises the palindromic sequence $TTCN_{(3/4)}GAA$ (Seidel et al., 1995). The transcriptional activation domain shows sequence diversity between STAT proteins, although the functional relevance for this is unclear.

The seven STAT proteins are cytoplasmically located until phosphorylation by a JAK. Phosphorylation of a key conserved tyrosine residue near the C-terminus leads to dimerisation via SH2 domains (Darnell et al., 1994). The activated dimer then translocates to the nucleus via importin α/β (McBride et al., 2002). The STAT dimer then binds to promoter regions of genes to drive transcription of these genes (Darnell et al., 1994). For example, IFNγ activates JAK1/JAK2 leading to the phosphorylation of STAT1. The STAT1 homodimer will then bind to gamma-activated site (GAS) motif and drive transcription. Gene knockout of STAT proteins has demonstrated their importance in immune development and function. (see table 1.3)

Table 1.3: Gene knockout of JAK and STAT proteins

JAK/STAT	**Phenotype**
JAK1	perinatal lethality due to neurologic defects, severe combined immunodeficiency
JAK2	*in utero* lethality due to absence of erythropoesis
JAK3	severe combined immunodeficiency
TYK2	normal development, increased susceptibility to infection
STAT1	impaired anti-viral response, increased tumours
STAT2	impaired anti-viral response
STAT3	embryonically lethal, refractory to IL-10 treatment
STAT4	defective Th1 differentiation
STAT5a	defective mammary gland development
STAT5b	impaired sexually dimorphic growth
STAT6	defective Th2 differentiation

1.10 IL-10

IL-10 is a key anti-inflammatory cytokine and was discovered in the 1990s. IL-10 is produced by many immune cells in response to inflammatory stimuli and then can act on multiple cell types to limit inflammation (Saraiva and O'Garra, 2010; Ouyang et al., 2011). The importance of IL-10 in resolving inflammation and preventing disease is underlined by the development of colitis in IL-10 deficient mice (Kühn et al., 1993). To further illustrate the role that IL-10 plays, it has been shown that mutations in IL-10 or the IL-10 receptors in humans leads to the early onset of severe colitis (Glocker et al., 2011). However, in some circumstances IL-10 can be detrimental. IL-10 has been shown to promote B cell viability and induce IgA secretion (Defrance et al., 1992; Rousset et al., 1992; Go et al., 1990). Interestingly, IL-10 neutralising antibodies protected severe combined immunodeficiency mice injected with PBMCs from SLE patients. Treatment with the IL-10 neutralising antibody led to reduced levels of autoantibodies, thus reducing the severity of the response (Llorente et al., 1995). Understanding the mechanisms controlling IL-10 production and how this varies in a cell- and stimulus-specific manner is therefore of great importance. The mouse Il10 promoter has been well studied (reviewed in Saraiva and O'Garra, 2010). Several transcription factors have been shown to bind to the promoter including NFχB, GATA3, STAT, MAF, CREB and SP1 (table 1.4). The importance of each transcription factor within different cell types and in response to different stimuli is not fully understood.

1.10.1 IL-10 transcription in T cells

1.10.1.1 Th2 cells

In T cells, several transcription factors have been identified as being important in IL-10 production. Th2 cells produce IL-4, IL-5, IL-13 and IL-10 and also promote antibody class switching in B cells (Mosmann et al., 1986; Fiorentino et al., 1989). IL-4 stimulates Th2 cell proliferation through the activity of NFAT, GATA3 and c-Maf. IL-4 stimulation induces IL-10 production via several mechanisms. NFAT is dephosphorylated and is translocated to the nucleus, where in complex with IRF4, it drives IL-10 transcription (Lee et al., 2009). IL-4 also has some indirect effects on IL-10 transcription by promoting

chromatin remodeling and histone modifications at the Il10 locus through up-regulation of GATA3 (Shoemaker et al., 2006; Chang et al., 2007b; Scheinman and Avni, 2009). The IL-4 dependent up-regulation of GATA3 is thought to occur via STAT6-mediated transcription of the GATA3 gene and through p38-mediated phosphorylation of GATA3 resulting in translocation to the nucleus (Ouyang et al., 2000; Maneechotesuwan et al., 2007).

c-Maf has also been linked to IL-10 producing Th2 cells, although this may be through an indirect effect by promoting IL-4 production (Ho et al., 1996; Kim et al., 1999). Th2 cells express the bZip transcription factor, NFIL3, at higher levels than Th1 cells and knockout of NFIL3 affects cytokine production in Th2 cells, notably decreasing IL-10 production (Kashiwada et al., 2011; Motomura et al., 2011). The effects of NFIL3 on IL-10 production in Th1 cells is less clear and the mechanism by which NFIL3 regulates IL-10 transcription is unknown.

1.10.1.2 Th1 cells

Under specific conditions, Th1 cells can also produce IL-10 (O'Garra and Vieira, 2007). *In vivo* this has been demonstrated during malaria, leishmaniasis and toxoplasmosis infections (Jankovic et al., 2007; Freitas do Rosário et al., 2012; Anderson et al., 2007). IL-10 producing Th1 cells can be generated *in vitro* through high doses of antigen in the presence of IL-12 (Saraiva et al., 2009). These stimuli led to prolonged ERK activation and active STAT4 dimers and results in IL-10 production, which also correlates with c-Maf expression. Co-stimulation of TCR with TGFβ induces a IFNγ+IL-10+ population of T cells and recruitment of SMAD4 to the IL10 promoter has been shown through ChIP assays (Huss et al., 2011).

1.10.1.3 Regulatory T Cells

Regulatory T cells can be classed into groups based on expression of Foxp3. CD4+Foxp3+ cells are the classical regulatory T cells (Treg) and CD4+Foxp3- cells are known as type 1 regulatory T cells (Tr1) (Brunkow et al., 2001; Groux et al., 1997; Vieira et al., 2004). CD4+Foxp3+ Tregs can secrete IL-10 and a population of Foxp3+IL-10+ cells can be

found in the periphery (Maynard et al., 2007). Interestingly, TGFβ promoted IL-10 production in CD4+Foxp3+ cells. Expression of IL-10 has been linked to levels of the transcriptional regulator Blimp-1, whilst knockout of Blimp-1 did not affect Treg development, it did prevent IL-10 secretion following TCR triggering (Cretney et al., 2011). ChIP assays showed that both IRF4 and Blimp-1 are recruited to the IL-10 locus and the importance of IRF4 was further underscored as IRF4 knockout T cells lack Blimp-1 expression and fail to develop into Tregs (Cretney et al., 2011). Blimp-1 is also important for IL-10 production from CD8 and Tr1 cells, although IL-10 production from Th2 cells derived *in vitro* from Blimp-1 knockout mice was normal (Sun et al., 2011; Iwasaki et al., 2013; Kallies et al., 2006).

Tr1 cells are characterised by high levels of IL-10 production along with differing amounts of IFNγ, IL-2 and IL-5. Tr1 cells can be derived from naive CD4 cells using various conditions, recently a strong indication for TGFβ and IL-27 in Tr1 development has been reported (Awasthi et al., 2007; Murugaiyan et al., 2009). The effects of IL-27 on Tr1 development are starting to be elucidated. Naive CD4 cells, cultured in the presence of anti-CD3, anti-CD8 and IL-27, required STAT1 and STAT3 for maximal IL-10 production (Iwasaki et al., 2013). The requirement of STAT3 for maximal IL-10 production can be linked to its role in the induction of egr2. Whilst a direct effect of egr2 on the IL-10 promoter was not shown, egr2 knockout results in ablated IL-10 production and loss of Blimp1 expression (Iwasaki et al., 2013). IL-27 also induces expression of the aryl hydrocarbon receptor (AhR). Knockdown of AhR using siRNA resulted in decreased IL-10 secretion from Tr1 cells, whilst AhR ligands increased IL-27-mediated IL-10 production (Apetoh et al., 2010). In the same study, it was shown that c-Maf siRNA also inhibited IL-10 expression and that both c-Maf and AhR were found at the IL-10 promoter in ChIP assays. The importance of AhR in Tr1 and IL-10 induction is also mirrored in human T cells (Gandhi et al., 2010).

Another affect of AhR ligands is to potentiate IL-21 production in IL-27 stimulated Tr1 cells and both AhR and c-Maf were bound to the IL-21 promoter (Apetoh et al., 2010). IL-21 acts as an autocrine stimulus for T cells and knockout of the IL-21 receptor decreased

IL-10 induction in Tr1 cells (Pot et al., 2009). Recently, a requirement for STAT3 in IL-21-induced IL-10 production was shown, although whether this is a direct effect on the IL-10 promoter or not is unknown (Spolski et al., 2009).

1.10.1.4 Th17 cells

Th17 cells are important in regulating bacterial and fungal infections but have been linked to the development of autoimmune diseases (Bi et al., 2007; Weaver et al., 2006). TGFβ and IL-6 are used to generate Th17 cells *in vitro* and these cytokines induce c-Maf expression, which correlates to IL-10 expression. STAT3 knockout does not affect TGFβ- and IL-6-induced IL-10 expression but blocked the effects of IL-27 preventing IL-10 induction (Xu et al., 2009). In addition to inducing c-Maf, TGFβ and IL-6 also induce egr2 expression, which interacts with the transcription factor BATF and prevents BATF binding to the IL-17 promoter (Miao et al., 2013). Although it can block IL-17 induction, whether egr2 can induce IL-10 expression, as in Tr1 cells, is unknown. BATF lacks a functional transcriptional activation domain and acts as both an activator and repressor of transcription (Murphy et al., 2013). BATF knockout blocks *in vitro* Th17 differentiation, possibly due to cooperation between BATF and IRF4 at important loci including IL-10 and Blimp-1 (Ciofani et al., 2012; Schraml et al., 2009; Li et al., 2012).

1.10.1.5 Cytotoxic T cells

Cytotoxic CD8+ T cells can produce IL-10 and this is evident in lung diseases triggered by influenza and other viruses (Palmer et al., 2010; Sun et al., 2009). Additionally, in a model of acute viral encephalitis, CD8+ T cells found in the CNS produced IL-10 (Trandem et al., 2011). The molecular mechanisms behind IL-10 production in CD8+ T cells are not well elucidated. However, small molecule inhibitors have implicated both ERK1/2 and p38 pathways (Trandem et al., 2011). Similarly to other T cells, IL-27 induces IL-10 secretion in CD8+ T cells. IL-27 and IL-2 act together to induce Blimp-1 and CTLs from Blimp-1 knockout mice secreted less IL-10 in an influenza infection model (Sun et al., 2011).

Further work will be required to identify the signaling pathways and transcription factors

that directly regulate IL-10 transcription downstream of both the TCR and IL-27. Also, comparisons between specific T cell subsets are required to identify general and specific mechanisms that regulate IL-10 transcription in T cells.

1.10.2 IL-10 production by B cells

Early *in vitro* studies demonstrated IL-10 transcription in B cells stimulated with anti-Ig and anti-CD40. This was a delayed response with transcription measured after 4 days, however, co-stimulation with IL-12 accelerated expression of IL-10 (Skok et al., 1999). B cells express numerous receptors including TLRs. TLR2, 4 and 9 agonists induce IL-10 production and MyD88 knockout ablates TLR-induced IL-10 in B cells (Sayi et al., 2011; Wagner et al., 2004; Agrawal and Gupta, 2011). The intracellular pathways downstream of TLR activation in B cells that result in IL-10 transcription are poorly understood.

TLR9 activation results in the activation of Bruton's tyrosine kinase (Btk). Btk knockout B cells do not secrete IL-10 although it is unclear what role Btk plays in inducing IL-10 (Lee et al., 2008). A similar role for Btk has been described in macrophages (Schmidt et al., 2006). EMSA data suggests reduced NFχB activity in Btk knockout cells, however deletion of p65RelA did not alter IL-10 production following TLR9 stimulation (Lee et al., 2008). TLR agonists can work in synergism with IFNα to induce greater amounts of IL-10 compared to TLR stimulation alone (Zhang et al., 2007; Giordani et al., 2009). Similarly, anti IgM antibodies potentiate IL-10 production from LPS-stimulated B cells. This increase is dependent on Ca^{2+} signaling through the ER Ca^{2+} sensors, stromal interaction molecule (STIM) 1 and 2 (Matsumoto et al., 2011).

1.10.3 IL-10 transcription in macrophages and dendritic cells

Macrophages and dendritic cells form part of the innate immune response and detect pathogens through PRRs such as the TLRs and C-type lectin receptors (Medzhitov, 2007; Kawai and Akira, 2010). These receptors initiate downstream signaling that activates MAPK and NFχB. IL-10 production downstream of these pathways has been well studied. All TLRs signal through the MyD88 adaptor, except for TLR3 which signals via TRIF. TLR4 recruits both adaptor molecules when activated (Akira and Takeda, 2004a,b). Both

MyD88- and TRIF-dependent signaling is able to induce IL-10 and knockout of MyD88 or TRIF causes decreased IL-10 production from LPS stimulated macrophages compared to wild-type cells (Boonstra et al., 2006). The specific contributions of MyD88 and TRIF is not clear yet, as only double knockout of MyD88 and TRIF will completely block activation of MAPK and NFκB (Yamamoto et al., 2003).

1.10.3.1 TLR-induced IL-10 transcription

Downstream of MyD88, TRAF6 is an important molecule in regulating MAPK and NFκB activation. The importance of TRAF6 is stimulus specific, as in response to CpG, a TLR9 agonist, TRAF6 knockout BMDMs do not induce IL-10 transcription, whilst LPS-stimulated BMDMs from TRAF6 deficient mice had normal IL-10 induction (Häcker et al., 2006). TRAF3 is, however, critical to IL-10 induction in response to both CpG and LPS, as well as the TRIF-dependent agonist poly(I:C) (Häcker et al., 2006). The mechanism of TRIF-dependent IL-10 induction is not fully understood. It has been proposed that TRAF3 modulates IL-10 induction as it controls production of type I interferon (Yamamoto et al., 2003). It has been suggested that sustained IL-10 production in macrophages in response to TLR4 stimulation is dependent on an IFNβ feedback loop (Chang et al., 2007a).

Further downstream of TLRs, both MAPK and NFκB are implicated in IL-10 induction. Inhibitors of the ERK1/2 pathway reduce IL-10 secretion from macrophages (Liu et al., 2006). Furthermore, knockout of Tpl2 or p105 (NFKB1), which are required for ERK1/2 activation by TLRs, reduced IL-10 production in response to TLRs (Banerjee et al., 2006). Expression of a Raf:ER fusion protein restores ERK1/2 activation in p105 KO mice by activating the Raf-MEK-ERK pathway, however this only partially restored the induction of IL-10. Whilst ERK1/2 activation has been restored, the limited IL-10 levels suggests that p105 may induce IL-10 production via additional mechanisms (Banerjee et al., 2006). In addition to binding to Tpl2, p105 can be partially processed leaving the p50 NFκB subunit. Using a combination of luciferase assays, ChIP and reconstitution of NFKB1 deficient cells with p50 protein, a direct role for p50 in inducing IL-10 transcription has been suggested (Cao et al., 2006).

Other NFκB family members have been implicated in IL-10 production. Another study suggested a macrophage-specific role for NFκB p65 in IL-10 gene expression (Saraiva et al., 2005). Using DNase I digestion, a potential site for NFκB was located in TLR-stimulated macrophages but not in PMA/ionomycin-stimulated Th2 cells (Saraiva et al., 2005). This study also showed that BAY 11-7082, a compound originally described as an IKK inhibitor, could reduce LPS-stimulated IL-10 transcription. The interpretation of results using this inhibitor is, however, complicated by the requirement of IKK to activate Tpl2 and thus ERK1/2 downstream of TLR signaling. In addition, a recent study has found that BAY 11-7082 acts by inhibiting the E3 ligases involved in Tak1 activation upstream of IKK and not as a direct IKK inhibitor (Strickson et al., 2013).

Four isoforms of p38 MAPK exist in mammalian cells, and p38α, γ and δ are important in macrophages (reviewed in Arthur and Ley, 2013). Knockout of p38α or treatment with p38α/β inhibitors reduces IL-10 secretion in macrophages (Kim et al., 2008a). The importance of p38α in regulating IL-10 has been further underlined by studies on DUSP1 knockout mice. DUSP1 is a dual specificity phosphatase that is involved in deactivating p38α signaling in response to LPS. Macrophages from DUSP1 knockout mice display prolonged p38α activation relative to wild type cells and, interestingly, they also exhibit increased IL-10 production (Salojin et al., 2006). A role for p38 γ and δ in the regulation of IL-10 has also recently been identified. p38 γ and δ are not affected by conventional p38 inhibitors and relatively little is known about their function. However, the use of p38γ/δ double knockout mice has shown that p38 γ and δ are required for normal LPS-induced IL-10 production (Risco et al., 2012). Deletion of p38γ/δ may indirectly affect IL-10 production as steady state levels of Tpl2 protein expression are reduced in the p38γ/δ double knockout mice. Consequently, macrophages from p38γ/δ double knockout mice fail to activate ERK1/2 downstream of TLR activation (Risco et al., 2012).

ERK1/2 and p38α have many targets including downstream kinases and several transcription factors and could therefore regulate IL-10 transcription via several potential mechanisms. Both ERK1/2 and p38α directly phosphorylate the transcription factor, SP1, and

numerous studies using IL-10 promoter reporter genes have shown that SP1 binding sites are required for inducing IL-10 transcription (Tan and Khachigian, 2009; D'Addario et al., 2006; Ma et al., 2001; Brightbill et al., 2000; Tone et al., 2000). ChIP assays have also demonstrated the recruitment of SP1 to the IL-10 promoter (Zhang et al., 2006). Thus the phosphorylation of SP1, or the related factor SP3, by ERK1/2 or p38 may be an additional mechanism by which ERK1/2 or p38 regulate IL-10 transcription. The precise mechanisms by which ERK1/2 and p38 MAPKs regulate IL-10 production are not fully elucidated, but as discussed below, the activation of downstream kinases is involved.

1.10.3.2 MSK1/2 and CREB mediated IL-10 transcription

A well established mechanism is the ability of ERK1/2 and p38 to activate the nuclear kinases, MSK1 and MSK2. In mice, double knockout of MSK1 and MSK2 or small molecule inhibitors of MSK reduced LPS-stimulated production of IL-10 by macrophages (Ananieva et al., 2008; Naqvi et al., 2012). Lower levels of IL-10 cause a less effective anti-inflammatory response. As a result of this, MSK1/2 knockout mice produce elevated levels of TNFα, IL-6 and IL-12 and are sensitized to endotoxic shock. MSKs control IL-10 production via the regulation of IL-10 mRNA transcription (Ananieva et al., 2008). MSKs have several targets that might explain their ability to regulate IL-10 transcription. MSKs can phosphorylate histone H3 on Ser10 and Ser28 (Dyson et al., 2005; Soloaga et al., 2003). Phosphorylation of histone H3 may affect the chromatin structure. Notably, it has been shown that ERK1/2, an upstream activator of MSK1/2, is required for H3 phosphorylation at the IL-10 promoter in response to stimulation of macrophages by immune complexes (Bettelli et al., 1998; Zhang et al., 2006).

Another target of MSKs is the transcription factor CREB, which is phosphorylated on Ser133 (Wiggin et al., 2002). In addition to binding sites for other transcription factors, the IL-10 promoter contains a CREB binding site (Gee et al., 2006). The importance of CREB to IL-10 induction has been demonstrated using mice with a Ser133Ala knockin mutation in the endogenous CREB gene. Mutation of the phosphorylation site on CREB resulted in lower IL-10 transcription compared to wild type cells in response to LPS or Zymosan (Ananieva et al., 2008; Elcombe et al., 2013). In addition to MSK, PKA can

also phosphorylate CREB at Ser133 and promote the recruitment of the co-activators CBP and p300, however PKA is only able to weakly activate IL-10 transcription (MacKenzie et al., 2013a). CBP/p300 recruitment is less evident following CREB phosphorylation on Ser133 by MSKs (Naqvi et al., 2014). This could indicate a requirement for additional inputs in CREB regulation downstream of TLR signaling. In addition to Ser133 phosphorylation, CREB has also been reported to be regulated by phosphorylation on other sites (Johannessen et al., 2004).

GSK3 can phosphorylate CREB at Ser139, although the functional consequence of this is less clear, with conflicting reports suggesting the activation or repression of transcription (Tyson et al., 2002; Tullai et al., 2007; Hu et al., 2006). In favour of GSK3 repressing CREB-mediated transcription, inhibition or knockdown of GSK3β has been found to increase IL-10 production in LPS stimulated monocytes (Martin et al., 2005). Knockdown of GSK3 resulted in increased association of CREB with CBP. A further study supported the repressive role of GSK3 as inhibition of GSK3 was found to promote IL-10 induction by IFNβ in dendritic cells (Wang et al., 2011).

1.10.3.3 MK2/3-mediated IL-10 transcription

In addition to MSKs, other MAPK activated kinases are thought to regulate IL-10 production. p38α activates the kinases MK2 and MK3 and double knockout of both MK2 and MK3 or knockout of MK2 alone in macrophages resulted in reduced IL-10 mRNA induction (Ehlting et al., 2011). Small molecule inhibitors suggested that MK2 might phosphorylate CREB downstream of TLRs (Mellett et al., 2011), however off-target effects of these inhibitors could not be excluded. Against this, knockout of MSK1/2 or MSK inhibitors, which do not target MK2/3, block CREB phosphorylation in response to TLR stimulation (Naqvi et al., 2012; Ananieva et al., 2008). MK2 therefore may act through regulating the activity of another transcription or via a post-transcription mechanism. MK2 is known to phosphorylate the mRNA binding protein TTP and as a result stabilise mRNAs targeted by TTP (Ronkina et al., 2010). IL-10 has been identified as a TTP target and MK2 has been found to regulate IL-10 mRNA stability (Stoecklin et al., 2008; Ehlting et al., 2011). An additional indirect role may account for the reduced IL-10

transcription caused by MK2 deficiency. MK2/3 are required to maintain p38α protein levels (Ronkina et al., 2007) and, therefore, a significant reduction in p38α protein levels in MK2/3 knockouts could result in the decreased IL-10 production observed.

1.10.3.4 Synergistic induction of TLR-induced IL-10 transcription

TLR-induced IL-10 production from macrophages can be synergistically increased by several stimuli, including PGE_2, β-glucans and IL-4. In the case of IL-4, a role for c-Maf has been identified as c-Maf is able to directly bind the IL-10 promoter (Cao et al., 2005). The regulation of c-Maf is not well understood but it has proposed that p38 can phosphorylate it (Sii-Felice et al., 2005). IL-4 was found to increase c-Maf expression and the ability of IL-4 to act in combination with LPS to induce IL-10 required c-Maf expression. In c-Maf knockout cells, the ability of LPS alone to induce IL-10 in macrophages was reduced, as was the synergistic effect of IL-4 and LPS (Cao et al., 2005).

PGE_2 increases the induction of IL-10 in response to LPS via its ability to increase cAMP and, therefore, activate PKA (Kim et al., 2011; MacKenzie et al., 2013a). In line with this, cAMP analogues or phosphodiesterase inhibitors, which increase cAMP levels, also increase IL-10 induction in response to TLR agonists (Feng et al., 2000; Eigler et al., 1998). PKA phosphorylates CREB on Ser133, but the effect of PGE_2 on IL-10 transcription was independent of Ser133 phosphorylation as shown in CREB Ser133Ala knockin macrophages (MacKenzie et al., 2013a; Clark et al., 2012). The molecular mechanism of the CREB Ser133-independent increase in response to PGE_2 was shown to be dependent on the CREB co-activator protein, CRTC3. In unstimulated cells, phosphorylated CRTC3 is retained in the cytoplasm through binding to 14-3-3 proteins. PKA phosphorylates the kinase SIK2 and this inhibits the ability of SIK2 to phosphorylate its substrate, CRTC3. Thus, CRTC3 becomes dephosphorylated and translocates to the nucleus, where it can induce transcription of CREB dependent genes including IL-10 (MacKenzie et al., 2013a; Clark et al., 2012). The regulation of CREB activity by MSKs and CRTCs is important in modulating transcription of CREB-driven genes as it has been shown that CREB is found at CRE elements before and during stimulation. In Raw264.7 cells, a macrophage cell line, CREB was found bound to CRE response elements within the COX2 promoter prior

to treatment with LPS (Kang et al., 2006). Therefore, CREB transcriptional is controlled by its interactions with proteins such as MSKs and CRTCs, rather than recruitment to CRE sites.

PGE_2 stimulates macrophages via the EP2 and EP4 receptors, which are GPCRs that elevate intracellular levels of cAMP by activating adenylate cyclase. Macrophages express multiple GPCRs and therefore GPCRs may function as a general mechanism by which the production of IL-10 by macrophages can be augmented. Recently, bile acids, acting via the GPCR, G protein-coupled bile acid receptor 1 (GPBAR1), have been shown to increase LPS induced IL-10 production (Haselow et al., 2013). Although this study implicated CREB downstream of GPBAR1 activation, it did not identify if this was a CREB Ser133 phosphorylation- or a CRTC3-dependent mechanism. Adenosine is another compound that can increase TLR-induced cytokine production and is known to act via GPCRs (reviewed in Haskó et al., 2007). The precise molecular mechanisms required to modulate macrophage cytokine production by adenosine are currently unclear, but could involve the cAMP-PKA pathway. In support of this, knockdown of CREB using shRNA blocked the ability of the adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA) to potentiate TLR-induced IL-10 production. Adenosine is also important for maximal IL-10 production by macrophages in response to heat killed *E. coli*, as IL-10 production was reduced by knockout of the A2A adenosine receptor (Csóka et al., 2007). The same study also demonstrated that knockout of C/EBPβ blocked adenosine-induced IL-10 production, which is supported by previous reporter gene studies showing that the IL-10 promoter contains functional C/EBP binding sites (Liu et al., 2003b).

C-type lectins are also able to modulate TLR induced cytokine production. Dectin-1, which is activated by β-glucan, was the first of these to be described (Kerrigan and Brown, 2011). Stimulation of Dectin-1 alone is sufficient to induce IL-10 transcription, but it is well established that it can act in concert with TLRs. Thus agonists, such as Zymosan, that activate both TLRs and Dectin-1 result in high levels of IL-10 transcription. Both the MAPK – MSK1/2 – CREB pathway as well as CRTC2 have been implicated in the mechanism that results in high IL-10 induction (Elcombe et al., 2013; Kelly et al., 2010; Slack

et al., 2007; Alvarez et al., 2009). Other C-type lectins including macrophage galactose lectin (MGL) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) have also been reported to promote IL-10 expression although the signaling downstream of these receptors is less clearly understood and additional studies are required (van Vliet et al., 2013; Gringhuis et al., 2007).

Macrophages express receptors that recognise phagocytic cells, but are also shared in pathogen recognition (Stuart and Ezekowitz, 2005). In contrast to pathogen recognition, however, phagocytosis of apoptotic cells results in an anti-inflammatory response characterised by IL-10, PGE_2 and TGFβ (Voll et al., 1997). Several studies have suggested that the failure to remove apoptotic cells and suppress the immune response contribute to the development of autoimmune diseases and chronic inflammation (Herrmann et al., 1998; Gal et al., 2000; Camenisch et al., 1999). LPS-activated PBMCs in the presence of apoptotic peripheral blood lymphocytes produce higher levels of IL-10 compared to PBMCs stimulated with LPS alone and a similar finding was reported for LPS-stimulated human monocytes in the presence of apoptotic neutrophils (Voll et al., 1997; Byrne and Reen, 2002). Apoptotic neutrophils alone failed to induce IL-10 production in unstimulated monocytes (Byrne and Reen, 2002). The molecular mechanism behind this increase has started to be elucidated, it has been shown that the production of IL-10 in response to apoptotic cells is dependent on CD36, p38 and the TALE homeoprotein Pre-B-cell leukemia homeobox 1 (Pbx1) (Chung et al., 2007). This suggests that IL-10 production from macrophages can be influenced not only by cytokines and other signaling molecules but by other signals including apoptotic cells.

1.10.3.5 Autocrine IL-10 signaling

Notably, IL-10 may promote its own transcription in macrophages and/or monocytes. Stimulation of monocyte-derived macrophages with IL-10 leads to an increase in IL-10 mRNA and resulted in increased IL-10 secretion (Staples et al., 2007). The same study suggested that this was a STAT3-dependent increase, as IL-10 promoter fragments with mutated STAT sites failed to drive luciferase reporters in response to IL-10 stimulation. A later study demonstrated that peritoneal macrophages from animals with a macrophage-

specific deletion of Stat3 gene lacked IL-10 production (Cheng et al., 2003).

1.10.4 IL-10 signaling

IL-10 acts via the IL-10 receptor to stimulate JAK-STAT signaling in its target cells (figure 1.4). The IL-10 receptor is a heterodimeric complex consisting IL-10R1 and IL-10R2 chains (Kotenko et al., 1997; Tan et al., 2001). IL-10R1 binds IL-10 with high affinity and is unique to the IL-10 receptor, while IL-10R2 also forms part of the receptors for IL-20, IL-22, and IL-28 (Yoon et al., 2010). Both receptor subunits are required for IL-10 dependent signaling and knockout of either IL-10R1 or IL-10R2 in mice is sufficient to block the anti-inflammatory effects of IL-10 (Pils et al., 2010; Spencer et al., 1998). Activation of the IL-10 receptor leads to the phosphorylation of JAK1 and Tyk2 (Finbloom and Winestock, 1995; Ho et al., 1995). It has been suggested that IL-10R1 recruits JAK1 and IL-10R2 binds Tyk2 (reviewed in Ouyang et al., 2011), although definitive evidence for the differential binding of JAK1 and Tyk2 to IL-10R1 and IL-10R2 is lacking.

Studies on JAK1 knockout mice have demonstrated a key role for JAK1 in mediating the effects of IL-10. JAK1 deletion is perinatally lethal, however, using macrophages cultured from JAK1 deficient embryos, it has been shown that JAK1 is essential for the ability of IL-10 to repress LPS-induced TNFα production (Rodig et al., 1998). Although, IL-10R1, IL-10R2 and JAK1 are required for IL-10 function, the role of Tyk2 in mediating the effects of IL-10 is less clear. Initial reports on Tyk2 knockout mice showed that phosphorylation of STAT3 in response to IL-10 was normal and that the anti-inflammatory effect of IL-10 was unaffected as measured by repression of TNFα production in macrophages (Shimoda et al., 2000; Karaghiosoff et al., 2000). A later study has found that Tyk2 deletion led to reduced STAT3 phosphorylation in response to IL-10 in macrophages, although STAT3 protein levels were also reduced in the Tyk2 deficient cells (Shaw et al., 2006). The role of Tyk2 in human cells is also unclear. JAK inhibitors with enhanced selectivity for JAK1 vs Tyk2 are much more effective at blocking IL-10 responses in human cells relative to Tyk2 selective inhibitors (Sohn et al., 2013). In contrast to this, PBMCs from a patient with an inactive mutant form of Tyk2 were reported to show a reduced ability for IL-10 to inhibit LPS induced TNFα production (Minegishi et al., 2006). These differences

Table 1.4: IL-10 transcription in immune cells

Cell type	Stimulus/stimuli	Signaling molecules/Transcription factor(s)	Effect on cells
T cells			
Th2	IL-4	NFAT/IRF4	increased IL-10
	IL-4	GATA3	increased IL-10 due to chromatin remodeling
	IL-4	c-Maf	possibly indirect effect, promotes IL-4 production
		NFIL3	increased IL-10
Th1	Antigen + IL-12	STAT4 (+ ERK1/2 activation)	increased IL-10
	TCR + TGFβ	SMAD4	increased IL-10 and IFNγ expression
Treg	TGFβ	?	increased IL-10
	TCR	Blimp1/IRF4	increased IL-10
Tr1	αCD3, αCD3 + IL-27	STAT1+STAT3	increased IL-10 + egr2 expression
		egr2	required for Blimp1 expression
		Blimp1	increased IL-10
	IL-27	AhR/c-Maf	increased IL-10
	AhR ligands	AhR/c-Maf	increased IL-10 + IL-21 expression
	IL-21	STAT3	increased IL-10
Th17	TGFβ + IL-6	c-Maf	increased IL-10
	TGFβ + IL-6	egr2/BATF/IRF4	important for Th17 differentiation
CD8+	IL-2 + IL-27	Blimp1	increased IL-10
B cells			
B10	αIg, αCD40 and IL-12	?	delayed IL-10 expression
	TLR9 agonist	Btk	increased IL-10 expression
	TLR agonist + IFNα	STAT?	synergistic induction
	LPS + αIgM	STIM1/2	αIgM potentiates LPS-induced IL-10 expression
Macrophages			
	LPS/Zymosan	NFκB	p50/p65 binding sites identified in Il10 promoter
	LPS/Zymosan	ERK/p38	activate MSKs and possible direct effects
	LPS/Zymosan	MSK/CREB	MSKs activate CREB and drives IL-10 expression
	LPS	MSKs	alter chromatin through targeting histone H3 and HMGN1
	LPS + PGE	PKA/SIK/CRTC3	syngeristic induction through CREB and CRTC3
	LPS	MK2/3	target TTP, IL-10 mRNA stability increased
	LPS + IL-4	p38/c-Maf	synergistic induction
	LPS + adenosine	CREB/C/EBP	synergistic induction
	IL-10	STAT3	autocrine induction
	LPS + apoptotic cells	CD36, p38 and Pbx1	synergistic induction

may demonstrate the relative importance of Tyk2 in IL-10 signaling in different species, genetic backgrounds or macrophage subtypes. In addition to other variables, other JAK isoforms may compensate for the loss of Tyk2 function in the Tyk2 knockout mice.

IL-10 binding to the receptor leads to JAK dependent phosphorylation of the receptor, which allows the recruitment of STATs. High concentrations of IL-10 can promote STAT1 and STAT3 recruitment and tyrosine phosphorylation, however STAT3 is thought to be the critical isoform for the anti-inflammatory effects of IL-10 (Riley et al., 1999; Meraz et al., 1996). JAK1 and Tyk2 phosphorylate STAT3 on tyrosine 705 leading to its dissociation from the receptor and the formation of an active homodimer. STAT3 dimers can then translocate to the nucleus where they will bind to STAT consensus sequences within promoter regions of their target genes. Similar to JAK1 deficient macrophages, STAT3 conditional knockout macrophages are also refractory to IL-10 treatment (Takeda et al., 1999). While STAT3 is required for the actions of IL-10, STAT3 phosphorylation alone does not explain how IL-10 represses cytokine production. For example, other cytokines such as IL-6 also induce STAT3 phosphorylation but do not reproduce the anti-inflammatory actions of IL-10. Precisely how IL-10 induced STAT3 phosphorylation affects cell function is not fully understood (Bazzoni et al., 2010; Hutchins et al., 2013; Murray, 2005). Several mechanisms have been suggested, including the induction of anti-inflammatory mediators, regulation of the transcription or mRNA stability of specific genes or modulation of intracellular signaling. It is likely that IL-10 will induce multiple events that allow the generation of anti-inflammatory response and more work is required to understand further the effects of IL-10.

1.10.5 Role of IL-10 *in vivo*

1.10.5.1 T-cell derived IL-10

IL-10 is critical to maintaining control of the immune system and loss of IL-10 exacerbates inflammation in a wide range of models (reviewed in Ouyang et al., 2011; Redford et al., 2011; Wilson and Brooks, 2011; O'Garra et al., 2008). Many types of immune cell have the potential to produce IL-10 and it is therefore necessary to understand what cell type is the major source in different disease contexts. The effect of cell type specific

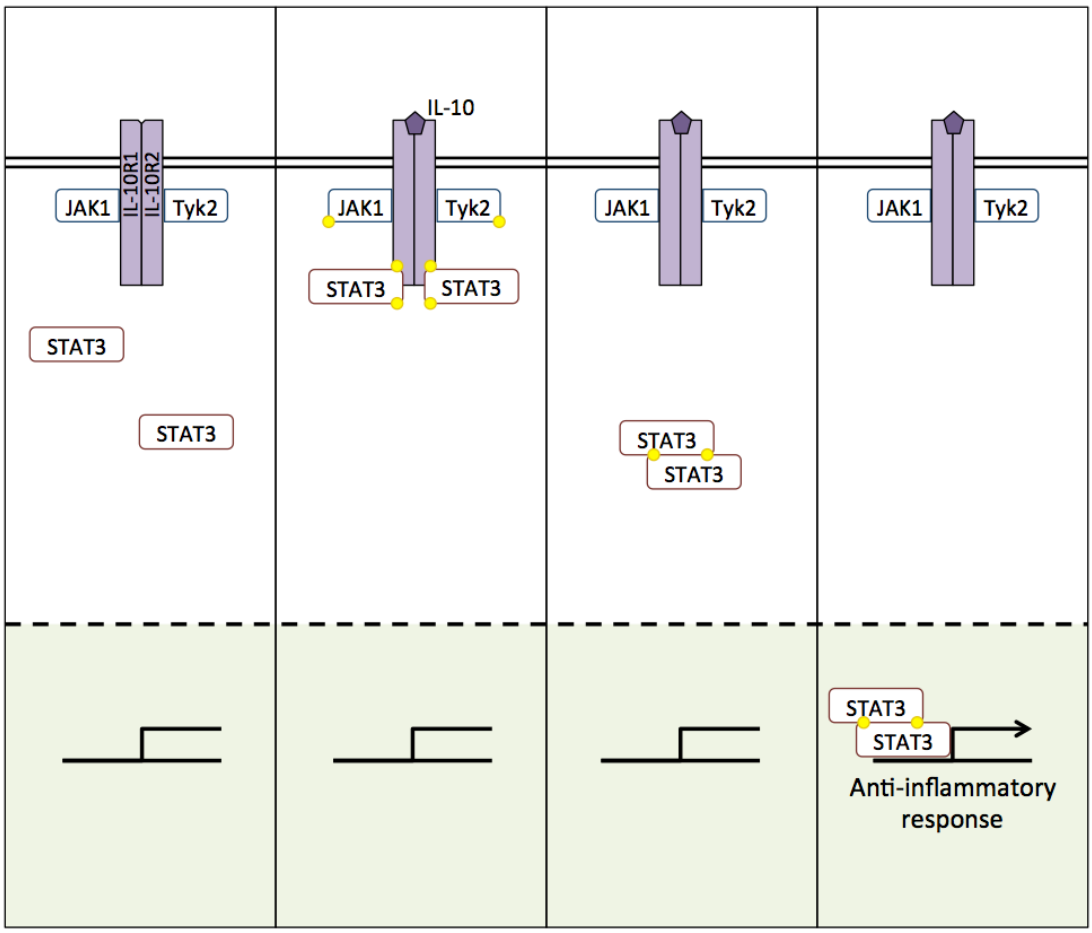

Figure 1.4: IL-10 signaling
IL-10 binds to IL-10R1 and IL-10R2 and leads to the activation of JAK1 and Tyk2. JAK1 and Tyk2 phosphorylate Tyr705 on STAT3. Phosphorylated STAT3 molecules dissociate from the receptor and form dimers. STAT3 dimers translocate to the nucleus and bind to promoters of genes involved in the anti-inflammatory response.

IL-10 or IL-10 receptor knockout has now been reported in several immune models. Total IL-10 knockout mice develop spontaneous inflammation in the gut (Kühn et al., 1993). Gnotobiotic IL-10 knockout mice do not develop colitis, demonstrating that IL-10 is important in controlling the host response to gut flora. The clinical relevance of IL-10 in colitis has been confirmed by mutations that block IL-10 function in humans resulting in the development of severe early onset colitis (Glocker et al., 2009, 2010). Original studies using reconstitution experiments and more recent studies using the deletion of IL-10 in either B cells, T cells or myeloid cells show that B cell or myeloid cell deletion of IL-10 does not result in the spontaneous development of colitis in mice, while deletion of IL-10 specifically in T cells does (Madan et al., 2009; Roers et al., 2004).

Conditional IL-10 knockouts have also demonstrated a role for T cell-derived IL-10 in hy-

persensitivity. T cell-specific deletion of IL-10 increased reaction to 2,4-dinitrochlorobenzene (DNCB) in contact hypersensitivity (Roers et al., 2004). Furthermore, Treg-specific IL-10 deletion increased contact hypersensitivity to 1-fluoro-2,4-dinitrobenzene (DNFB) and allergic lung inflammation in response to sensitization to ovalbumin (Rubtsov et al., 2008). T cell-specific deletion of IL-10 has demonstrated the importance of T cells as a source of IL-10 in combating infections. Interestingly, IL-10 can have beneficial or detrimental effects and the balance between this is dependent on the model (Ouyang et al., 2011; Redford et al., 2011; Wilson and Brooks, 2011; O'Garra et al., 2008). As IL-10 is anti-inflammatory, the loss of IL-10 can cause an enhanced immune reaction, which results in faster pathogen clearance. Alternatively, loss of IL-10 may allow excessive inflammation and therefore the development of cytokine storms resulting in tissue damage or mortality (Saraiva and O'Garra, 2010; O'Garra et al., 2008).

1.10.5.2 B-cell and myeloid cell derived IL-10

B cells have been shown to produce IL-10 in several *in vivo* models including i.p. injection of LPS, EAE, colitis and infection (reviewed in Kalampokis et al., 2013; Vitale et al., 2010). Only a small proportion of B cells can produce IL-10 and these cells are known as B10 cells. *In vivo*, B10 cells appear to play important roles as demonstrated in experimental autoimmune encephalomyelitis (EAE), a mouse model for multiple sclerosis. EAE has both an induction phase with active disease, followed by a remission phase in which disease activity reduces. Mice with a disruption in the Ig μ heavy chain (μMT), have a lack of B cells and develop a non-remitting form of EAE. This suggest an important role for B cells in this model and, significantly, transfer of wild type B cells restored remission. However, B cells from IL-10 deficient mice were unable to do this (Fillatreau et al., 2002). Another study has shown that total IL-10 knockout results in an increased severity of EAE (Bettelli et al., 1998).

Total IL-10 knockout leads to an increased sensitivity to endotoxic shock induced by LPS and elevated levels of pro-inflammatory cytokines such as TNFα, IL-12 and IL-6 were also observed. Using Vert-X IL-10 reporter mice, B cells were shown to be the major source of splenic IL-10 in LPS treated mice, while in the blood, CD11b+ myeloid cells

were the major source of IL-10 (Madan et al., 2009). Deletion of IL-10 in myeloid cells increased lethality and TNFα production relative to wild type mice following i.p. injection of LPS. Deletion of IL-10 in T cell or B cells, however, did not sensitize mice to LPS; thus demonstrating the differing contribution of individual cell types to the model studied (Madan et al., 2009; Siewe et al., 2006). Interestingly, the effect of myeloid specific IL-10 deletion was not as severe as the total IL-10 knockout, which suggests another IL-10-producing cell is important in this model.

1.10.5.3 Mast cell-derived IL-10

Mast cells have also been found to produce IL-10 *in vivo*. Mast-cell derived IL-10 is important in the context of chronic bladder infection (Chan et al., 2013). In this study, reconstitution of mast cell-deficient mice with IL-10-deficient bone marrow-derived mast cells (BMMCs) led to lower bacterial persistence compared to control mice. A similar decrease in bacterial persistence was evident in Mcpt5-cre IL-10 fl/fl (a mast-cell specific deletion of IL-10) and $IL10^{-/-}$ mice. This study demonstrated that mast cells are a major source of IL-10 within the bladder and that mast cell-derived IL-10 allows a chronic infection to be established.

The importance of IL-10 in regulating the immune response and promoting resolution is underlined by the detrimental effect that loss of IL-10 has in inflammatory and disease models. Therefore it is important to understand the mechanisms that govern the regulation of IL-10 transcription and how different signals can modulate the transcription of IL-10.

1.11 Type I interferons

Type I interferons were described originally as being responsible for the ability of a viral infection to generate resistance to infection with a different virus (Isaacs and Lindenmann, 1957; Nagano and Kojima, 1958). IFNα and IFNβ are the most widely expressed type I interferons (Decker et al., 2005). Type I interferons are often produced in response to viral nucleic acids sensed by cytosolic receptors such as RIG-I or endosomal receptors such as TLR3 and TLR7 (Kawai and Akira, 2010). Recognition of nucleic acids by the

appropriate TLR leads to the activation of IRF3 or IRF7 and results in the transcription of type I interferon genes. Notably, IFNβ can drive a positive feedback loop by inducing IRF7 expression which results in the transcription of type I interferon genes (Marié et al., 1998). More recently, it has been shown that type I interferons can be produced downstream of NOD-like receptors (Pandey et al., 2009; Watanabe et al., 2010). NOD1 and NOD2 recognise peptides from bacterial cell walls and initiate signaling via Rip2 leading to NFχB activation. Additionally, RIP2 can interact with TRAF3 and activates TBK1 resulting in IRF7 activation and the transcription of IFNβ. Type I interferons are crucial to the immune response and induce transcription of a subset of genes known as interferon-stimulated genes (ISGs).

1.11.1 IFNβ signaling

Type I interferons signal through a heterodimeric receptors consisting of the IFNAR1 and IFNAR2 chains (Taniguchi and Takaoka, 2001; Trinchieri, 2010). IFNβ binds to the type I interferon receptor and thus activates Tyk2 and JAK1, leading to recruitment and tyrosine phosphorylation of STAT1 and STAT2 molecules (Li et al., 1996, 1997). STAT1 and STAT2 molecules will recognise phosphorylated tyrosine residues through their SH2 domains and form a heterodimer. STAT1-STAT2 heterodimers form a complex with IRF9 and then translocate to the nucleus. This complex is known as IFN-stimulated gene factor 3 (ISGF3) and induces transcription of ISGs (figure 1.5).

1.11.2 IFNβ in the immune response

Type I interferons are crucial in anti-viral responses and are also induced during bacterial infections. However, type I interferons can also play detrimental roles in some contexts (reviewed in Yao et al., 2013; Asselah, 2012; Rönnblom and Eloranta, 2013; Inoue and Shinohara, 2013; Banchereau and Pascual, 2006). IFNβ will induce transcription of the ISGs which include PKR, CXCL10, CCL2 and CCL7 (Schoggins and Rice, 2011; Trinchieri, 2010). The expression of this subset of genes is designed to increase the ability of cells to detect and eliminate viruses. It will also enhance leukocyte recruitment to promote pathogen clearance. Type I interferon signaling has also been implicated in several diseases. In systemic lupus erythematosus, overproduction of IFN leads to peripheral

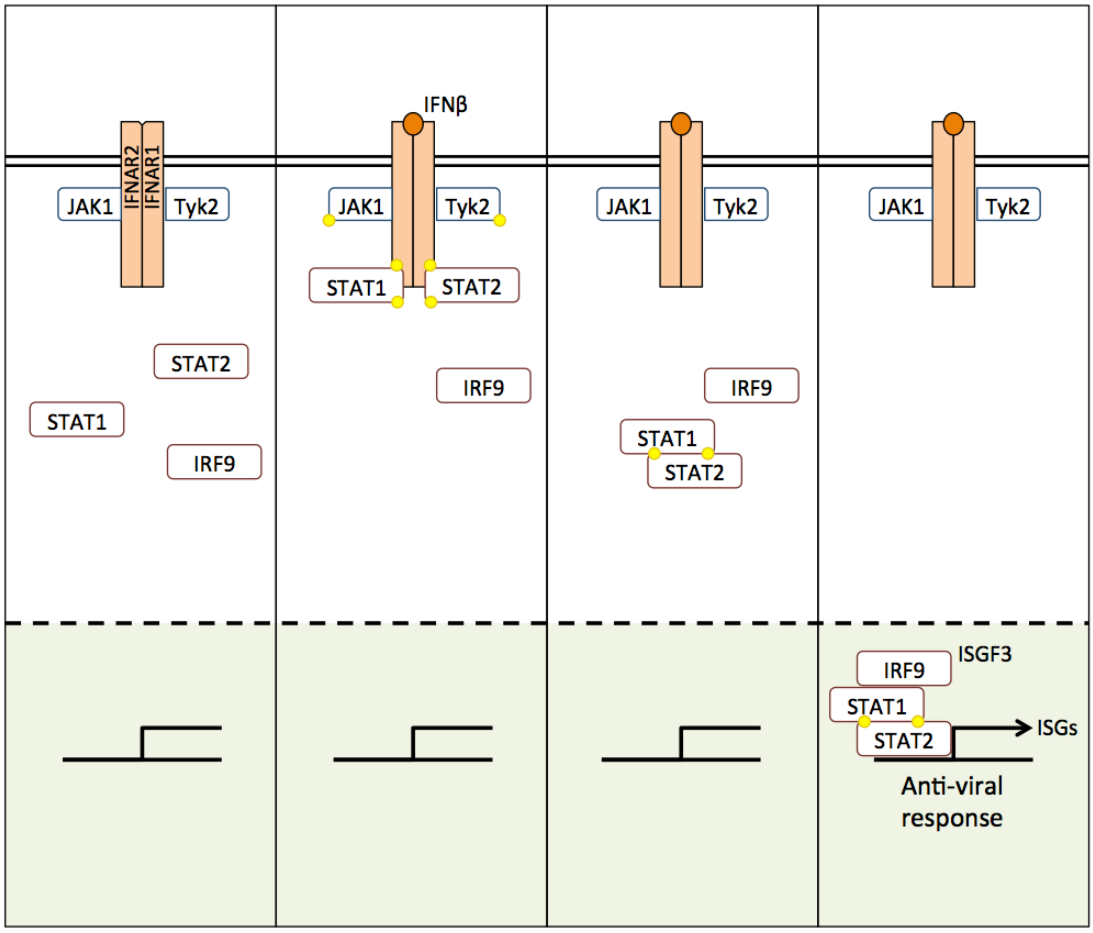

Figure 1.5: Type I interferon signaling
Type I interferons bind to the type I IFN receptor and this leads to activation of JAK1 and Tyk2. JAK1 and Tyk2 then phosphorylate STAT1 on Tyr701 and STAT2 on Tyr689. STAT1 and STAT2 dissociate from the receptor and form a dimer. In the cytoplasm, they interact with IRF9 to form the complex known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 translocates to the nucleus and binds to promoters of target genes involved in the anti-viral response.

tolerance breakdown (reviewed in Banchereau and Pascual, 2006). This is caused by the activation of myeloid dendritic cells, which leads to the activation of auto reactive T cells and expansion of auto reactive B cells. An increase of apoptotic cells, which can be captured by DCs, leads to an amplification of this autoimmune reaction. Recent treatment strategies have targeted blocking IFNα, its receptor or the plasmacytoid DC responsible for producing large amount of IFNα (Kirou and Gkrouzman, 2013).

1.11.3 Effects of type I interferon on TLR signaling

Type I interferons are induced downstream of several TLRs including TLR4. Intraperitoneal injection of LPS, which activates TLR4, causes endotoxic shock and, dependent on dose, lethality. It has been shown that deficiency in type I interferons reduces LPS-

induced lethality in mice (Karaghiosoff et al., 2003). Similar results are seen with mice lacking STAT1 and Tyk2 which are activated downstream of type I IFN signaling (Kamezaki et al., 2004). Interestingly, mice lacking the type I IFN receptor have increased mortality in a model of cecal ligation and puncture (CLP)-induced sepsis (Kelly-Scumpia et al., 2010). In contrast, STAT1- (and to some extent Tyk2-) deficient mice are protected from CLP-induced sepsis (Herzig et al., 2012). Deletion of STAT1 may block signaling downstream of type II interferons as well as type I interferons, thus complicating interpretation of the results. Blocking type I interferon signaling leads to abnormal responses and underlines their role in regulating TLR signaling. Recently, the importance of IFNβ was demonstrated for immunity to *C.albicans* (del Fresno et al., 2013). Dectin-1 stimulation initiated IFNβ production in a Syk-IRF5 dependent manner. Deficiency of the type I interferon receptor reduced survival following infection.

1.11.4 JAK inhibitors

Cytokines control the growth and differentiation of haematopoietic cells as well as regulating the immune response. It is also becoming clear that cytokines play an important role in immune-mediated diseases but also in other pathologies, including atherosclerosis and cancer. Therefore, the ability to therapeutically target the action of cytokines would have health benefits. Most cytokines signal via JAK/STAT pathways and with the ability to design specific inhibitors of kinases, the four JAK family members became attractive targets (Kontzias et al., 2012).

1.11.4.1 Ruxolitinib

Ruxolitinib (INCB018424) entered clinical trials in 2007 and has 3.3nM and 2.8nM IC50 against JAK1 and JAK2, respectively (Mesa et al., 2012; Quintás-Cardama et al., 2010). It has 6-fold selectivity over Tyk2 (19nM) and much higher selectivity over JAK3 (428nM) (Quintás-Cardama et al., 2010). Ruxolitinib is currently used to treat myeloproliferative disorders and is also undergoing clinical trials now as a treatment for plaque psoriasis (Punwani et al., 2012; Fridman et al., 2011). In the phase III clinical trials for use against myelofibrosis, Ruxolitinib reduced spleen volume, improved symptoms and improved overall survival compared to placebo (Harrison et al., 2012; Verstovsek et al., 2012).

1.11.4.2 Tofacitinib

Tofacitinib (CP690550) was developed by Pfizer and is now approved for the treatment of RA (Burmester et al., 2013; Fleischmann et al., 2012b,a; van der Heijde et al., 2013; Yamaoka and Tanaka, 2014). Tofacitinib is described as a selective JAK1/JAK3 inhibitor with IC50 of 2.2nM for JAK3 (Ghoreschi et al., 2011). In trials, Tofacitinib improved the ACR (American College of Rheumatology) score by 20% in 67% of patients compared to 25% who received a placebo. Tofacitinib is also being trialled for use against psoriasis and improved the psoriasis area and severity index (PASI) score by more than 75% in 25-67% of patients, depending on dose, compared to only 2% of patients receiving placebo (Ports et al., 2013; Mamolo et al., 2013; Boy et al., 2009). The effectiveness of JAK inhibition is also being examined in the treatment of inflammatory bowel disease (IBD) (Vuitton et al., 2013; Coskun et al., 2013) Tofacitinib is FDA approved but has side effects including neutropenia that may be linked to JAK2 inhibition (Gupta et al., 2010; Salgado et al., 2013).

1.11.4.3 Other JAK inhibitors for inflammatory diseases

Other JAK inhibitors are being used in clinical trials. Ruxolitinib and Tofacitinib have been approved for clinical use. Three other compounds, Baricitinib, Decernotinib and Filgotinib, are in clinical development. Baricitinib is developed by Incyte who developed Ruxolitnib and has a similar chemical structure (Fridman et al., 2010; Clark et al., 2014). It has a similar specificity for JAK1/2 over JAK3. Baricitinib has shown efficacy in a rodent model of arthritis and can block IL-6 and IL-23 signaling. Baricitinib has completed phase 2 studies for RA. Decernotinib is being developed for RA and is currently in phase II trials. Decernotinib is a potent JAK3 inhibitor with low nM IC50 for JAK3. Decernotinib was shown to improve histological scores and reduce ankle swelling and paw weight in a rat model of collagen-induced arthritis (Fleischmann, 2012; Clark et al., 2014). Filgotinib is being developed by Galapagos and has completed a phase II trial for RA (Norman, 2012; Van Rompaey et al., 2013). Filgotinib shows selectivity for JAK1 over the other JAKs (Clark et al., 2014).

1.11.5 IRF3 and IRF7

IRF3 and IRF7 are highly homologous and act as important regulators of type I interferon gene expression (Honda et al., 2006). IRF3 is constitutively expressed and resides in the cytosol in resting cells. Viral infection stimulates the phosphorylation, dimerisation and nuclear translocation of IRF3 (Lin et al., 1998; Sato et al., 1998b; Yoneyama et al., 1998). Phosphorylation at Ser386 is thought to be required for activation and results in either the phosphorylated serine interacting with a hydrophobic pocket on another IRF3 molecule or the introduction of conformational change which allows IRF dimerisation (Mori et al., 2004; Takahasi et al., 2003; Qin et al., 2003). Once dimerised and in the nucleus, IRF3 homodimers or IRF3/IRF7 heterodimers will bind to target DNA sequence and recruit the coactivators CBP and p300 (Lin et al., 1998; Sato et al., 1998b; Yoneyama et al., 1998).

IRF7 is expressed at low levels and is induced by type I interferon signaling (Marié et al., 1998; Sato et al., 1998a). Similarly to IRF3, IRF7 becomes phosphorylated following viral infection and thus results in dimerisation and nuclear translocation. IRF7 can efficiently induce transcription of both IFNα genes and the IFNβ gene, whereas IRF3 preferentially activates the IFNβ gene (Marié et al., 1998; Sato et al., 1998a). IRF7 exhibits a very short half life of approximately 0.5-1hr as it is subject to ubiquitin-mediated proteasomal degradation (Sato et al., 2000; Yu et al., 2005). This rapid turnover of IRF7 may act to curtail the induction of type I interferon gene expression. Both IRF3 and IRF7 can be activated downstream of cytosolic PRRs such as RIG-I and from transmembrane receptors such as TLR3.

1.12 Aims

The aim of this thesis was to identify pathways that regulate the production of IL-10. Transcription of IL-10 is controlled by several transcription factors including CREB and NF$\varkappa$B. Other pathways may regulate the production of IL-10 in macrophages. The importance of IFNβ-mediated feedback was examined on IL-10 production. Additionally, the role of IFNβ-mediated feedback was investigated on MCP-1 production. The transcription factor, MEF2D, has been implicated in IL-10 transcription in T cells (Liopeta

et al., 2009) and its role in macrophage function was examined. Lastly, the importance of sphingosine kinase 1, a marker of the regulatory macrophage phenotype, was examined in Zymosan-stimulated macrophages and its role in regulating IL-10 production in response to Zymosan stimulation.

Chapter 2

Materials and Methods

2.1 Materials

All chemicals were obtained from Sigma or VWR unless otherwise stated. All solutions were prepared using deionised water (MilliQ system, Millipore) and were autoclaved at 121°C, 15 psi for 20 minutes where appropriate.

2.2 Common solutions

Common solutions used throughout the work are listed in table 2.1.

2.3 Animals

IFN$\alpha\beta$R knockout mice were obtained from Dr Anne O'Garra (National Institute of Medical Research). IL-10 knockout mice were obtained from the Jackson Laboratory. IFN$\alpha\beta$R knockout mice and IL-10 knockout mice were backcrossed with C57BL/6J mice for a minimum of 12 generations. MEF2D KO mice were generated through crossing a floxed allele with Bal1, to generate total MEF2D knockout mice (See Appendix E- MEF2D targeting strategy). MEF2D knockout mice have been backcrossed onto C57Bl/6J for at least 6 generations. ERK5 mice were obtained from Dr Cathy Tournier (University of Manchester). ERK5 cKO mice were generated through crossing a floxed allele with Vav, to generate a conditional ERK5 knockout mice. All mice were maintained in specific

Table 2.1: Common solutions

Solution	
5x Sample Buffer	250 mM Tris-HCl pH 6.8 32.5% (v/v) Glycerol 5% (w/v) Sodium Dodecyl Sulphate 5% (v/v) 2-β-Mercaptoethanol
SDS-PAGE Running Buffer	25 mM Tris 192 mM Glycine 0.1% (w/v) SDS
SDS-PAGE Separating Gel	0.375 M Tris-HCl pH 8.6 10% (w/v) 29:1 Acrylamide:Bis-acrylamide 0.01% (w/v) Sodium Dodecyl Sulphate 0.034% (w/v) Ammonium Persulphate 0.08% (v/v) Temed
SDS-PAGE Stacking Gel	0.124 M Tris-HCl pH 6.8 3.73% (w/v) 29:1 Acrylamide:Bis-acrylamide 0.01% (w/v) Sodium Dodecyl Sulphate 0.085% (w/v) Ammonium Persulphate 0.1% (v/v) Temed
Transfer Buffer	48 mM Tris 39 mM Glycine 20% (v/v) Methanol
Tris Buffered Saline and Tween (TBS-T)	50 mM Tris pH 7.5 150 mM NaCl 0.1% (v/v) Tween-20
Triton Lysis Buffer	50 mM Tris-HCl pH 7.5 1 mM EDTA 1 mM EGTA 1 % (v/v) Triton X-100 1 mM Sodium Orthovanodate 50 mM Sodium Fluoride 5 mM Sodium Pyrophosphate 10 mM Sodium β-Glycerophosphate 0.27M Sucrose 0.1% 2-β-Mercaptoethanol 1x protease inhibitor tablet (Roche)

pathogen free conditions and in line with United Kingdom and European Union regulations. Work was approved by local ethical review and was carried out under the authority of a Home Office project license.

All routine animal care and biopsies were undertaken by the staff of the Transgenic Unit, University of Dundee. All mouse colony maintenance and breeding was organised by Dr. Arthur. PCR genotyping of mouse biopsies was undertaken by Julia Carr (MRC PPU, University of Dundee) and Dr. Arthur.

2.4 Cell culture

2.4.1 Isolation and culture of BMDMs

Bone marrow-derived macrophages (BMDMs) were derived from adult mice. Mice were sacrificed and femurs removed under sterile conditions. The femurs were flushed with sterile PBS to remove the bone marrow. The bone marrow suspension was passed through a 100 µm cell strainer (BD Biosciences) and then centrifuged at 900 rpm for 5 minutes. The supernatant was aspirated and the pellet resuspended in 20 ml of BMDM media (DMEM (Gibco) with 10% heat inactivated FBS (Biosera), 5 mM L-Glutamine (Gibco), 100 U/ml penicillin G (Gibco), 100 µg/ml streptomycin (Gibco), 0.25 µg/ml amphotericin (Gibco) and 5 ng/ml M-CSF (R&D Systems)). The cell suspension was plated onto two 10 cm bacteriological grade plates and incubated at 37°C in 5% CO_2 for seven days.

After seven days, the media was aspirated and the plates washed with PBS. The cells were then detached with PBS containing 0.5 mM EDTA and scraped. The cell suspension was centrifuged at 900 rpm for 5 minutes and the supernatant aspirated. The pellet was resuspended in BMDM media and replated in 6-well or 12-well plates. Cells were incubated overnight at 37°C in 5% CO_2. Cell counts were performed using Trypan Blue (Gibco) and a haemocytometer.

2.4.2 Culture of Raw 264.7 and HeLa cells

Raw 264.7 and HeLa cells were cultured in DMEM containing 10% (v/v) FBS (Biosera), 2 mM L-Glutamine, (Gibco), 100 U/ml penicillin G (Gibco), 100 µg/ml streptomycin (Gibco) in 75 cm^2 flasks for routine maintenance at 37°C in 5% CO_2. Cells were replated in 10 cm plates or 6 well plates for experiments.

2.4.3 Stimulation of cells

Cells were incubated with or without inhibitors at the final concentration indicated in table 2.2 for 1 hour before stimulation. Inhibitors were dissolved in DMSO and added to the culture medium. Cells were stimulated for various times with various agonists: 100 ng/ml LPS (Sigma, from *Escherichia coli* 026:B6), 1 µg/ml Pam3CSK4 (Invivogen), 2

μM CPG (Invivogen), 10 μg/ml poly(I:C), 1 μg/ml R848, 200 μg/ml Zymosan (Sigma), 10 μg/ml Curdlan (Sigma), 1 μM S1P, 10 μM DHS1P, 100 ng/ml EGF or 0.5M Sorbitol. Recombinant mouse IL-10 (R&D Systems) was added at 100 ng/ml and recombinant mouse IFNβ (PBL interferon) at 500 U/ml. IL-10 neutralising antibody (provided by Anne O'Garra, National Institute of Medical Research) was used at 2.5 μg/ml. Culture media was aspirated or collected for cytokine analysis and cells were lysed for protein or RNA as described in section 2.5 or 2.6.

Table 2.2: Small molecule inhibitors

Name	Target	Conc.	Stock Conc.	Source	Reference
Ruxolitinib	JAK1/2	0.5 μM	0.5 mM	Selleck Chemicals	(Quintás-Cardama et al., 2010)
Tofacitinib	JAK1/3	5 μM	5 mM	Selleck Chemicals	(Manshouri et al., 2008)
SK1-I	SphK1	10 μM	10 mM	Enzo Life Sciences	(Paugh et al., 2008b)
SK1 II	SphK1/2	10 μM	10 mM	Merck	(French et al., 2003)
PF-543	SphK1	5 μM	5 mM	Millipore	(Schnute et al., 2012)
THI	S1P lyase	5 μM	5 mM	Sigma	(Schwab et al., 2005)
ERK5-IN-1	ERK5	5 μM	5 mM	Nathanael Gray	(Deng et al., 2013)

2.5 Lysis for protein

Cells were washed twice with ice-cold PBS and then lysed using Triton Lysis buffer (table 2.1). Cell lysates were collected on ice using a cell scraper and transferred to a 1.5 ml tube. Lysates were centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatants were transferred to a new 1.5 ml tube and stored at -80°C.

2.5.1 Determination of protein concentration by Bradford assay

Clarified lysate was diluted where appropriate and 5 μl added to 250 μl of Coomassie Bradford reagent (Thermo) in a 96-well plate in triplicate. The absorbance at 595 nm was read against a blank sample using a Versamax tunable microplate reader (Molecular Devices) and Softmax pro 4.0 software. The protein concentration was calculated using the average absorbance against a standard curve of known BSA concentrations.

2.6 Lysis for RNA and RNA isolation

Culture medium was aspirated and RLT lysis buffer (a proprietary lysis buffer for lysing cells and tissues prior to RNA isolation) with 1% (v/v) 2-β-Mercaptoethanol added. Cells

were collected using a scraper and the lysate added to a QIAshredder column (Qiagen). The column was centrifuged at 13,000rpm for 2 minutes and the column and lysate stored at -80°C. Total RNA was extracted from the lysate using RNeasy micro kit (Qiagen) following the manufacturer's instructions. RNA was eluted in 15 µl of RNase free water and stored at -80°C.

2.6.1 Determination of RNA concentration and reverse transcription

1.5 µl of the eluate was quantified using Nanodrop 1000 (Thermo Scientific) as per the manufacturer's instructions. Absorbance at 260 nm was read and concentration determined using the following equation:

$$A = \varepsilon bc$$

where A is the absorbance, ε is the extinction coefficient (0.025 $(\mu g/ml)^{-1}$ cm^{-1}), b is the length of the light path and c is the concentration of the RNA in the solution.

0.5 to 1 µg of RNA was reverse transcribed using iScript (Bio-Rad) in a 20 µl reaction volume containing 4 µl iScript reaction mix and 1 µl iScript reverse transcriptase as per the manufacturer's instructions. Samples were incubated at 25°C for 5 minutes, 42°C for 30 minutes and then 85°C for 5 minutes. Samples were diluted 1:10 with RNase free water and stored at -20°C.

2.7 Real Time-Quantitative PCR

2.7.1 qPCR reaction and cycling conditions

Quantitative PCR was performed in 96- or 384-well plates (BioRad) using a SYBR green based detection system, SsoFast EvaGreen Supermix (BioRad), with a CFX 96 or CFX 384 thermal cycler (BioRad). Each reaction compromised 6.6 µl SYBR, 2.4 µl nuclease free water, 0.5 µl 10 µM sense primer, 0.5 µl 10 µM antisense primer and 4 µl cDNA. Samples were heated to 95°C for 30 seconds and then underwent 40-50 cycles of 95°C for 1 second and 60°C for 25 seconds. This was followed by 95°C for 1 minute, 65°C for 1 minute then 0.5°C increments every 5 seconds to calculate a melt curve.

2.7.2 qPCR analysis

Each sample was quantified in duplicate and an average cycle threshold value taken. 18S ribosomal RNA values were used for normalisation. The relative mRNA levels were calculated using the following equation:

$$Relative\,mRNA\,level = \frac{E_u^{(ct_{uc}-ct_{us})}}{E_r^{(ct_{rc}-ct_{rs})}}$$

where E is efficiency of PCR, Ct is the threshold cycle, u is the mRNA of interest, r is the 18S ribosomal RNA, s is the sample and c is the unstimulated control.

2.7.3 qPCR primers

Primers were designed using Beacon Designer 7.0 software programme and custom synthesised by Invitrogen. The primers were reconstituted to 100 µM in RNase free water and then diluted to 10 µM for use. All primers were stored at -20°C. Primer sequences are shown in table 2.3.

Table 2.3: qPCR primers

Primer Name	Sense	Antisense
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
IL-10	CCCTTTGCTATGGTGTCCTTTC	GATCTCCCTGGTTTCTCTTCCC
Arg-1	CTCCAAGCCAAAGTCCTTAGAG	AGGAGCTGTCATTAGGGACATC
COX2	AATATCAGGTCATTGGTGGAGAGG	TCAGACCAGGCACCAGACC
Interferon β	GGAAAAGCAAGAGGAAAGATTGAC	CCACCATCCAGGCGTAGC
IL-1b	GACGGACCCCAAAAGATGAAGG	GTGATACTGCCTGCCTGAAGC
IL-6	TTCCATCCAGTTGCCTTCTTG	AGGTCTGTTGGGAGTGGTATC
IL-12a (p35)	TATCTCTATGGTCAGCGTTCC	TGGTCTTCAGCAGGTTTCG
IL-12b (p40)	TCATCAGGGACATCATCAAACC	TGAGGGAGAAGTAGGAATGGG
IL-27 (p28)	GAGGAGGACAAGGAGGAAGAGG	GGGAGTGAAGGAGCTGGTAGC
LIGHT	CTGCATCAACGTCTTGGAGA	GATACGTCAAGCCCCTCAAG
Nur77	CCTGTTGCTAGAGTCTGCCTTC	CAATCCAATCACCAAAGCCACG
MCP-1	TTTGAATGTGAAGTTGACCCGTAAATC	TCACTGTCACACTGGTCACTCC
A20	ACTGGAATGACGAATGG	CTTCTGAGGATGTTGCT
DUSP1	CCACAGGACACCGCACAAG	AGCGAAGAAGGAGCGACAATC
DUSP2	CGTGCCGTGGTGCTGGATG	TGAAACCGCCTCGCAAGAAGC
DUSP5	CTGAGTGCTGTGTGGATGTG	TAGGCGACGCTGAGAACG
c-Jun	CGCCTCGTTCCTCCAGTC	ACGTGAGAAGGTCCGAGTTC
SPHK1	ACAGCAGTGTGCAGTTGATGA	GGCAGTCATGTCCGGTGATG
TNFα	CAGACCCTCACACTCAGATCATC	GGCTACAGGCTTGTCACTCG
EBI3	GCCGCTCCCCTGGTTA	CAATGAAGGACGTGGATCTGGTC

2.8 Cytokine secretion determination by Luminex-based assay

Culture supernatant was collected following stimulation and was stored at -80°C. Samples were thawed and secreted cytokines measured by the Luminex-based Bioplex assay (BioRad) as per the manufacturer's instructions.

2.9 Detection of protein by western blotting

2.9.1 Resolution of protein samples by SDS-PAGE

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed using the ATTO system (ATTO corporation). Samples were denatured by adding 5x sample buffer containing fresh 0.1% (v/v) 2-β-Mercaptoethanol and heated for 10 mins at 95°C. 30 µg of protein of each sample was loaded onto 10% Tris-glycine gels with Tris-glycine running buffer. Gels were run at a constant voltage of 100V for 180 minutes. Solutions required for polyacrylamide gels are shown in table 2.1.

2.9.2 Electrophoretic transfer of protein from SDS PAGE gels

Proteins were transferred from SDS-PAGE gels onto nitrocellulose membranes using Mini Trans-Blot Electrophoretic Transfer Cell (BioRad). Blotting pads and nitrocellulose membranes were equilibrated in transfer buffer prior to use. The gel sandwich was set up as followed from cathode side to anode side: blotting pad, Whatman 3 MM paper, gel, nitrocellulose membrane, Whatman 3 MM paper, blotting pad. The gel sandwich was placed in the Transfer Cell and completely submerged in transfer buffer. The transfer was carried out at 100V for 1 hour.

2.9.3 Western blotting

Membranes were blocked by incubation in TBS-T containing 5% (w/v) dried milk powder at room temperature. The membranes were subsequently incubated overnight at 4°C in primary antibody diluted in TBS-T containing 5% (w/v) dried milk powder. The antibodies used are listed in table 2.4. Membranes were washed three times in TBS-T and

then incubated with horseradish-peroxidase conjugated secondary antibody at room temperature for 1 hour. Secondary antibodies were used at 1:2000 (anti-Rabbit IgG), 1:2000 (anti-Goat IgG) 1:1000 (anti-Mouse IgG) and 1:2000 (anti-Sheep IgG). Immunoreactive proteins were visualised using ECL reagent (Amersham GE) according to the manufacturer's instructions and by subsequent exposure to X-ray film (Konica Minolta), which was developed and fixed in an automatic processor.

Table 2.4: Antibodies

Protein	Epitope	Concentration	Source	#
ERK1/2	Total	1:1000	Cell Signaling	9102
ERK1/2	phospho (T202/Y204)	1:1000	Cell Signaling	9101
ERK5	Total	1:2000	Sigma	E1523
p38	Total	1:1000	Cell Signaling	9212
p38	phospho (T180/Y182)	1:1000	Cell Signaling	9211
JNK	phospho (T183/Y185)	1:1000	Invitrogen	44682G
STAT1	Total	1:1000	Cell Signaling	9172
STAT1	phospho (Y701)	1:1000	Cell Signaling	9167
STAT2	phospho (Y689)	1:1000	Abcam	53132
STAT3	Total	1:1000	Cell Signaling	4904
STAT3	phospho (Y705)	1:1000	Cell Signaling	9131
IκBa	Total	1:1000	Cell Signaling	4814
IκBa	phospho (S32)	1:1000	Cell Signaling	2859
p105	phospho (S933)	1:1000	Cell Signaling	4806
COX2	Total (M-19)	1:1000	Santa Cruz	sc-1747
Akt	Total	1 mg/ml	DSTT	S742B/2nd
Akt	phospho (T308)	1 mg/ml	Cell Signaling	9275
Akt	phospho (S473)	1 mg/ml	Cell Signaling	9271
A20	Total	1:1000	Cell Signaling	5630
MEF2D	Total	1:1000	BD Transduction	610774
MEF2D	phospho (S121)	1 mg/ml	DSTT	R3084/3rd

2.10 Immunoprecipitation

Protein G sepharose slurry was centrifuged at 14,000 rpm for 1 min at 4°C and the supernatant aspirated. Protein G sepharose was then washed twice with triton lysis buffer. 1 µg of antibody was added for each immunoprecipitation and coupled to the beads for 1 hour at 4°C on a shaking platform. Antibody-coupled protein G sepharose was then washed three times with triton lysis buffer and then 0.2-0.5 mg of protein lysate was added. Lysate and coupled antibody was incubated for 3 hours at 4°C on a shaking platform. Immunoprecipitations with phospho antibodies also contained 10 µg of dephospho-peptide per 1 µg of antibody at the coupling stage. Immunoprecipitation reactions were washed twice in lysis buffer and then resuspended in 2x sample buffer. Samples were then heated at

95°C for 10 minutes prior to SDS-PAGE and western blotting analysis.

2.11 Lipid mass spectrometry (performed by Jeremy C. Allegood, School of Medicine, Virginia Commonwealth University)

2.11.1 Materials

Internal standards were purchased from Avanti Polar Lipids (Alabaster, AL). Internal standards were added to samples in 20 μL ethanol:methanol:water (7:2:1) as a cocktail of 500 pmol each. Standards for sphingoid bases and sphingoid base 1-phosphates were 17-carbon chain length analogs: C17-sphingosine, (2S,3R,4E)-2-aminoheptadec-4-ene-1,3-diol (d17:1-So); C17-sphinganine, (2S,3R)-2-aminoheptadecane-1,3-diol (d17:0-Sa); C17-sphingosine 1-phosphate, heptadecasphing-4-enine-1-phosphate (d17:1-So1P); and C17-sphinganine 1-phosphate, heptadecasphinganine-1-phosphate (d17:0-Sa1P). Standards for N-acyl sphingolipids were C12-fatty acid analogs: C12-Cer, N-(dodecanoyl)-sphing-4-enine (d18:1/C12:0); C12-Cer 1-phosphate, N-(dodecanoyl)-sphing-4-enine-1-phosphate (d18:1/C12:0-Cer1P); C12-sphingomyelin, N-(dodecanoyl)-sphing-4-enine-1-phosphocholine (d18:1/C12:0-SM); and C12-glucosylceramide, N-(dodecanoyl)-1-β-glucosyl-sphing-4-eine. The HPLC grade solvents (chloroform, # EM-CX1050; and methanol, # EM-MX0475, as well as formic acid (ACS grade, # EM-FX0440-7), were obtained from VWR (West Chester, PA). For LC-MS/MS analyses, a Shimadzu LC-20 AD binary pump system coupled to a SIL-20AC autoinjector and DGU20A3 degasser coupled to an ABI 4000 quadrupole/linear ion trap (QTrap) (Applied Biosystems, Foster City, CA) operating in a triple quadrupole mode was used. Q1 and Q3 was set to pass molecularly distinctive precursor and product ions (or a scan across multiple m/z in Q1 or Q3), using N2 to collisionally induce dissociations in Q2 (which was offset from Q1 by 30-120 eV); the ion source temperature set to 500 °C.

2.11.2 Extraction of sphingolipids

Cells were collected by scraping and transferred into 13 x 100 mm borosilicate tubes with a Teflon-lined cap (catalog #60827-453, VWR, West Chester, PA). Then 1 mL of CH_3OH and 0.5 mL of $CHCl_3$ were added along with the internal standard cocktail (500 pmol of

each species dissolved in a final total volume of 20 μl of ethanol:methanol:water 7:2:1). The contents were dispersed using an ultra sonicator at room temperature for 30 s. This single phase mixture was incubated at 48°C overnight. After cooling, 75 μl of 1 M KOH in CH_3OH was added and, after brief sonication, incubated in a shaking water bath for 2 h at 37°C to cleave potentially interfering glycerolipids. The extract was brought to neutral pH with 6 μl of glacial acetic acid, then the extract was centrifuged using a table-top centrifuge, and the supernatant was removed by a Pasteur pipette and transferred to a new tube. The extract was reduced to dryness using a Speed Vac. The dried residue was reconstituted in 0.5 ml of the starting mobile phase solvent for LC-MS/MS analysis, sonicated for ca 15 sec, then centrifuged for 5 min in a tabletop centrifuge before transfer of the clear supernatant to the autoinjector vial for analysis.

2.11.3 LC-MS/MS of sphingoid bases, sphingoid base 1-phosphates and complex sphingolipids

These compounds were separated by reverse phase LC using a Supelco 2.1 (i.d.) x 50 mm Ascentis C18 column (Sigma, St. Louis, MO) and a binary solvent system at a flow rate of 0.5 mL/min with a column oven set to 35°C. Prior to injection of the sample, the column was equilibrated for 0.5 min with a solvent mixture of 95% Moble phase A1 (CH_3OH/H_2O/HCOOH, 58/41/1, v/v/v, with 5 mM ammonium formate) and 5% Mobile phase B1 (CH_3OH/HCOOH, 99/1, v/v, with 5 mM ammonium formate), and after sample injection (typically 40 μL), the A1/B1 ratio was maintained at 95/5 for 2.25 min, followed by a linear gradient to 100% B1 over 1.5 min, which was held at 100% B1 for 5.5 min, followed by a 0.5 min gradient return to 95/5 A1/B1. The column was re-equilibrated with 95:5 A1/B1 for 0.5 min before the next run.

2.12 SILAC and Mass Spectrometry (Mass spectrometry performed by Matthias Trost, University of Dundee)

Raw 264.7 cells were labeled with amino acids synthesised with stable isotopes of carbon, nitrogen and hydrogen. Cells were grown in DMEM media containing exclusively ‘light’ or ‘medium’ forms of arginine and lysine: the ‘light’ amino acids contain the common

isotopes of hydrogen, carbon and nitrogen and the 'medium' amino acids contain 6 13C atoms and 4 deuterium (2H) atoms respectively. Cells were passaged at least five times in the SILAC media to ensure complete labelling of amino acids within the cell. Cells were stimulated with LPS for 30 minutes, washed with ice cold PBS and lysed in 8M urea. Protein levels were determined by Bradford assay (section 2.5.1). Equal amounts of protein from each SILAC condition were mixed. The cysteine residues in the protein sample were reduced by incubation in 75 μL 10 mM dithiothreitiol/0.1 M ammonium biocarbonate at 65 ºC for 45 min and then alkylated using 75 μL 50 mM iodoacetamide at room temperature for 20 min. The samples were then washed in 50% methanol/0.1M ammonium bicarbonate to remove any remaining reducing and alkylating reagents. The soluble proteins were then digested with trypsin. Phospho-peptides were then enriched by sequential hydrophilic chromatography followed by Fe^{3+}-immobilized metal affinity chromatography. Phospho-peptides were measured by tandem mass spectroscopy on Thermo Fisher Scientific LTQ Orbitrap Velos instrument set to perform top-15 data-dependent collision-induced dissociation analysis in the 350–1600 m/z range using a resolution of 60,000 for the precursor scan and a minimal intensity for sequencing of 10,000 counts. Monoisotopic precursor selection was used, and +1 as well as unassigned charge states were excluded from sequencing. Dynamic exclusion was set to a repeat count of 2 within 30 s, with exclusion duration of 90 s and an exclusion mass width of 10 ppm. The relative amounts of equivalent peptides bearing differently labeled amino acids were analyzed using MaxQuant.

2.13 Chromatin Immunoprecipitations

Wildtype BMDMs were stimulated with 500 U/ml of IFNβ for 30 mins. At harvest, cells were cross-linked with 1% formaldehyde at room temperature for 10 mins. Glycine was added to a final concentration of 0.125M for 5 mins at room temperature. Cells were washed twice with 10 mL of ice-cold PBS and then scraped into 2mL ice cold PBS before being centrifuged at 1000 rpm in an Avanti benchtop centrifuge at 4ºC for 10 mins. The supernatant was removed and the pellet was resuspended in 0.5 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml

aprotinin) and left on ice for 10 mins. Samples were then sonicated at 4ºC, 8 times 15 seconds on, 30 seconds off, using a VibraCell (Sonics) sonicator at 50% power. Supernatant was recovered by centrifugation at 12,000 rpm for 10 mins at 4ºC.

Protein was normalised to the least concentrated sample using Bradfords reagent before being diluted 10 fold in dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1). Samples were then pre-cleared for 2 hours at 4ºC with 2 μg of sheared salmon sperm DNA (Sigma) and 20 μL of protein G-Sepharose (50% slurry). At this stage, 10% of the material was kept and stored at -20ºC as input material. Immunoprecipitations were performed overnight with specific antibodies - αSTAT1 and αIgG antibodies from Cell Signaling - (1μg per IP), with the addition of BRIJ-35 (Merck Biosciences) detergent to a final concentration of 0.1%. All the required Protein G-Sepharose beads (30 μL per IP of 50% slurry in dilution buffer) were diluted to 1400 μL in dilution buffer and aliquoted equally, into low adhesion tubes (StarLab, E1415-1510), among the number of samples required. The beads were spun down (1,000rpm, 30 sec at 4ºC) and supernatant discarded. The immune complexes were captured by addition of the IP solution to the beads along with 2 μg salmon sperm DNA for 1 hour at 4ºC. The immunoprecipitates were washed sequentially for 5 mins each at 4ºC in 1mL Wash Buffer 1 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), 1mL Wash Buffer 2 (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and 1mL Wash Buffer 3 (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Beads were washed twice with Tris-EDTA (TE) buffer (10mM Tris-HCl, pH 7.5 and 1mM EDTA) and eluted with 140 μL of Elution Buffer (1% SDS, 0.1 M NaHCO3). To reverse the crosslinks, samples, including the stored inputs, were incubated in 0.2M NaCl at 65ºC overnight. Supernatants were then incubated for 1 h at 45ºC with Proteinase K (20 μg each, 40mM Tris-HCl pH6.5, and 10mM EDTA). DNA was cleaned using PCR purification columns (Qiagen) according to the manufacturers' instructions, into a final volume of 40 μL.

Analysis of ChIP was performed by qPCR. STAT1 or IgG ChIP DNA from either unstimulated or IFNβ-stimulated cells was analyzed to test for the presence of STAT1 tar-

get sequences in the promoter regions of CCL2 (Forward- CACTTCCTGGAAACACC-CGA and Reverse- CTTGGTGCCAAGGAGTAGCA) and a region in the GAPDH with no known STAT binding site (Forward- AGTGCCAGCCTCGTCCCGTAGACAAAATG and Reverse- AAGTGGGCCCCGGCCTTCTCCAT). ChIP data was calculated as percentage of input DNA for each sample.

Table 2.5: ChIP buffers

Solution	
1x Lysis Buffer	
	1% SDS
	10mM EDTA pH 8.1
	50mM Tris-HCl pH 8.1
	1mM PMSF
1x Dilution Buffer	
	1% Triton X-100
	2mM EDTA pH 8.1
	150mM NaCl
	20mM Tris-HCl pH 8.1
1x Wash Buffer 1	
	0.1% SDS
	1% Triton X-100
	2mM EDTA pH 8.1
	20mM Tris-HCl pH 8.1
	150mM NaCl
1x Wash Buffer 2	
	0.1% SDS
	1% Triton X-100
	2mM EDTA pH 8.1
	20mM Tris-HCl pH 8.1
	500mM NaCl
1x Wash Buffer 3	
	0.1M LiCl
	1% NP-40
	1% Deoxycholate
	1mM EDTA
	10mM Tris-HCl pH 8.1

2.14 Statistical Analysis

Student's T test or ANOVA was performed as appropriate between groups. A p value > 0.05 is indicated as ns. Significant results are indicated as follows; * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$).

Chapter 3

Results- Role of IFNβ signaling in IL-10 production

JAKs have been associated with inflammatory diseases such as Rheumatoid Arthritis (RA) and Crohn's Disease (Ports et al., 2013; Yamaoka and Tanaka, 2014; Vuitton et al., 2013; Mesa et al., 2012). The importance of JAKs in the context of inflammation and immune signaling is therefore in need of further elucidation to allow the development of specific and less harmful treatments for inflammatory diseases such as RA.

Several cytokines can act in an autocrine or paracrine manner to modulate the macrophage response. IL-10 is an important anti-inflammatory cytokine and promotes the resolution of inflammation via JAK/STAT signaling (Ouyang et al., 2011). Interferon β is an important mediator of the antiviral response and also activates JAK/STAT signaling (van Boxel-Dezaire et al., 2006; Stetson and Medzhitov, 2006). The action of JAKs and the effects of JAK inhibitors has been studied extensively within the adaptive immune system (Ghoreschi et al., 2011; Egwuagu, 2009; Oyamada et al., 2009; Mahmud et al., 2013; Ross et al., 2007). However the contribution of JAKs within the innate immune response is less clearly defined. Macrophages, in response to TLR stimulation, will produce and secrete both IL-10 and IFNβ, which can signal via JAK/STAT to modulate the inflammatory response. To examine the role of JAKs in LPS-stimulated BMDMs, a clinical JAK inhibitor, Ruxolitinib, was used.

3.1 *In vitro* specificity of Ruxolitinib and ability to block JAK/STAT signaling

Ruxolitinib was first tested at 1 and 0.1 μM against an in-house kinase panel which contains JAK2 to determine its selectivity. Ruxolitinib strongly inhibited JAK2 (2% remaining activity) at both concentrations (figure 3.1). It also inhibited TrkA by 68% and at 1 μM had moderate effects on MARK3, PHK and IRAK1. To further examine the specificity of Ruxolitinib, IC50s were calculated for JAK2, MARK3, TrkA and IRAK1. Ruxolitinib inhibited JAK2 with an IC50 of 8nM (figure 3.2). While IC50 values for TrkA (192nM), IRAK1 (565nM) and MARK3 (617nM) were over 20 fold higher.

IC50 values for Ruxolitinib were then determined against each of the JAK family members. Ruxolitinib showed similar IC50s for JAK1, JAK2 and Tyk2 with a ~10 fold higher IC50 for JAK3 (figure 3.3). Ruxolitinib is reported in the literature as a JAK1/2 inhibitor and this data supports these reports (Quintás-Cardama et al., 2010).

Next, the ability of Ruxolitinib to block STAT activation downstream of IL-10 and IFNβ signaling in BMDMs was examined. Both IL-10 and IFNβ activate JAK1 and Tyk2, downstream of IL-10R and the type I interferon receptor respectively (Finbloom and Winestock, 1995; Ho et al., 1995; Velazquez et al., 1992; John et al., 1991). At high concentrations, both STAT1 and STAT3 can become phosphorylated in response to IL-10. However, STAT3 seems to be the dominant isoform for mediating IL-10 effects (Takeda et al., 1999). IL-10 signaling leads to phosphorylation of tyrosine 705 on STAT3 by JAK1 and Tyk2 (Ananieva et al., 2008; Takeda et al., 1999; Shaw et al., 2006; Karaghiosoff et al., 2000). IFNβ signaling will cause phosphorylation of tyrosine 701 on STAT1 by JAK1 and Tyk2 (Schindler et al., 1992; John et al., 1991; Velazquez et al., 1992). IL-10 stimulation for 30 minutes leads to strong phosphorylation of Y705 on STAT3 (figure 3.4). Ruxolitinib was capable of blocking tyrosine phosphorylation of STAT3 downstream of IL-10 stimulation at 0.5 μM. IFNβ stimulation for 30 minutes induced strong tyrosine phosphorylation of STAT1. This phosphorylation event was also blocked by the addition of 0.5 μM of Ruxolitinib.

Macrophages secrete both IL-10 and IFNβ in response to LPS and this will lead to STAT phosphorylation. It has previously been shown that IL-10 is required for the phosphorylation of STAT3 on Tyr705 downstream of LPS signaling (Ananieva et al., 2008). WT

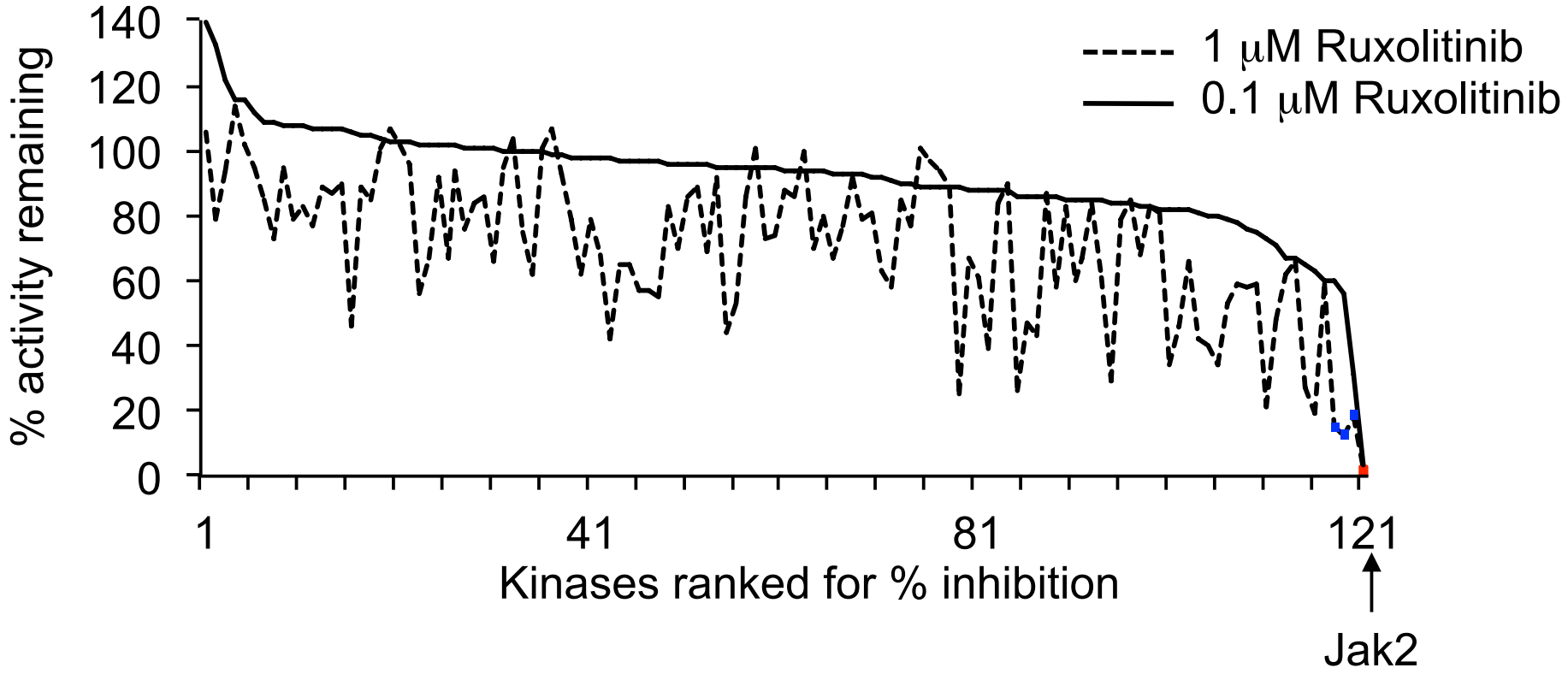

Figure 3.1: Kinase profiling of Ruxolitinib
Ruxolitinib was screened against 121 kinases *in vitro* at 1 and 0.1 μM by the International Center for Kinase Profiling using an ATP competitive assay. Kinases were ranked for remaining activity at 0.1 μM. Red marker indicates JAK2, blue markers indicate MARK3, IRAK1 and TrkA.

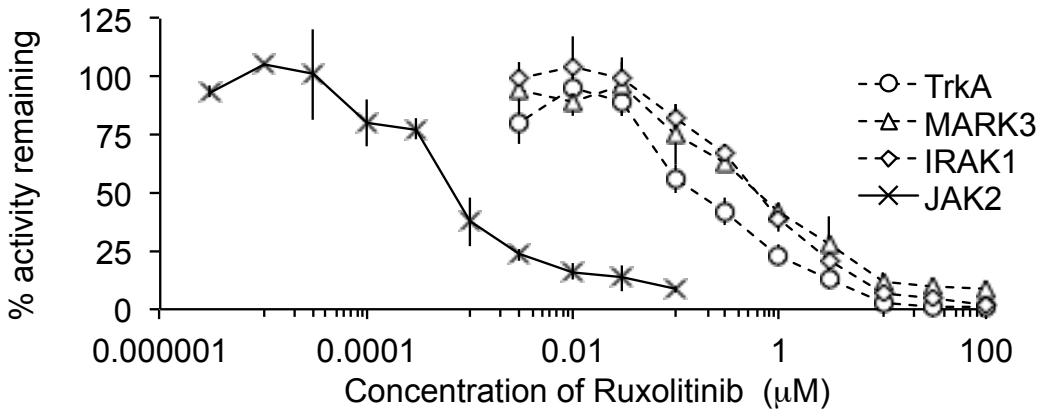

Figure 3.2: IC50s for Ruxolitinib against TrkA, MARK3, IRAK1 and JAK2
Ruxolitinib was screened against TrkA, MARK3, IRAK1 and JAK2 by the International Center for Kinase Profiling to calculate IC50s.

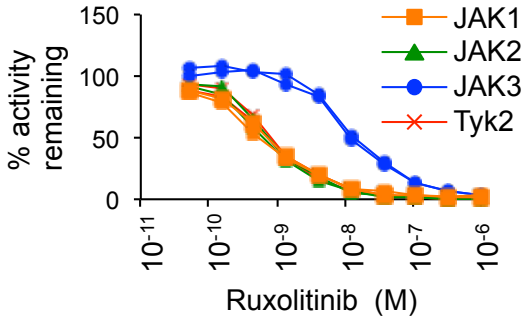

Figure 3.3: Ruxolitinib IC50s against each JAK family member
IC50s were calculated for Ruxolitinib against each JAK family member by Reaction Biology (MA, USA).

and IFNαβR KO macrophages were stimulated with LPS and levels of phosphorylated STAT1 and STAT3 were measured to examine which isoforms were affected by loss of IFNβ signaling. LPS stimulation in wildtype BMDMs induced phosphorylation of both STAT1 and STAT3 at 2 hours (figure 3.5). IFNαβR KO BMDMs did not demonstrate any phosphorylation of STAT1 showing a requirement for IFNβ feedback, additionally they also exhibit decreased levels of STAT1 total protein. IFNαβR KO macrophages demonstrated normal STAT3 phosphorylation at 2 hours, but at later time points (6 hours) show reduced phosphorylation of STAT3 compared to wild-type controls.

LPS stimulation of macrophages causes the production of cytokines including IFNβ and IL-10, which will activate STAT1 and STAT3 respectively (Ananieva et al., 2008 and figure 3.5). LPS stimulated macrophages were pre-treated with Ruxolitinib and the effects on STAT phosphorylation measured. LPS treatment of macrophages leads to the activation of MAPK pathways as demonstrated by phosphorylation of ERK1/2, p38 and JNK after 20 minutes (figure 3.6). TLR4 signaling also induced the activation of NFκB as

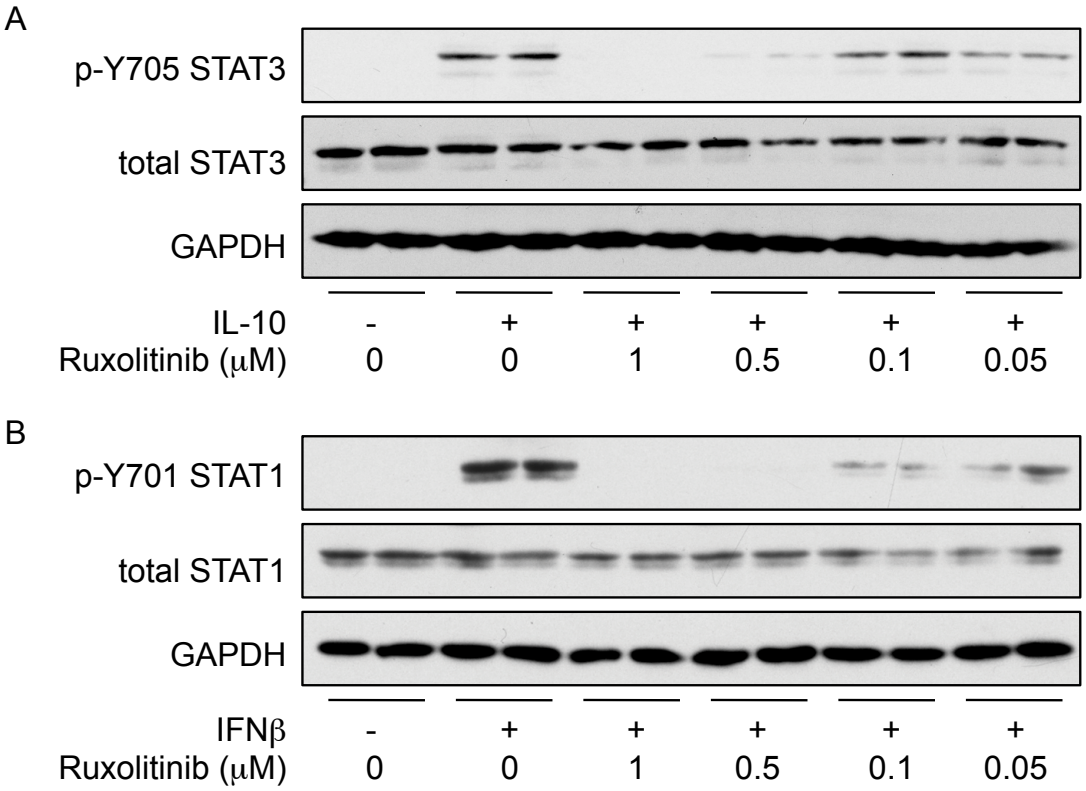

Figure 3.4: Ruxolitinib blocks IL-10 and IFNβ signaling
Bone marrow-derived macrophages were pre-treated with the indicated concentrations of Ruxolitinib for 1 hour before stimulation with A) 100 ng/mL of IL-10 for 30 minutes or B) 500 U/mL IFNβ for 30 minutes. Cells were lysed in triton lysis buffer and lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with antibodies to A) phospho STAT3 (Y705), STAT3 and GAPDH or B) phospho STAT1 (Y701), STAT1 and GAPDH. Results are representative of two independent experiments.

judged by degradation of IκBα, which is degraded in response to phosphorylation by IKK, and by the phosphorylation of p105 after 20 minutes. These effects were not affected by Ruxolitinib. LPS stimulation induces transcription and secretion of multiple cytokines including IL-10 and IFNβ and this leads to STAT phosphorylation from 2 hours. At 0.5 μM, Ruxolitinib blocked activation of STAT1 and STAT3 judged by tyrosine phosphorylation, at Y701 and Y705 respectively, in response to LPS figure 3.6.

3.2 Effects of Ruxolitinib on IL-10 transcription and secretion

Ruxolitinib could block LPS-induced STAT phosphorylation either by blocking JAK signaling downstram of IL-10 or IFNβ or by inhibiting the secretion of these cytokines by LPS stimulated macrophages. The effects of Ruxolitinib on IL-10 transcription and secretion were therefore examined. LPS stimulation of cells led to induction of IL-10 transcription from 1 hour that peaked at 4 hours before decreasing to basal around 24 hours.

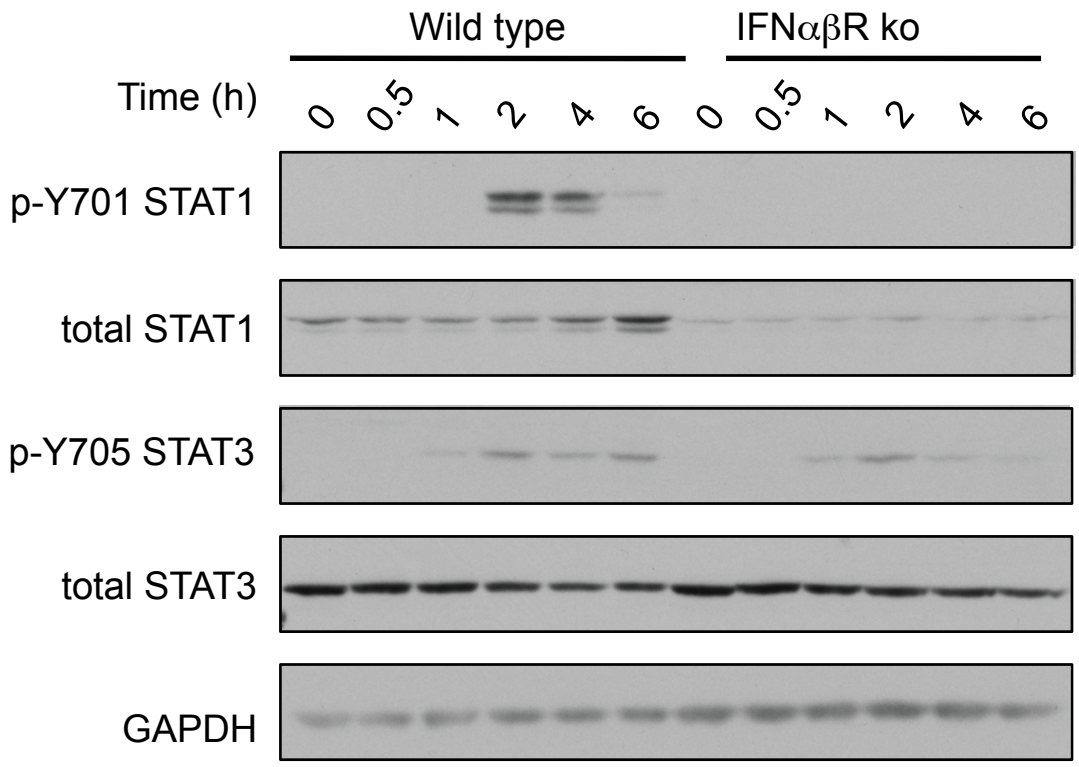

Figure 3.5: Deletion of type I IFN receptor blocks LPS-induced STAT1 phosphorylation
Bone marrow was isolated from wild-type and IFNabR KO mice. Bone marrow-derived macrophages were stimulated with 100 ng/mL of LPS. Cells were lysed in triton lysis buffer and lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with antibodies to phospho STAT1 (Y701), STAT1, phospho STAT3 (Y705), STAT3 and GAPDH. Results are representative of three independent experiments

Ruxolitinib pre-treatment of BMDMs prior to LPS stimulation did not affect IL-10 transcription at early time points (1 hour), however IL-10 mRNA levels were decreased at later time points (4, 8 and 16 hours) (figure 3.7).

The IL-10 promoter contains potential STAT binding sites (Benkhart et al., 2000; Staples et al., 2007; Ziegler-Heitbrock et al., 2003). Autocrine IL-10 mediated STAT3 phosphorylation downstream of LPS and therefore Ruxolitinib treatment could have disrupted a positive feedback pathway that sustains IL-10 transcription. To determine if this was occurring, an anti-IL-10 neutralising antibody was used in combination with LPS stimulation to specifically block the function of IL-10. LPS stimulation for 2 hours induced IL-10 mRNA transcription comparable to that seen in previous experiments. Treatment with the neutralising antibody did not affect IL-10 transcription compared with LPS stimulation alone (figure 3.8), nor did treatment with an isotype control affect IL-10 transcription. To validate that the IL-10 neutralising antibody had blocked IL-10 signaling, TNFα mRNA levels were also measured. LPS alone induced TNFα transcription which was strongly in-

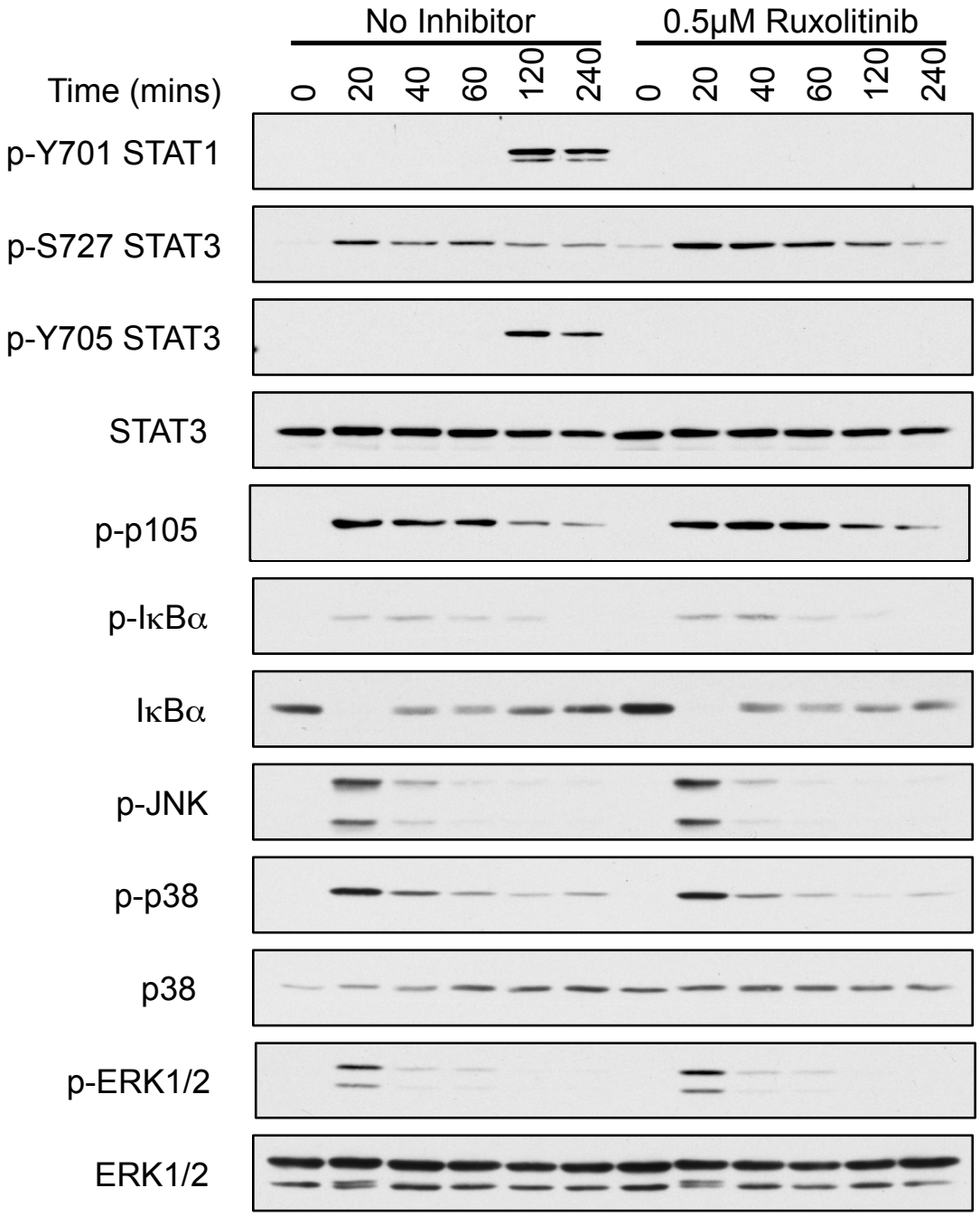

Figure 3.6: Ruxolitinib blocks LPS-induced STAT phosphorylation
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 100 ng/mL of LPS. Cells were lysed in triton lysis buffer and lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with antibodies to phospho STAT1 (Y701), phospho STAT3 (Y705), phospho STAT3 (S727), STAT3, phospho p105, phospho IκBa, IκBa, phospho JNK, phospho p38, p38, phospho ERK1/2 and ERK1/2. Results are representative of three independent experiments.

creased in the presence of the neutralising antibody. The isotype control did not increase TNFα mRNA levels (figure 3.8).

Another effect of Ruxolitinib was to block the phosphorylation of STAT1 at Y701 in response to LPS (figure 3.6) suggesting that the IFNβ feedback loop was being blocked

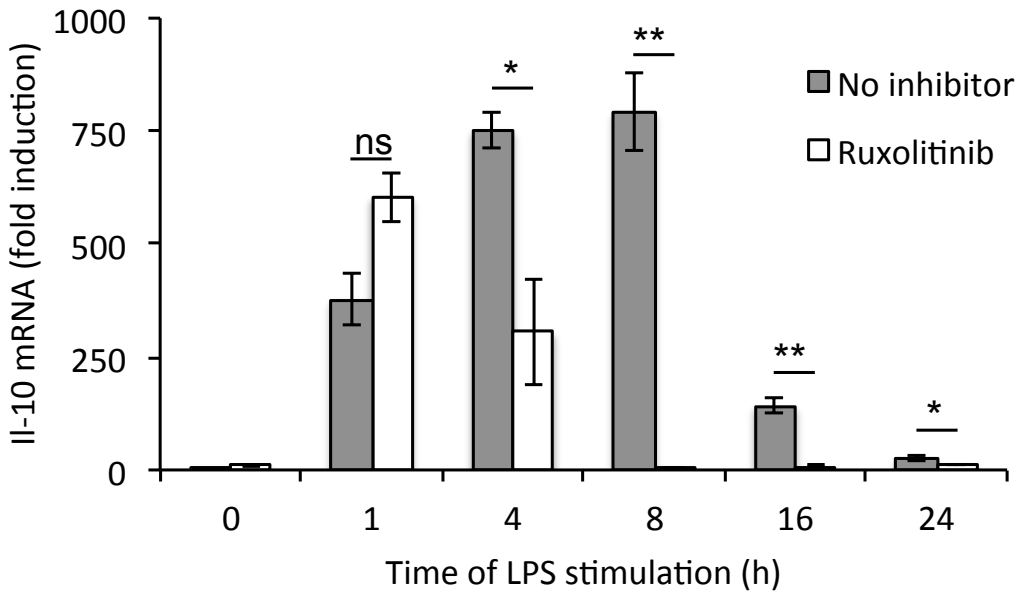

Figure 3.7: Ruxolitinib prevents sustained IL-10 transcription
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

(see figure 3.5). To investigate whether the IFNβ feedback loop was involved in IL-10 transcription, BMDMs were cultured from wild-type and type I IFN receptor (IFNαβR) KO mice and stimulated with LPS. In WT cells, LPS stimulation once again induced a similar pattern of IL-10 transcription from 1 hour leading to a peak between 4 and 8 hours before decreasing to basal at 24 hours. Similarly to treatment with Ruxolitinib (figure 3.7), IL-10 transcription was unaffected at early time points (1 hour) by deletion of the type I interferon receptor (figure 3.9). Interestingly, at later time points, IL-10 transcription was reduced in the IFNαβR KO BMDMs compared to their wild-type counterparts. This indicates that IL-10 transcription is sustained by the IFNβ feedback loop and would be consistent with the effect of Ruxolitinib treatment on IL-10 induction.

3.3 The role of IFNβ signaling in IL-10 transcription

The above results would suggest that IFNβ sustains LPS-induced IL-10 transcription. In order to test if IFNβ could directly activate the IL-10 promoter, wild-type primary macrophages were stimulated with IFNβ in the presence or absence of the JAK inhibitor,

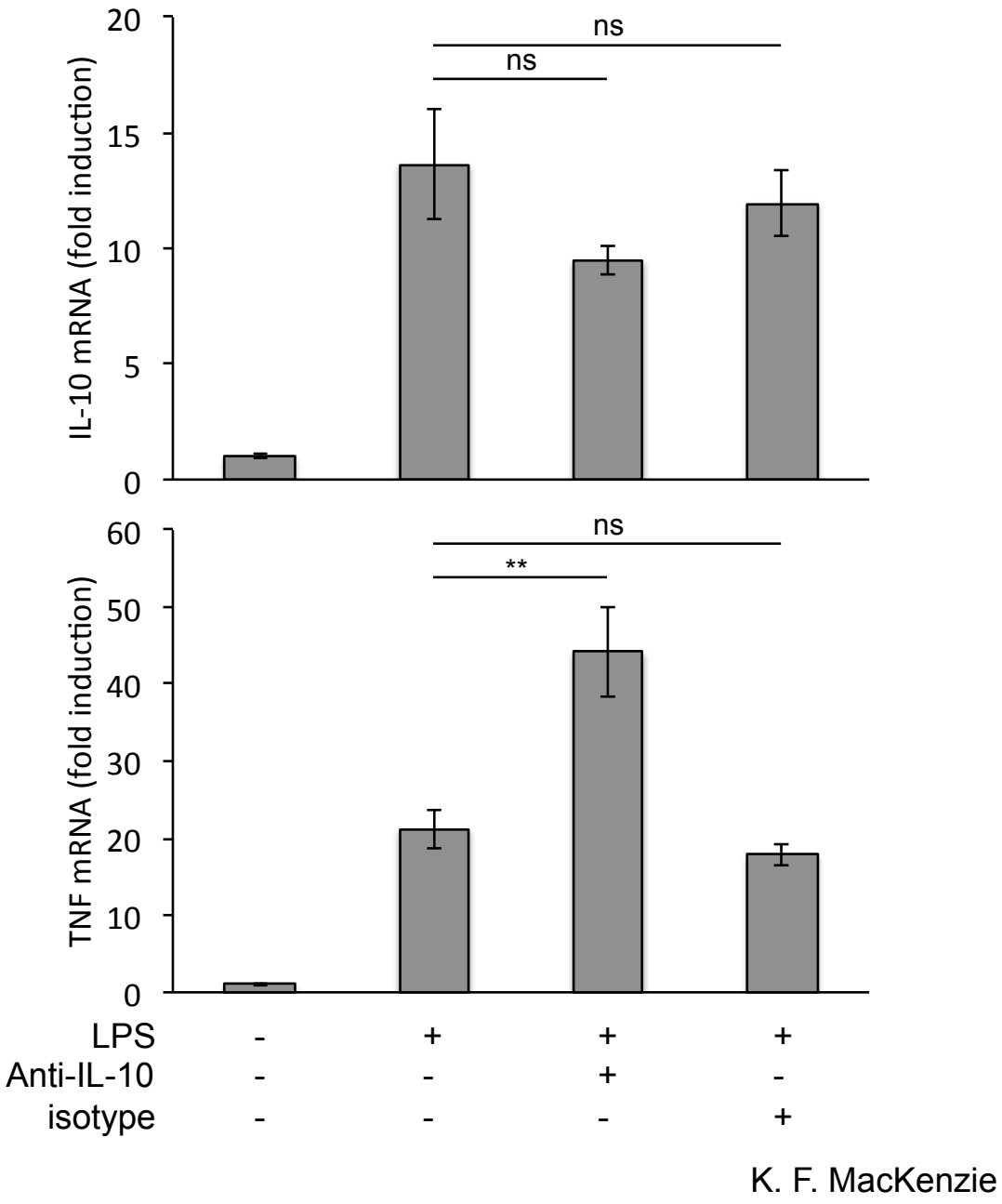

Figure 3.8: IL-10 feedback does not drive IL-10 transcription
Bone marrow-derived macrophages were treated with 100 ng/mL of LPS or a combination of LPS and anti-IL-10 neutralising antibody or an isotype control for 2 hours for IL-10 measurement and TNFα measurement. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 and TNFα mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

Ruxolitinib. IFNβ stimulation induced IL-10 mRNA from 2 hours up until 12 hours. This induction was blocked by Ruxolitinib, suggesting that IFNβ is mediating its effects on IL-10 transcription via the JAK/STAT pathway (figure 3.10). IFNβ induction of IL-10 transcription appears to be delayed suggesting a requirement for an intermediate.

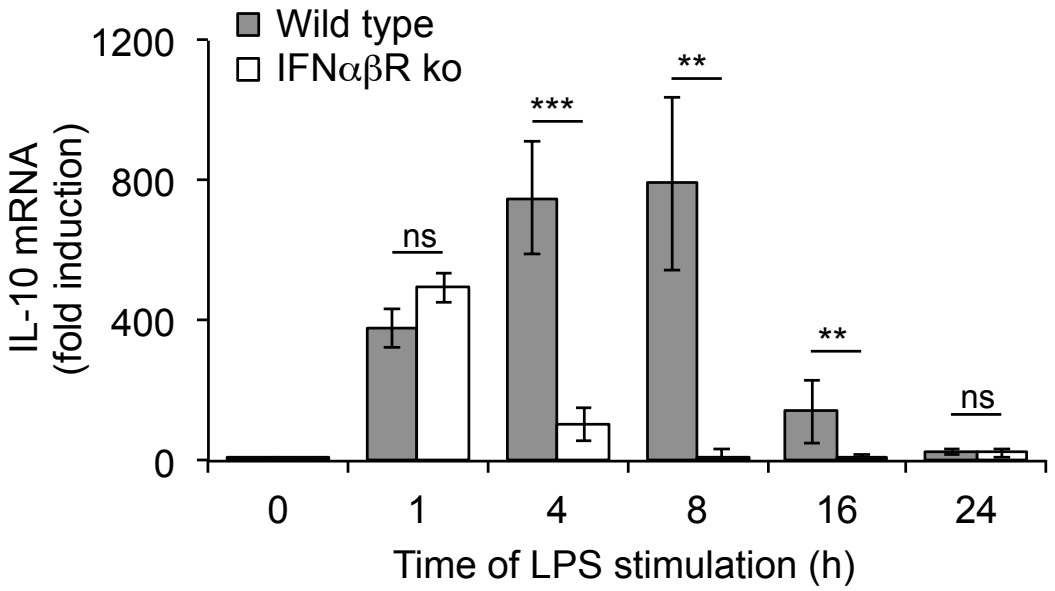

Figure 3.9: Type I IFN receptor deficiency causes loss of sustained IL-10 transcription
Bone marrow was isolated from wild-type and IFNαβR KO mice. Bone marrow-derived macrophage were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

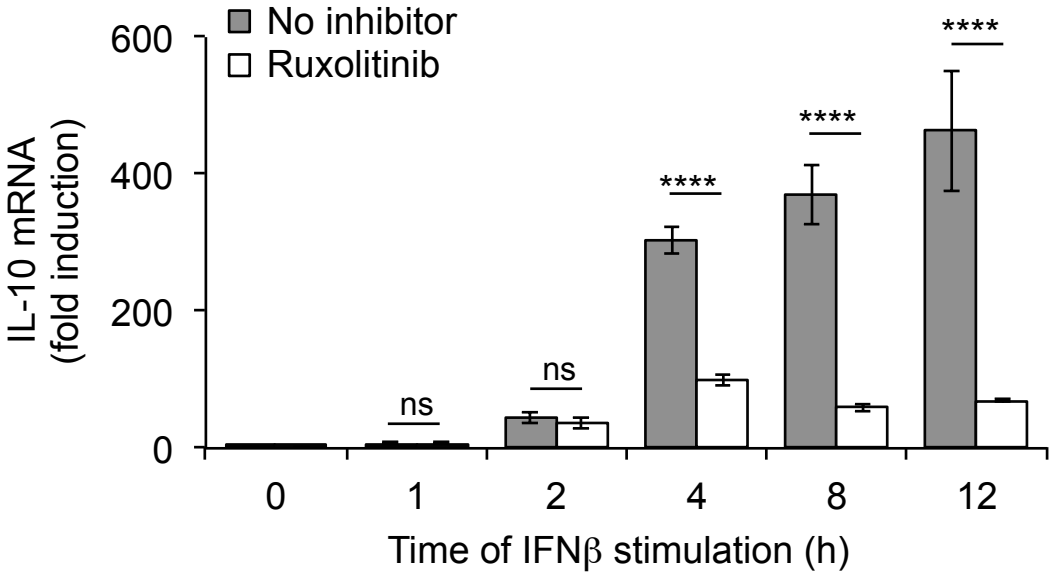

Figure 3.10: IFNβ stimulates IL-10 transcription
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 500 U/mL of IFNβ. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

The role of IL-27 in IL-10 transcription has been investigated and results have been published suggesting it may be a necessary intermediate for IL-10 transcription (Iyer et al.,

2010; Molle et al., 2010; Fitzgerald et al., 2013). IL-27 is a dimeric protein, consisting of p28 and EBI3, and a member of the IL-12 family. To understand the role of IL-27 in regulating IL-10 transcription, mRNA levels of the two IL-27 subunits were measured in IFNβ stimulated primary macrophages. IL-27(p28) mRNA is induced in response to IFNβ and this occured with similar kinetics to that of IL-10 suggesting that IL-27 is not required for IL-10 transcription in response to IFNβ (figure 3.11a). Induction of IL-27(p28) is blocked by the JAK inhibitor, Ruxolitinib. EBI3 was not induced by IFNβ stimulation (figure 3.11b).

The importance of JAKs in the IFNβ feedback control of IL-10 production downstream of TLR4 was demonstrated when wild-type and IFNαβR KO BMDMs were stimulated with LPS in the presence or absence of Ruxolitinib. LPS stimulation of wildtype BMDMs led to secretion of IL-10 into the culture media, which peaked at 8 hours (figure 3.12). Treatment of wildtype macrophages with Ruxolitinib did not affect initial IL-10 secretion at 4 hours but did reduce IL-10 secretion at 8, 16 and 24 hours. IFNαβR KO BMDMs produced similar levels of IL-10 to wildtype macrophages at 4 hours, however at later time points the type I interferon receptor deficient macrophages secreted lower levels of IL-10 compared to wild type macrophages. In fact, IFNαβR KO macrophages produced similar amounts of IL-10 to Ruxolitinib-treated wildtype BMDMs. Ruxolitinib treatment of IFNαβR KO BMDMs did not affect IL-10 secretion compared to LPS stimulation alone of the knockout macrophages. This suggests that the effects seen with Ruxolitinib upon IL-10 transcription are due to blocking an IFNβ-mediated feedback loop.

3.4 Blocking JAK signaling abolishes the IL-10 mediated negative feedback loop

Reduced levels of IL-10 and/or a block in IL-10 signaling may result in reduced suppressive effects on pro-inflammatory cytokines such as TNFα, IL-6 and IL-12. Wild-type and IFNαβR KO BMDMs were stimulated with LPS in the presence or absence of Ruxolitinib and transcription and secretion of pro-inflammatory cytokines was measured. At 0.5 μM, Ruxolitinib pre-treatment enhanced the transcription and secretion of several pro-inflammatory cytokines, namely TNFα, IL-6 and IL-12 in response to LPS compared to LPS-alone treatment (figure 3.13). IFNαβR KO BMDMs demonstrated similar levels of pro-inflammatory cytokines to wildtype controls except in the case of IL-6 suggesting

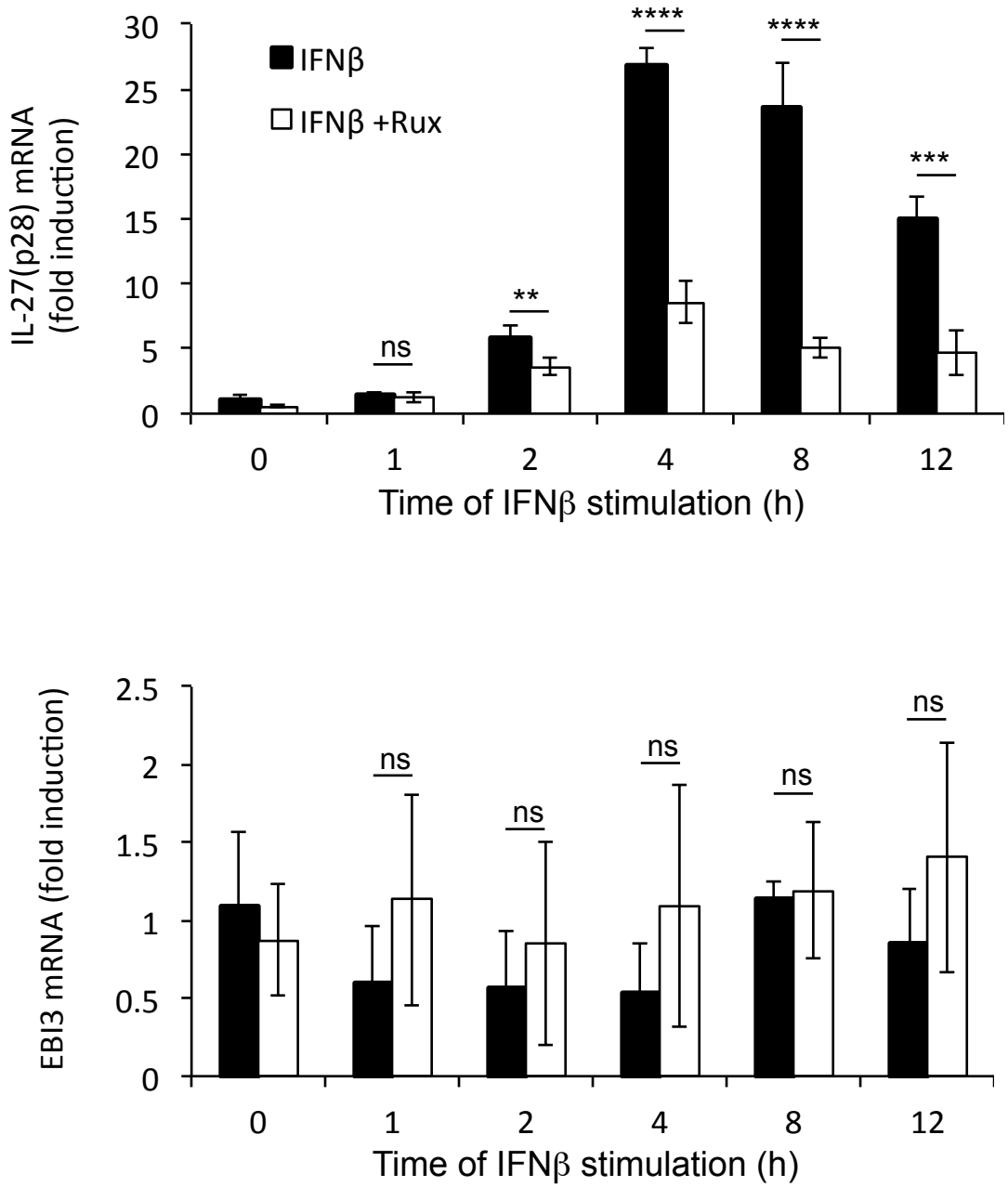

Figure 3.11: IFNβ induces IL-27 (p28) mRNA with similar kinetics to IL-10
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 500 U/mL of IFNβ. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-27(p28) and EBI3 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

a requirement for IFNβ in IL-6 transcription. Ruxolitinib treatment strongly increased levels of TNFα, IL-6 and IL-12 in IFNαβR KO BMDMs. This indicates that although, Ruxolitinib blocks both IL-10 and IFNβ feedback, the effects of Ruxolitinib treatment on TNFα and IL-12 levels are due to inhibition of IL-10-STAT3 signaling. The block of IFNβ by Ruxolitinib has minimal effects on TNFα and IL-12 levels as deletion of the

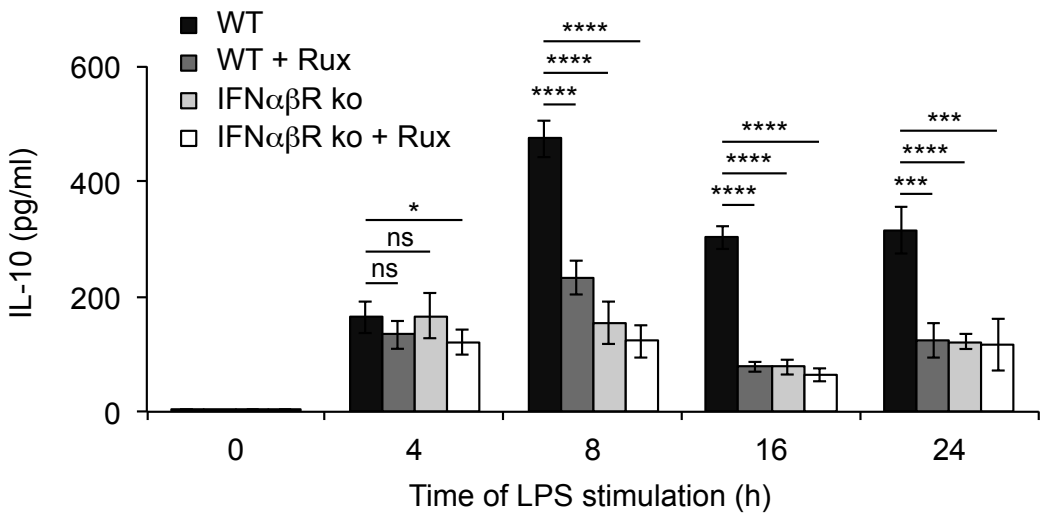

Figure 3.12: Type I IFN receptor deficiency causes reduced IL-10 secretion at late time points
Bone marrow was isolated from wild-type and IFNαβR KO mice. Bone marrow-derived macrophage were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 100 ng/mL of LPS. Culture supernatant was collected and IL-10 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

type I interferon receptor only affects IL-10-STAT3 signaling at late time points, whilst IL-10-STAT3 signaling at early time points is required for reducing TNFα and IL-12 levels. However, IL-6 levels in Ruxolitinib pre-treated IFNαβR KO macrophages did not increase to the same degree as wild-type cells, further suggesting a role for IFNβ feedback in maintaining IL-6 transcription.

To further investigate this requirement, WT macrophages were stimulated with IFNβ and IL-6 mRNA measured. IFNβ stimulated IL-6 transcription from 1 hour, peaking at 8 hours (figure 3.14). This induction was blocked by the JAK inhibitor, Ruxolitinib. This data along with that seen in the IFNαβR KO cells suggests that although IFNβ and IL-10 pathways are important in modulating IL-6 transcription, other JAK-dependent pathways may also contribute.

3.5 Comparison of Tofacitinib and Ruxolitinib

In addition to Ruxolitinib, other JAK inhibitors are available and some are in clinical use, such as Tofacitinib. While Ruxolitinib showed selectivity for JAK2 within the panel of 121 kinases screened, off-target effects can not be ruled out, therefore a second inhibitor was used to corroborate the observed results. To determine if Tofacitinib was suitable

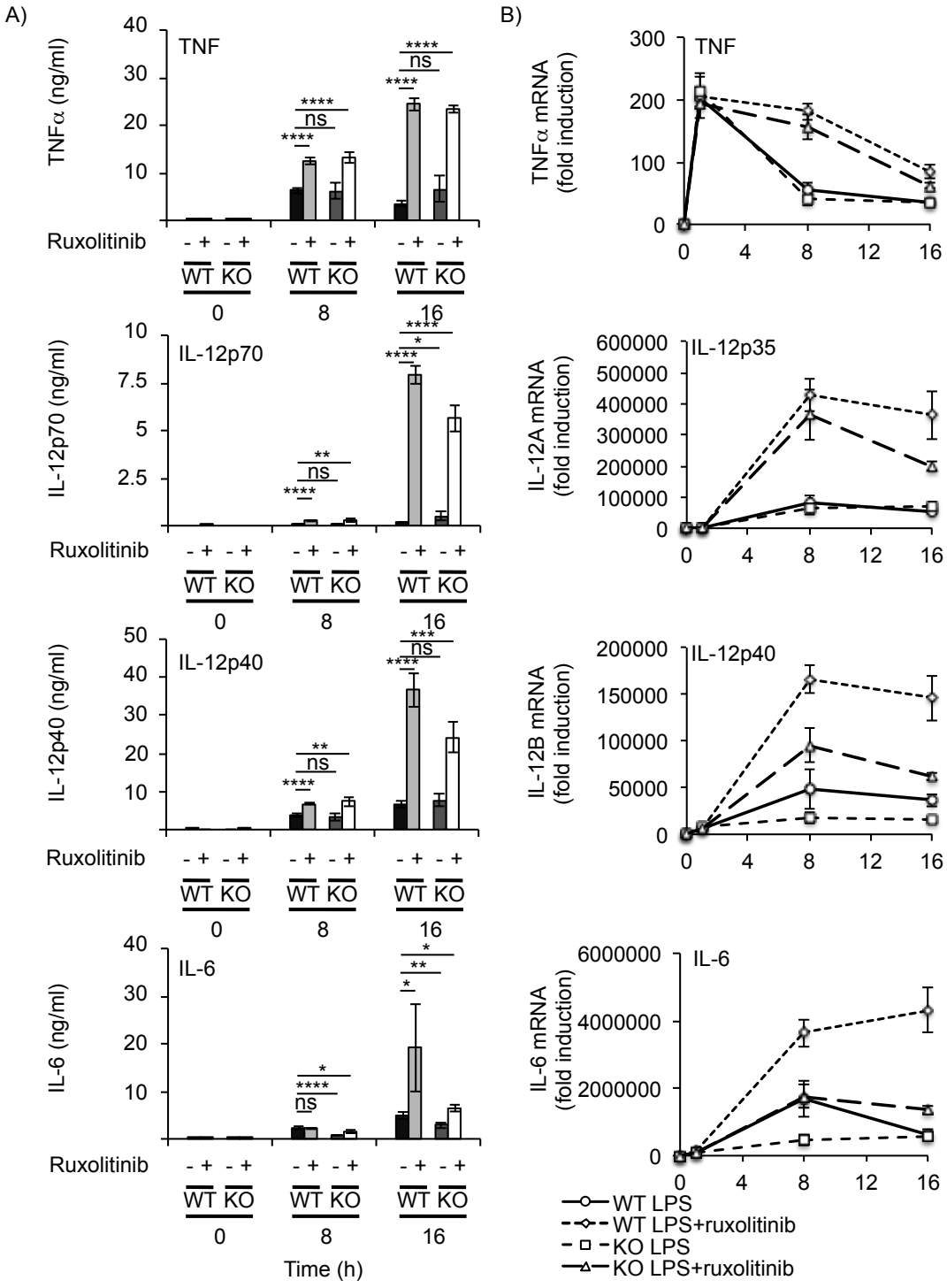

Figure 3.13: Ruxolitinib leads to enhanced pro-inflammatory cytokine transcription and secretion in response to LPS

Bone marrow-derived macrophages from WT and IFNαβR KO mice were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 100 ng/ml LPS. A) Culture supernatant was collected and TNFα, IL-12(p70), IL-12(p40) and IL-6 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. B) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for TNFα, IL-12(p35), IL-12(p40) and IL-6 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

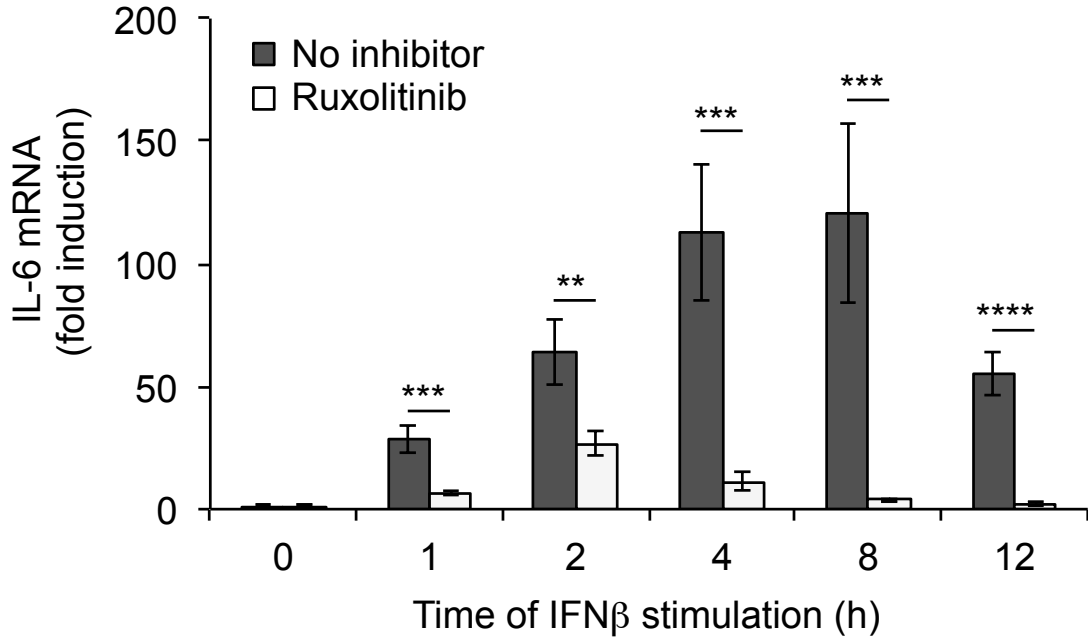

Figure 3.14: IFNβ induces IL-6 mRNA transcription
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 500 U/mL of IFNβ. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-6 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

for selectively blocking JAK activity, a kinase screen was performed. Tofacitinib was screened at 1 μM and 0.1 μM. Tofacitinib strongly reduced JAK2 activity (figure 3.15) and did not strongly target other kinases present in the kinase panel.

Next, the ability of Tofacitinib to block different JAK family members was measured through calculation of IC50s performed by Reaction Biology (MA, USA). Tofacitinib demonstrates similar IC50s for JAK1/3 with higher IC50s for JAK2 and Tyk2 (figure 3.16a). The comparative IC50s for Ruxolitinib and Tofacitinib for each JAK family member are shown (figure 3.16b), this demonstrates that Ruxolitinib and Tofacitinib have similar potency against JAK1 but differing potencies against JAK2, JAK3 and Tyk2.

To test whether Tofacitinib effectively blocked JAK activity within cells, BMDMs were incubated with increasing concentrations of Tofacitinib for 1 hour before stimulation with 100 ng/ml IL-10 for 30 minutes. IL-10 induces STAT3 phosphorylation of Y705 through activation of JAK1/Tyk2 (figure 3.17a). 5 μM of Tofacitinib was sufficient to block phosphorylation of STAT3 in response to IL-10 stimulation. IFNβ stimulation of macrophages also activates JAK1/Tyk2 and leads to phosphorylation of STAT1 at Y701 (figure 3.17b).

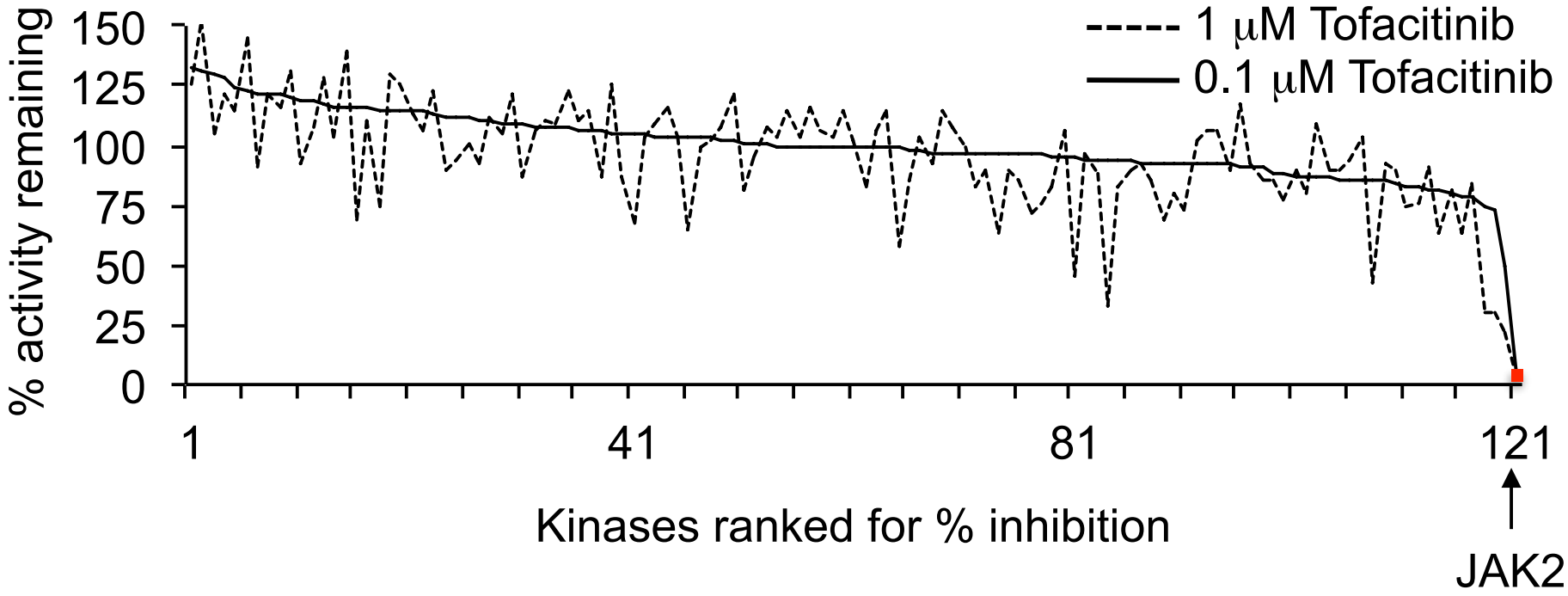

Figure 3.15: Kinase profiling of Tofacitinib
Tofacitinib was screened against 121 kinases *in vitro* at 1 and 0.1 μM by the International Center for Kinase Profiling using an ATP competitive assay. Kinases were ranked for remaining activity at 0.1 μM. Red marker indicates JAK2.

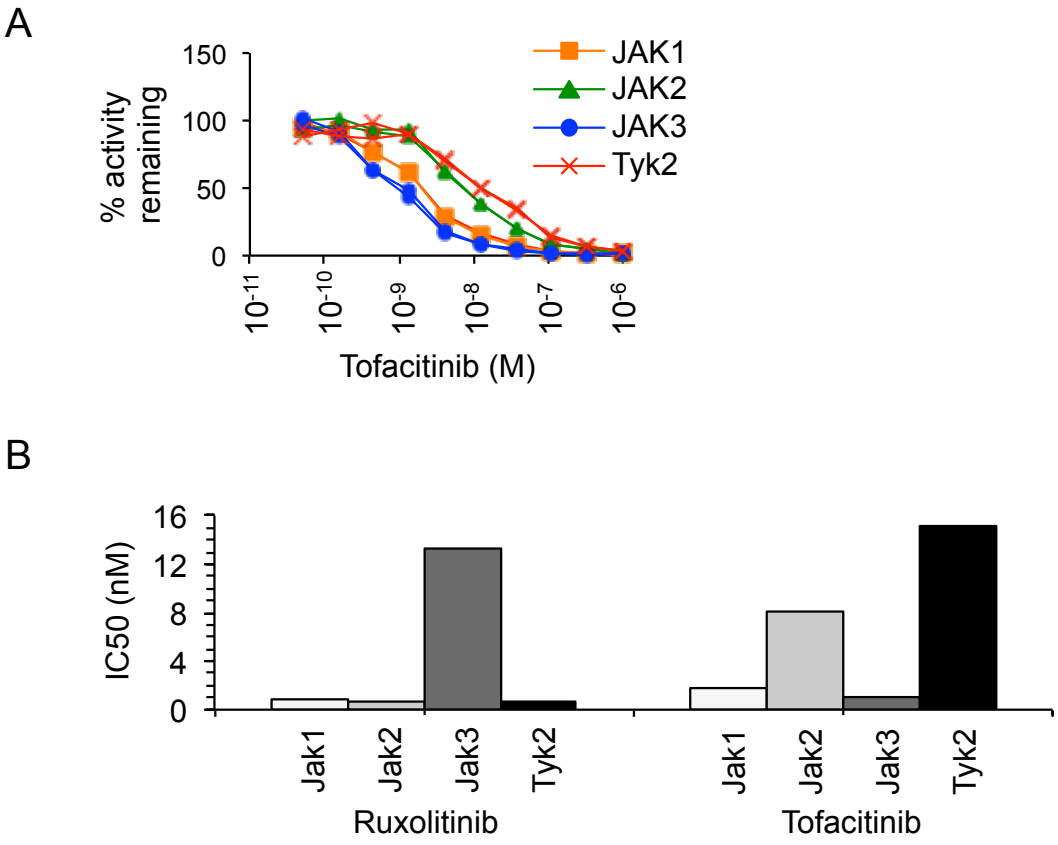

Figure 3.16: Tofacitinib IC50s against JAK family members
IC50s were calculated for (A) Tofacitinib against each JAK family member by Reaction Biology (MA, USA). Relative IC50s for Ruxolitinib and Tofacitinib against each JAK family member are shown (B).

In response to IFNβ, 0.5 μM Tofacitinib was sufficient to block tyrosine phosphorylation of STAT1. Based on IC50 data obtained which showed that Tofacitinib and Ruxolitinib had differing IC50s for Tyk2 whilst their IC50s for JAK1 are comparable, the higher concentration required to block IL-10 signaling suggests that IL-10 signaling is potentially more Tyk2-dependent. Although inhibitor data has suggested the importance of Tyk2 in IL-10 signaling, studies using Tyk2-deficient mice have demonstrated a redundant role for Tyk2 in IL-10 signaling (Karaghiosoff et al., 2000; Shimoda et al., 2000).

In order to see if Tofacitinib had effects on IL-10 transcription that were consistent with those observed with Ruxolitinib treatment, BMDMs were pre-incubated with 5 μM Tofacitinib prior to LPS stimulation for a timecourse of 16 hours. LPS induced IL-10 transcription, which peaked around 4 hours before declining at 8 and 16 hours (figure 3.18a). Tofacitinib treatment did not strongly decrease IL-10 transcription at 1 hour, but led to marked reduction from 4 hours onward. IL-10 secretion was also quantified and was strongly induced by LPS at 8 and 16 hours (figure 3.18b). In line with the mRNA data,

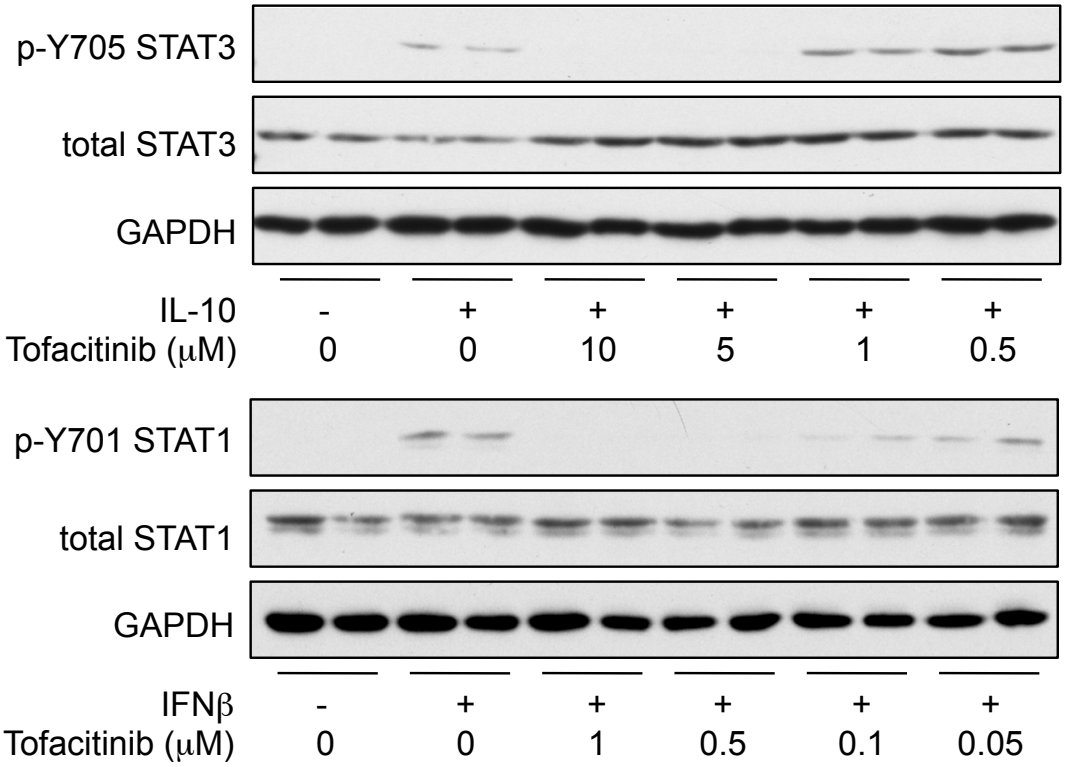

Figure 3.17: Tofacitinib blocks IFNβ and IL-10 signaling
Bone marrow-derived macrophages were pre-treated with the indicated concentrations of Tofacitinib for 1 hour before stimulation with A) 100 ng/mL of IL-10 or B) 500 U/mL IFNβ. Cells were lysed in triton lysis buffer and lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with antibodies to A) phospho STAT3 (Y705), STAT3 and GAPDH or B) phospho STAT1 (Y701), STAT1 and GAPDH. Results are representative of two independent experiments.

Tofacitinib reduced IL-10 secretion at 8 and 16 hours.

Due to the block of IL-10-STAT3 signaling, pro-inflammatory cytokines levels should be increased when BMDMs are pre-treated with Tofacitinib. LPS stimulation of macrophages induced TNFα, IL-6 and IL-12 (p40) and IL-12(p70) from 8 to 24 hours (figure 3.19). Tofacitinib treatment strongly increased pro-inflammatory cytokine secretion from 8 hours onwards, correlating with the effects seen on pro-inflammatory cytokine secretion with Ruxolitinib treatment.

3.6 IFNβ signaling sustains expression of LPS-induced MCP-1

Several reports have suggested that JAK/STAT, especially IFNβ, signaling is important in expression of β-chemokines (Fantuzzi et al., 2001; Nakano et al., 2012; Iida et al., 2011; Nyquist et al., 2010; Kok et al., 2009). MCP-1 is a β-chemokine which is strongly induced in macrophages stimulated with LPS. I therefore examined the role of IFNβ or IL-10 me-

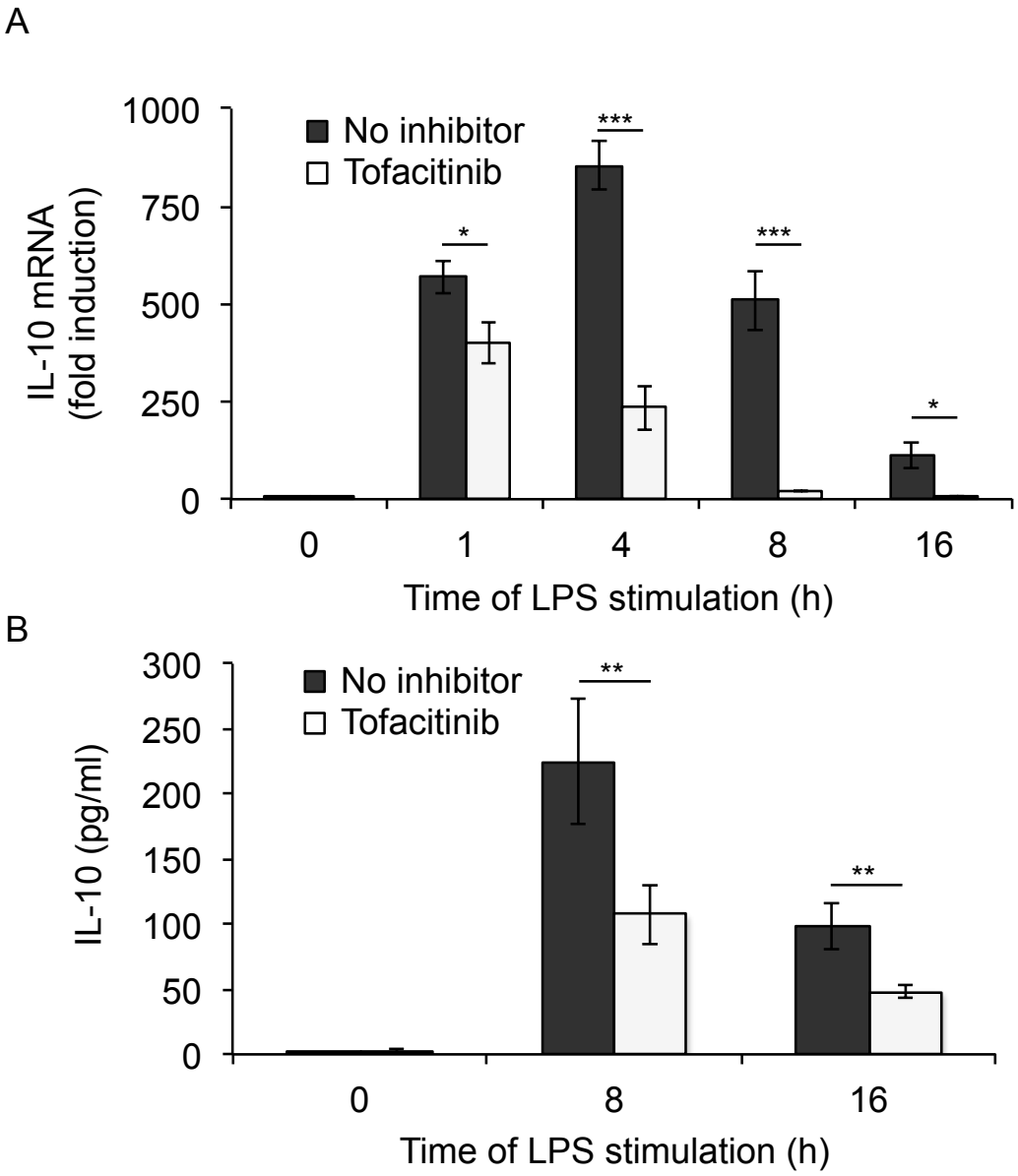

Figure 3.18: T**ofacitinib reduces LPS-induced IL-10 transcription and secretion at late time points**
Bone marrow-derived macrophages were pre-treated with 5 μM of Tofacitinib for 1 hour before stimulation with 100 ng/mL of LPS. A) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA. B) Culture supernatant was collected and IL-10 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

diated feedback control of MCP-1 production. The importance of LPS-induced IFNβ on MCP-1 levels was examined. Wildtype and IFNαβR KO macrophages were stimulated with LPS in the presence or absence of Ruxolitinib. LPS induced transcription of MCP-1 in wildtype cells from 1 hour until 24 hours, peaking at 4 hours (figure 3.20a). Treatment of wildtype cells with Ruxolitinib prior to LPS stimulation resulted in increased transcription at 1 hour compared to macrophages treated with LPS alone. However, from 4 hours

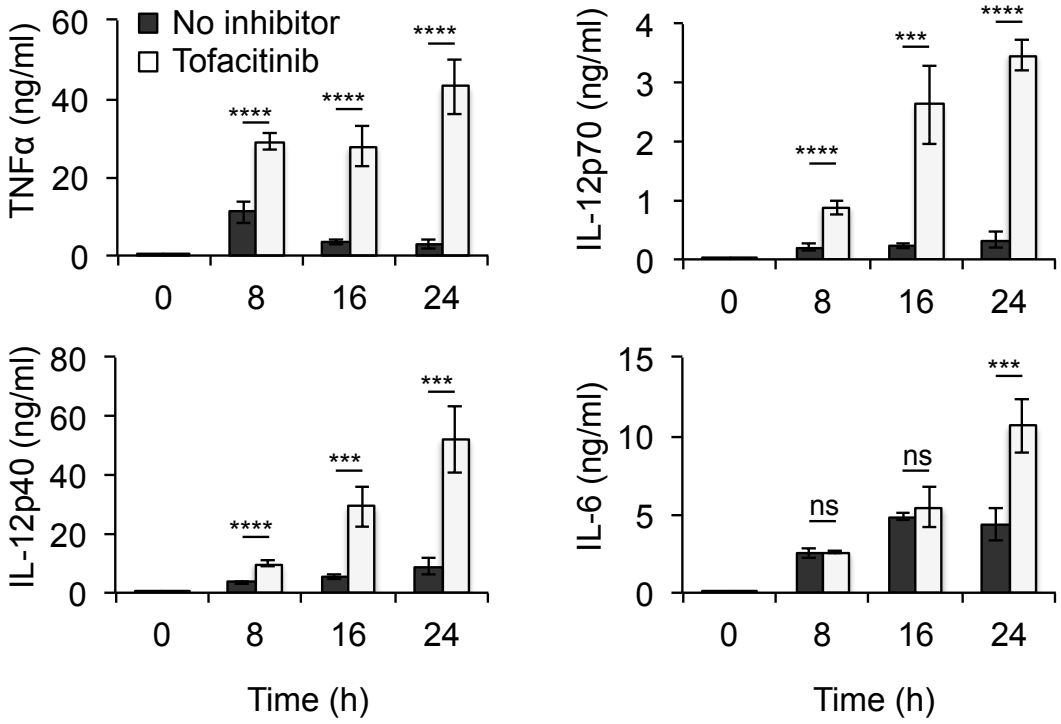

Figure 3.19: Tofacitinib treatment increases pro-inflammatory cytokine secretion in response to LPS Bone marrow-derived macrophages were pre-treated with 5 μM of Tofacitinib for 1 hour before stimulation with 100 ng/mL of LPS. Culture supernatant was collected and cytokine secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

onwards, treatment with Ruxolitinib resulted in decreased transcription of MCP-1 compared to macrophages treated with LPS alone. IFNαβR KO macrophages had a similar transcriptional profile to wildtype macrophages pretreated with Ruxolitinib with a peak at 1 hour and mRNA levels near to basal from 8 hours onwards. Ruxolitinib treatment of IFNαβR KO macrophages did not alter the induction of MCP-1 in response to LPS, suggesting that IFNβ signaling through IFNαβR is responsible for maintaining MCP-1 transcription.

MCP-1 protein levels were also measured following LPS treatment in the presence or absence of Ruxolitinib in wildtype and IFNαβR KO BMDMs. LPS stimulated secretion of MCP-1 from wildtype BMDMs that increased throughout the 24 hour timecourse (figure 3.20b). Treatment with Ruxolitinib did not affect the initial secretion of MCP-1 at 4 or 8 hours, however at 16 and 24 hours, Ruxolitinib treatment caused a strong reduction in MCP-1 levels compared to wildtype controls. Deletion of the IFNαβR, similar to Ruxolitinib, did not affect the initial production of MCP-1 in response to LPS. At 16 and 24

hours following LPS stimulation, IFNαβR KO macrophages secreted reduced amounts of MCP-1 compared to LPS-stimulated wildtype macrophages. Treatment of IFNαβR KO BMDMs with Ruxolitinib prior to LPS stimulation did not alter MCP-1 secretion compared to LPS-stimulated IFNαβR KO BMDMs. In line with the mRNA data, MCP-1 requires IFNβ signaling for sustained production in response to LPS stimulation.

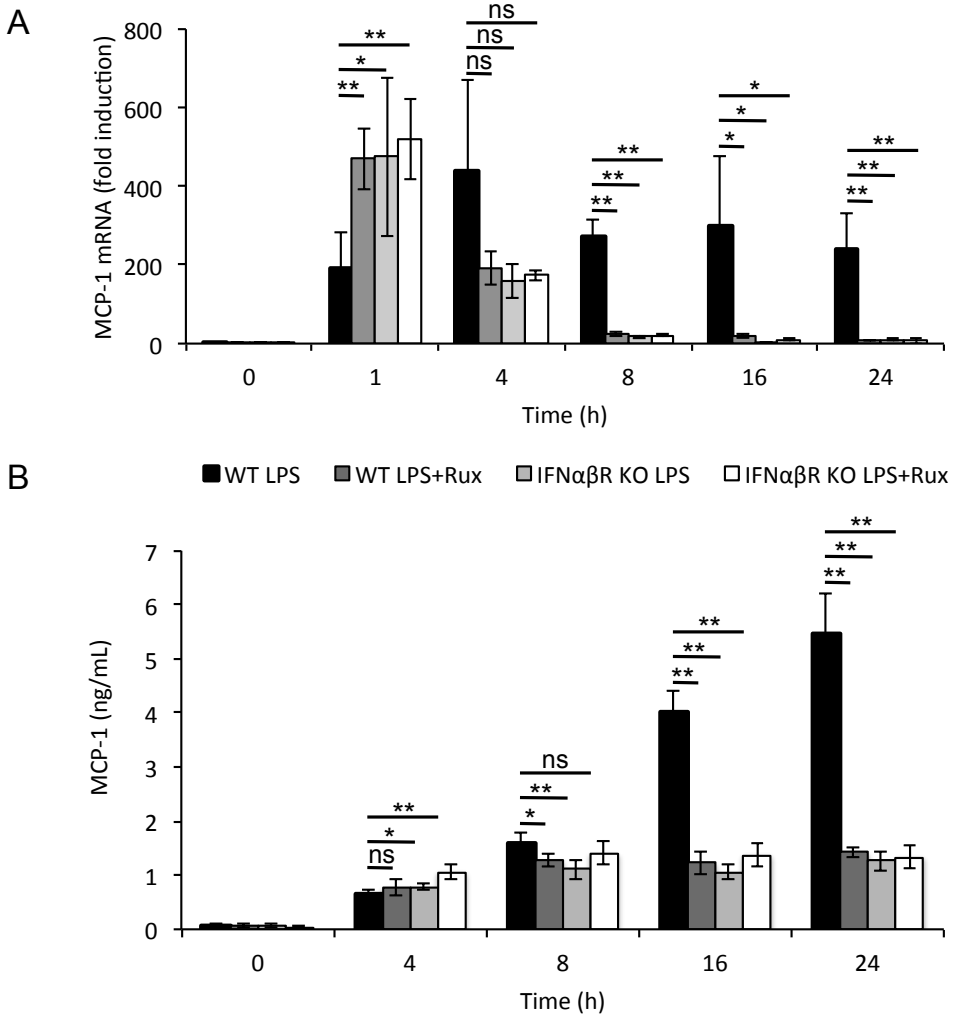

Figure 3.20: IFNβ signaling sustains expression of LPS-induced MCP-1
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 100 ng/mL of LPS. A) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for MCP-1 mRNA. B) Culture supernatant was collected and MCP-1 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

3.7 IFNβ induces MCP-1 in a JAK-dependent manner

The above data suggests a requirement for IFNβ to maintain MCP-1 expression following LPS stimulation. To determine if this was a direct activation of the MCP-1 promoter by IFNβ, macrophages were stimulated with IFNβ in the presence or absence of Ruxolitinib. IFNβ induced MCP-1 expression after 1 hour of stimulation and expression remained relatively constant across the 12 hour timecourse (figure 3.21a). Treatment with Ruxolitinib, blocked the induction of MCP-1 by IFNβ. IFNβ treatment promoted secretion of MCP-1 at 4 hours and MCP-1 levels were increased at 8 and, again, at 12 hours (figure 3.21b). In line with the mRNA data, treatment with Ruxolitinib blocked production of MCP-1 in response to IFNβ.

3.8 STAT1 binds to the MCP-1 promoter in response to IFNβ

IFNβ induces activation of JAK1 and Tyk2 and the phosphorylation of STAT1 and STAT2. IFNβ can initiate transcription of MCP-1, although whether this is STAT-dependent is unclear. Interestingly, STAT1 phosphorylation is lost in LPS-stimulated macrophages from IFNαβR KO mice, which produce decreased levels of MCP-1 in response to LPS (figure 3.5). To determine whether deletion of the IFNαβR also blocked phosphorylation of STAT2 in LPS-stimulated macrophages, BMDMs were isolated from wildtype and IFNαβR KO mice and stimulated with LPS for 6 hours. STAT2 phosphorylation was induced by LPS after 2 hours of stimulation and was still present after 6 hours (figure 3.22). Phosphorylation of STAT2 at Tyr689 was not detectable in BMDMs isolated from IFNαβR KO mice.

Furthermore, a STAT binding site is present within the MCP-1 (ccl2) promoter (figure 3.23a). To determine if IFNβ-induced MCP-1 expression was through activation of STAT1 and recruitment to the MCP-1 promoter, macrophages were stimulated with IFNβ for 30 mins and STAT1 chromatin immunoprecipitations performed. Upon stimulation with IFNβ, a marked increase in STAT1 was found at the MCP-1 promoter compared to an IgG negative control and in the unstimulated state with STAT1 and IgG (figure 3.23b). STAT1 and IgG showed no increase in recruitment to the gapdh promoter in resting or IFNβ-stimulated cells (figure 3.23c).

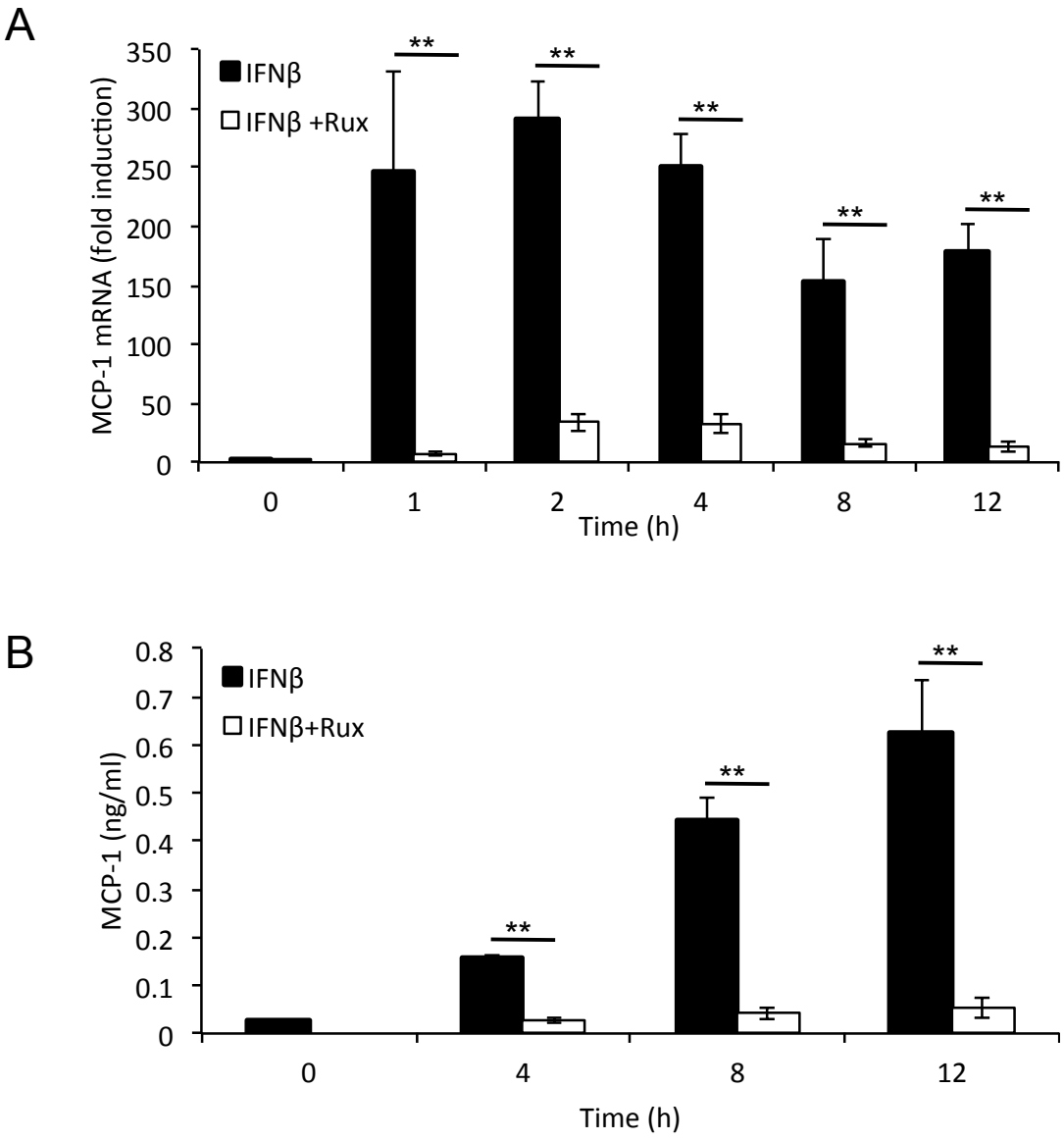

Figure 3.21: IFNβ induces MCP-1 expression and secretion
Bone marrow-derived macrophages were pre-treated with 0.5 μM of Ruxolitinib for 1 hour before stimulation with 500 U/ml IFNβ. A) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for MCP-1 mRNA. B) Culture supernatant was collected and MCP-1 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

3.9 MCP-1 production downstream of TLR3 requires IFNβ feedback signaling

Poly(I:C) activates TLR3 signaling and stimulates biphasic production of IFNβ, which is dependent on type I IFN signaling (Honda et al., 2006; Enesa et al., 2012; Marié et al., 1998). The effect of poly(I:C) on MCP-1 expression was therefore examined. As expected, poly(I:C) strongly induced IFNβ transcription in a biphasic manner which was

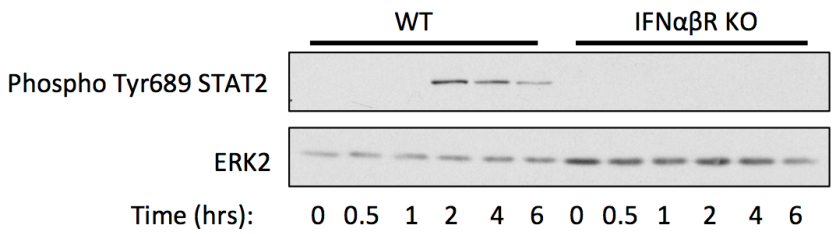

Figure 3.22: STAT2 phosphorylation requires IFNβ-mediated feedback signaling in response to LPS Bone marrow was isolated from wild-type and IFNαβR KO mice. Bone marrow-derived macrophages were stimulated with 100 ng/mL of LPS. Cells were lysed in triton lysis buffer and lysates resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with antibodies to phospho STAT2 (Y689) and ERK2. Results are representative of two independent experiments.

absent in the IFNαβR knockout cells (figure 3.24).

Macrophages stimulated with poly(I:C) initiated transcription of MCP-1, which was sustained throughout the 24 hour timecourse (figure 3.25a). Poly(I:C) stimulation led to lower mRNA levels of MCP-1 compared to LPS stimulation (see figure 3.20). IFNαβR KO macrophages stimulated with poly(I:C) induced MCP-1 mRNA expression, which was similar to that seen in wildtype macrophages. However, at later time points, MCP-1 mRNA levels were strongly decreased compared to wildtype cells. Poly(I:C) also induced MCP-1 secretion at 8 hours and was increased at 24 hours. Deletion of IFNαβR in macrophages caused reduced levels of MCP-1 secretion in response to poly(I:C) compared to wildtype macrophages (figure 3.25b).

3.10 Discussion

The importance of JAKs in the development of immune cells and in regulating the adaptive immune response has been well studied. The role of JAK signaling within the innate immune response is less well understood. TLR activation leads to the rapid production of numerous cytokines and chemokines which initiate and control the inflammatory response required to combat infection. The production of these cytokines and chemokines is dependent on many signaling pathways including MAPK, NFκB, IRF and autocrine pathways. For example, both IL-10 and IFNβ establish autocrine pathways which are mediated by JAK/STAT signaling. IL-10 establishes an anti-inflammatory response that results in the suppression of TNFα, IL-6 and IL-12 (Fiorentino et al., 1991; Murray, 2005; Hutchins et al., 2013). IFNβ has been shown to mediate a feedback pathway in dendritic

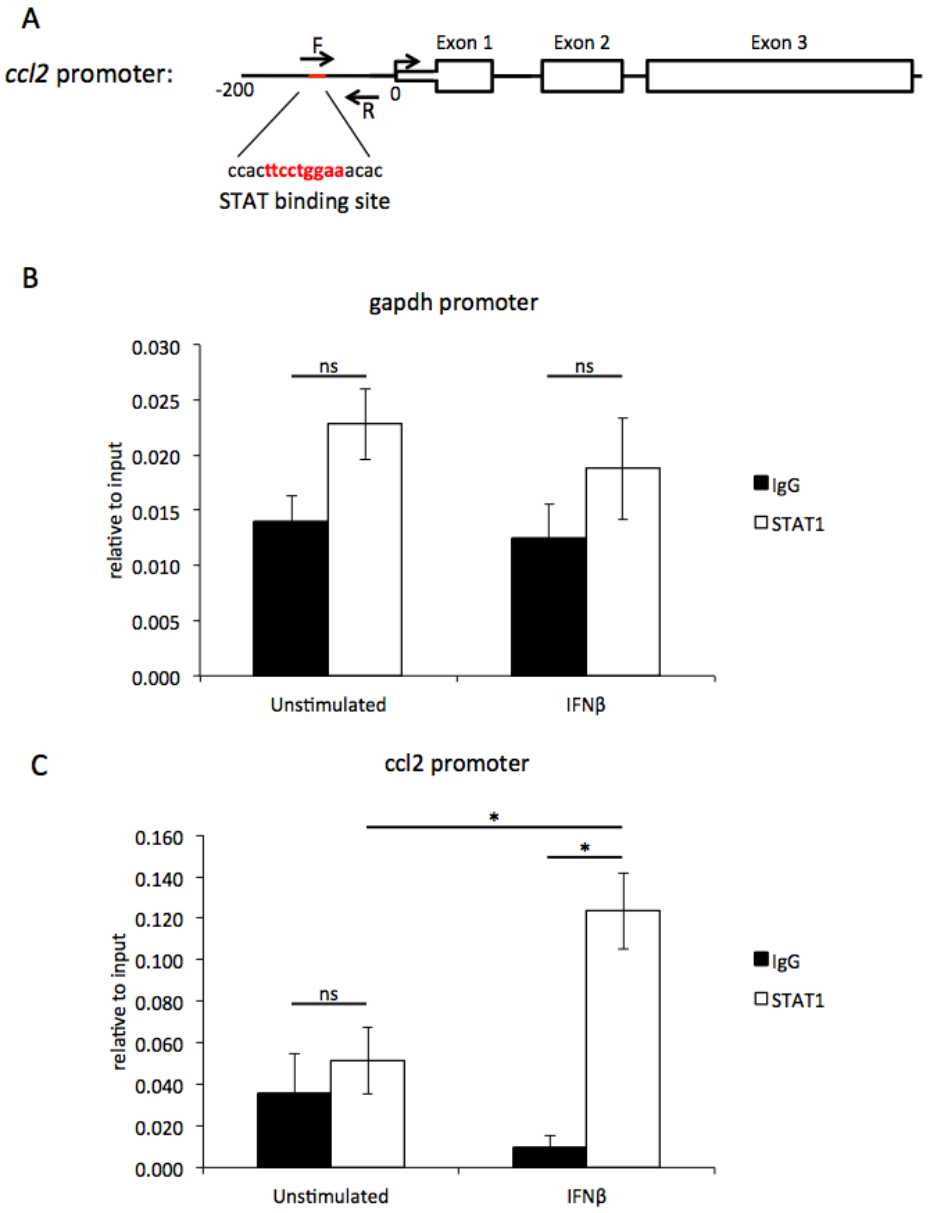

Figure 3.23: IFNβ stimulation recruits STAT1 to the MCP-1 promoter
A) To analyse STAT1 recruitment to the MCP-1 promoter by ChIP, qPCR using primers designed to cover a potential STAT-binding site in the ccl2 (MCP-1) promoter were designed. BMDMs were isolated from wild-type mice and stimulated with 500 U/ml IFNβ for 30 min. Cells were cross-linked and STAT1 or control IgG immunoprecipitations performed. ChIP signals relative to input for a region of B) GAPDH gene and C) the STAT binding region of the ccl2 promoter are shown. Error bars represent the s.e.m. of stimulations from 4 independent cultures of BMDMs.

cells, required to maintain IL-12 transcription (Gautier et al., 2005). The results presented in this chapter demonstrate a requirement for IFNβ to maintain transcription of IL-10 in response to TLR4 activation with LPS. Furthermore, IFNβ was shown to directly induce IL-10 mRNA levels.

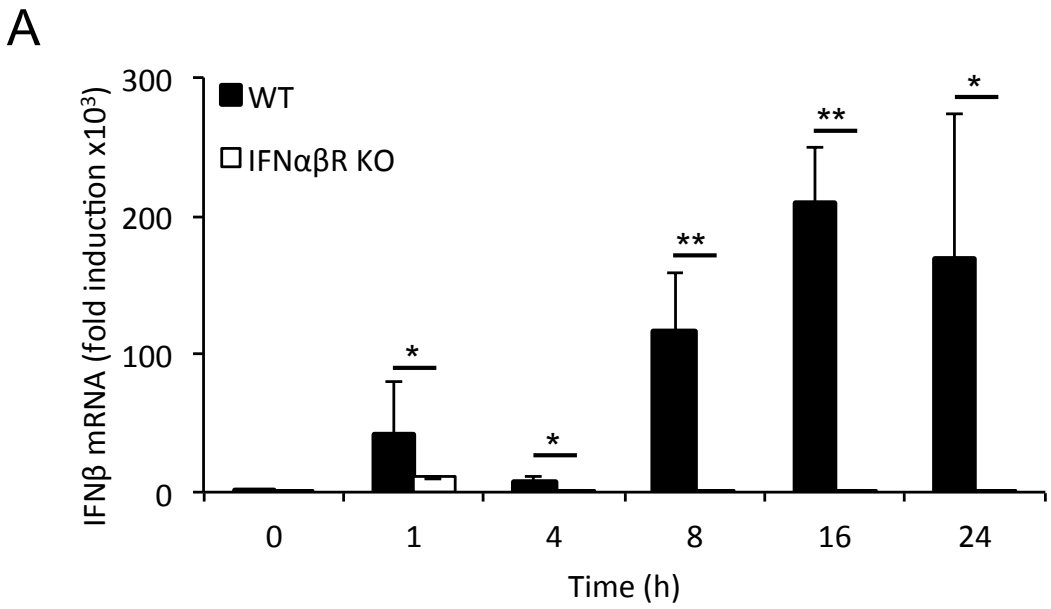

Figure 3.24: Biphasic induction of IFNβ by poly(I:C) requires the type I interferon receptor
Bone marrow-derived macrophages from WT and IFNαβR KO mice were stimulated with 10 μg/ml poly(I:C). Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IFNβ mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

3.10.1 IFNβ-mediated feedback pathway sustains IL-10 transcription

TLR4 is a potent producer of IFNβ, other TLRs expressed by macrophages do not all produce similar levels of IFNβ when stimulated. Therefore it is unclear if the IFNβ-mediated feedback loop is required to sustain transcription of IL-10 in response to other TLR agonists. Equally, it is unknown whether IFNβ maintains IL-10 transcription in response to agonists for other PRRs.

In addition to IL-10 and IFNβ, LPS-stimulated macrophages produce other cytokines that will activate JAK/STAT signaling. Therefore, the effects seen with Ruxolitinib treatment on LPS-stimulated macrophage cytokine production could be linked to blocking JAK/STAT signaling mediated by other cytokines and stimuli. However, the data above suggests that IL-10 and IFNβ are the predominant feedback mechanisms within TLR-stimulated macrophages. Deletion of IL-10 results in a loss of STAT3 phosphorylation at Tyr705 and deletion of the type I interferon receptor, which will block IFNβ signaling, caused loss of STAT1 phosphorylation at Tyr701, suggesting that STAT1 and STAT3

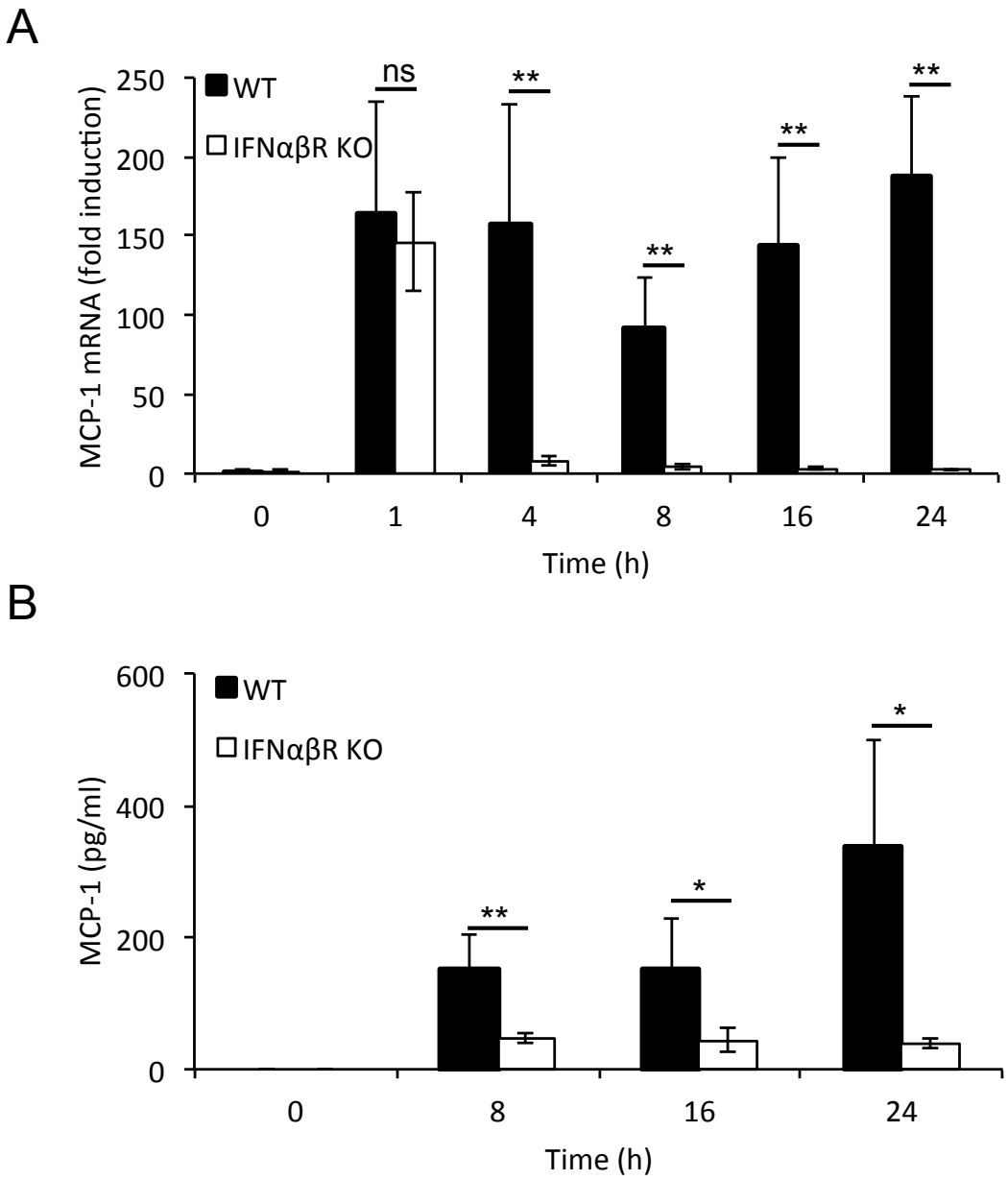

Figure 3.25: Poly(I:C)-induced MCP-1 expression requires biphasic IFNβ induction
Bone marrow-derived macrophages from WT and IFNαβR KO mice were stimulated with 10 μg/ml poly(I:C). A) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for MCP-1 mRNA. B) Culture supernatant was collected and MCP-1 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

primarily mediate effects of IFNβ and IL-10 respectively (Ananieva et al., 2008 and figure 3.5).

Interestingly, deletion of IFNαβR did not affect the initial phase of IL-10 transcription nor did treatment with Ruxolitinib (figure 3.7 and figure 3.9). Other signaling pathways and transcription factors have been implicated in regulating the initial transcription of IL-10, including CREB, Sp1 and NFκB (Saraiva et al., 2005; Brightbill et al., 2000; Ma et al., 2001; Elcombe et al., 2013).

The results above demonstrate a requirement for IFNβ in sustaining IL-10 transcription in response to TLR4 stimulation. IFNβ will activate STAT1 downstream of the type I receptor via JAK1 and Tyk2. STAT1 could therefore be directly binding to the IL-10 promoter and regulating its transcription. In line with this, treatment of LPS-stimulated cells with Ruxolitinib resulted in the loss of STAT1 phosphorylation which correlated with loss of sustained IL-10 transcription (figure 3.6). Equally, deletion of the type I interferon receptor caused a similar loss of STAT1 phosphorylation, which correlated with decreased IL-10 transcription at late time points (figure 3.5). Therefore active STAT1 could be required for maintaining IL-10 transcription. However, the requirement for an intermediate between IFNβ signaling and sustaining IL-10 transcription cannot be ruled out. One study has suggested a role for IL-27, a member of the IL-12 cytokine family, in the induction of IL-10 in response to IFNβ (Iyer et al., 2010). Contrary to this study, the induction of IL-27 in response to IFNβ occurred with the same kinetics as those of IL-10 (see figure 3.10 and figure 3.11), which suggests that IL-27 may not be required for IL-10 transcription in response to IFNβ.

3.10.2 Ruxolitinib affects the production of pro-inflammatory cytokines

Importantly, deletion of the type I interferon receptor does not affect the negative feedback loop of IL-10 that represses the pro-inflammatory cytokine TNFα. Macrophages from IFNαβR KO mice do not display elevated levels of TNFα compared to wildtype controls (figure 3.13). This correlates with the result that deletion of IFNαβR does not affect the initial transcription or secretion of IL-10. Early release of IL-10 is responsible for the negative feedback loop, which is partly mediated by phosphorylated STAT3 downstream of IL-10R (Takeda et al., 1999; Riley et al., 1999). In line with this, the Ruxolitinib-induced increase in TNFα level in LPS-stimulated macrophages was not affected by IFNαβR KO.

Deletion of IFNαβR moderately affected IL-12(p35) and IL-12(p40) transcription and this was reflected in secreted levels of IL-12(p40) and the dimer of the p35 and p40 subunits, IL-12(p70). Interestingly, a study in dendritic cells has identified a role for IFNβ in maintaining IL-12 transcription (Gautier et al., 2005). Using mice deficient in IFNαβR or STAT1, they demonstrated that IFNβ maintained transcription of both IL-12(p35) and IL-12(p40) in response to TLR agonists. Whilst the changes in IL-12 transcription affected secretion of the IL-12(p70) dimer, levels of IL-12(p40) were unaffected. This study suggested that IL-12 mRNA was affected more than protein levels by IFNβ signaling, which is in agreement with results presented above.

IL-6 is also repressed by IL-10 and treatment with Ruxolitinib increased IL-6 levels in wildtype macrophages by blocking IL-10 signaling. In contrast to TNFα, knockout of the type I receptor strongly reduced IL-6 transcription and secretion from LPS-stimulated macrophages (figure 3.13). In addition to this, deletion of the type I IFN receptor reduced the increase caused by Ruxolitinib treatment of LPS-stimulated macrophages. These results suggested that IFNβ can positively regulate IL-6 transcription. A direct role for IFNβ stimulating IL-6 transcription was also demonstrated (figure 3.14). These results are supported by other studies that demonstrate a role for IFNβ in maintaining IL-6 transcription. In IFNβ knockout macrophages, array profiling demonstrated that IL-6 was less strongly induced in response to LPS compared to wildtype macrophages (Thomas et al., 2006). In addition to this, the importance of the IFN feedback loop was also demonstrated for IL-6 in dendritic cells (Yasuda et al., 2007).

JAK inhibitors, such as Ruxolitinib and Tofacitinib, are proposed to be effective in the treatment of a range of autoimmune diseases (O'Shea et al., 2013b). An important part of the therapeutic benefit achieved is through blocking JAK signaling within the adaptive immune system. Interestingly, both Ruxolitinib and Tofacitinib result in increased pro-inflammatory cytokine secretion from bone marrow-derived macrophages stimulated with LPS, due to inhibition of IL-10 signaling (figure 3.13 and figure 3.19). Ruxolitinib has been shown to prevent DC maturation and function by affecting cytokine levels and their ability to activate T cells (Heine et al., 2013). *In vivo* it has been shown to reduce

cytotoxic T cell responses following OVA/CpG injection into mice and delay clearance of an adenovirus. Tofacitinib has been shown to elevate levels of IL-10 and reduce those of the pro-inflammatory cytokines, TNFα, IL-6 and IL-12 in response to i.p. injection of LPS into DBA/1J mice (Ghoreschi et al., 2011). This is in contrast to the results seen in isolated BMDMs, where Tofacitinib treatment elevates pro-inflammatory cytokine levels (figure 3.19). This may be due to the contribution of multiple cell types to the inflammatory response and whose cytokine production may be regulated in different ways by JAKs compared to macrophages.

In vivo, macrophages would be primed by IFNγ, whilst isolated macrophages are naive prior to LPS stimulation (Hu et al., 2005). JAK inhibitors would block this priming and thereby reduce the pro-inflammatory potential of macrophages. Equally, IFNβ can prime macrophages, which would also be blocked by JAK inhibitors (Thomas et al., 2006; Hu et al., 2008). Deletion of IFNβ, STAT1 or Tyk2 renders mice less sensitive to LPS-induced endotoxic shock (Thomas et al., 2006; Karaghiosoff et al., 2000).

Lastly, the concentration of Tofacitinib is important and directly relates to its ability to block IL-10 and/or IFNβ signaling. Whether the concentration used during the *in vivo* studies compares to the dose used in BMDMs to block both IL-10 and IFNβ signaling is unclear. This could partially explain the discrepancy between *in vivo* and *in vitro* effects on pro-inflammatory cytokine production as the *in vivo* dose of Tofacitinib may have been unable to block the anti-inflammatory effects of IL-10.

3.10.3 Comparison of Ruxolitinib and Tofacitinib

Ruxolitinib and Tofacitinib are structurally distinct inhibitors of JAKs and have similar effects on pro-inflammatory cytokine secretion from LPS-stimulated macrophages. Notably, Tofacitinib inhibited IFNβ signaling more potently than IL-10 signaling, whereas Ruxolitinib inhibited both signaling pathways at a similar concentration. Both IFNβ and IL-10 signal through JAK1 and Tyk2. The results above show that Tofacitinib has similar IC50s for JAK1 and JAK3, but higher IC50s for JAK2 and Tyk2 (figure 3.16). Interestingly, some studies have noted that a higher concentration of Tofacitinib is required to

block STAT1 and STAT3 phosphorylation in response to IL-6 (Ghoreschi et al., 2011; Rosengren et al., 2012). IL-6 is reported to signal through JAK1, JAK2 and Tyk2. It could be suggested that Tyk2 is more important in phosphorylation of STAT3 compared to STAT1 phosphorylation. However, studies with Tyk2 knockout mice have shown normal phosphorylation of STAT3 in response to IL-10 but decreased IFNβ-induced STAT1 phosphorylation (Karaghiosoff et al., 2000; Shimoda et al., 2000).

3.10.4 Transcription of MCP-1 is sustained by the IFNβ-mediated feedback pathway

The importance of the IFNβ-mediated feedback pathway in modulating the production of cytokines and chemokines from LPS-stimulated macrophages was further underlined by the requirement for IFNβ and STAT1 signaling in inducing transcription of MCP-1, a β-chemokine (figure 3.20, figure 3.23). Macrophages also require the IFNβ-mediated feedback pathway to sustain transcription of MCP-1 in response to poly(I:C), a TLR3 agonist. Ruxolitinib blocks direct activation of MCP-1 transcription by IFNβ.

IFNβ can activate both STAT1 and STAT2, which can form a complex with IRF9. Deletion of the type I receptor blocks both STAT1 and STAT2 phosphorylation in response to LPS. The results above demonstrate that STAT1 is recruited to the ccl2 (MCP-1) promoter in response to IFNβ. In line with this, other studies have identified STAT binding sites within the ccl2 promoter (Zhou et al., 1998; Shyy et al., 1990). STAT2 may also be important in regulating transcription of MCP-1. In a model of colitis, mice deficient for STAT2 produced less MCP-1 (Gamero et al., 2010). Other studies have suggested roles for STAT4 and STAT6 respectively in regulating MCP-1 transcription (Iida et al., 2011; Tang et al., 2011), however my work suggests a dominant role for STAT1 and/or STAT2.

3.10.5 The importance of MCP-1 in viral infection

Interestingly, MCP-1 is induced by IFNβ, which is important for promoting an anti-viral response. This might suggest that MCP-1 is important for combating viral infections. Several studies have examined mice deficient for MCP-1 or, its receptor, CCR2 in viral

infection models. In a model of influenza pneumonia, WT mice have elevated pulmonary MCP-1 levels. Notably, MCP-1 KO mice suffered from an increased viral load along with diminished influx of macrophages and granulocytes. MCP-1 deficient mice also exhibited increased pro-inflammatory mediators such as TNFα and IL-6 (Dessing et al., 2007). MCP-1- and CCR2-deficient mice were infected with murine cytomegalovirus (CMV) and showed reduced accumulation of macrophages and NK cells compared to wildtype mice. In addition to reduced immune cell trafficking, mice lacking either MCP-1 or CCR2 had increased viral titers (Hokeness et al., 2005). Following West Nile virus infection, knockout of CCR2 caused reduced monocyte accumulation in the brain and increased mortality from encephalitis (Lim et al., 2011). These studies demonstrate a clear role for MCP-1 in the anti-viral response and therefore suggests that the prolonged transcription and secretion of MCP-1 promoted by the IFNβ-mediated feedback pathway is important for an appropriate and effective anti-viral response.

3.10.6 Conclusion

LPS-stimulated macrophages produce a number of cytokines and chemokines as part of the inflammatory response. Regulation of these inflammatory mediators is important to promote resolution and prevent tissue damage from excessive or chronic inflammation. Numerous pathways control the production of IL-10, an important inflammatory cytokine. The data presented in this chapter demonstrate that an IFNβ-mediated feedback pathway is required for sustained transcription and secretion of IL-10. Additionally, IFNβ can directly activate IL-10 transcription and this induction can be blocked by the JAK inhibitor Ruxolitinib. Treatment of wildtype cells with Ruxolitinib will increase pro-inflammatory cytokine production by inhibiting the IL-10-mediated anti-inflammatory response. Deletion of the type I IFN receptor did not increase secretion of pro-inflammatory cytokines, TNFα and IL-12. However, IL-6 transcription was reduced in type I IFN receptor-deficient macrophages. A structurally distinct JAK inhibitor, Tofacitinib, also had similar effects on IL-10 and pro-inflammatory cytokine production. Notably, Tofacitinib required a 10-fold higher concentration to block IL-10 signaling compared to IFNβ, whereas Ruxolitinib inhibited both pathways with similar potency.

In addition to regulating IL-10 transcription, the IFNβ-mediated feedback pathway was also required for sustained MCP-1 transcription. Deletion of IFNαβR from macrophages reduced MCP-1 mRNA and protein levels at late time points following LPS and poly(I:C) stimulation. Similarly to IL-10, MCP-1 transcription was directly induced in response to IFNβ in a JAK-dependent manner. Furthermore, in response to IFNβ, STAT1 was shown to be recruited to the ccl2 promoter.

Together these results demonstrate the importance of the IFNβ-mediated feedback pathway in promoting an appropriate and effective immune response figure 3.26. This feedback pathway is important in the resolution of inflammation by maintaining IL-10 transcription and the migration of immune cells by promoting MCP-1 transcription in response to bacterial and viral ligands.

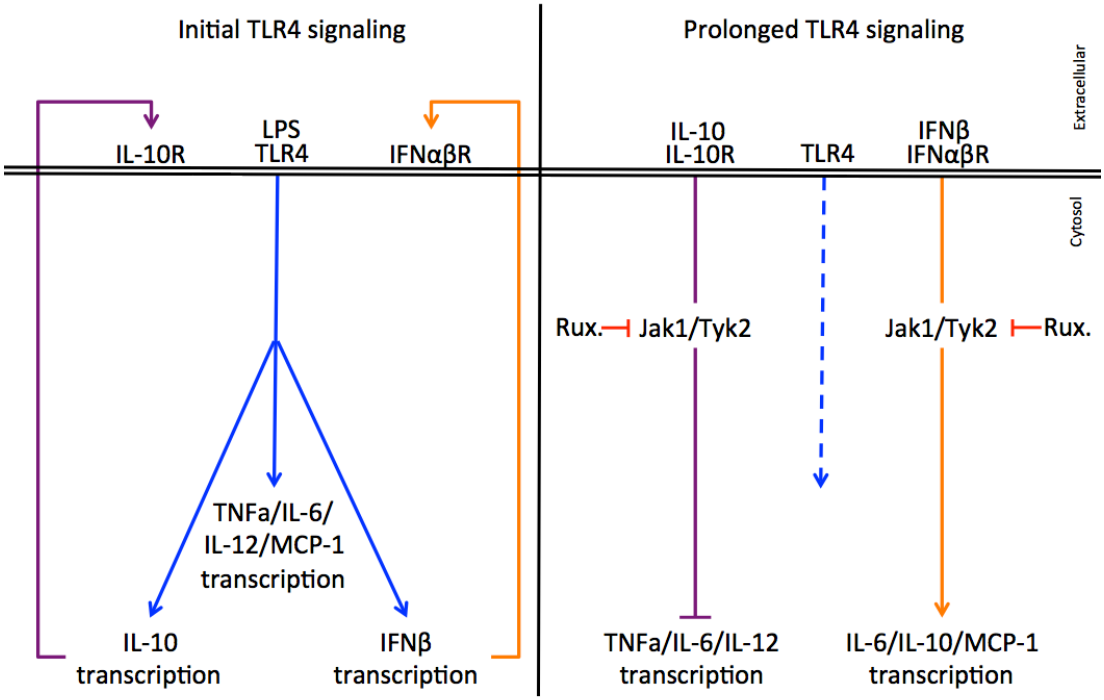

Figure 3.26: Importance of the type I interferon-mediated feedback loop in LPS-stimulated macrophages
In response to LPS, the transcription of TNF, IL-6, IL-12, MCP-1, IL-10 and IFNβ is induced. IL-10 can feedback through the IL-10R and repress transcription of TNF, IL-6 and IL-12. IFNβ also establishes a feedback loop that enhances the sustained transcription of IL-6, IL-10 and MCP-1. Ruxolitinib, the JAK inhibitor, blocks the actions of both IL-10 and IFNβ feedback pathways resulting in enhanced secretion of TNF, IL-6 and IL-12. Secretion of IL-10 and MCP-1, in response to LPS, is reduced at late time points in the presence of Ruxolitinib.

Chapter 4

Results- MEF2D negatively regulates IL-10 transcription

4.1 Myocyte enhancer factor 2

The MEF2 (myocyte enhancer factor 2) transcription factor family has been implicated in immune cell development but a role in the context of TLR signaling has not yet been identified (Black and Olson, 1998; Potthoff and Olson, 2007). MEF2 factors are evolutionary conserved and function as part of an ancient network regulating muscle cells. MEF2 factors recognise a conserved sequence (YTA$(A/T)_4$TAR) in many muscle-specific genes and loss of function studies have demonstrated the importance of MEF2 factors in muscle gene expression during embryogenesis. In addition, MEF2 factors have also been shown to respond to mitogenic signals, while their expression and function is controlled by numerous inputs to modulate the transcriptional programme produced.

4.1.1 MEF2 structure and function

MEF2 was originally described as binding an A/T rich DNA sequence in the muscle creatine kinase enhancer (Gossett et al., 1989). This A/T rich sequence was recognized to be conserved and found in many skeletal and cardiac muscle genes (Bassel-Duby et al., 1992; Muscat et al., 1992). Early studies showed that mutation of the binding site strongly reduced expression of these genes and that ectopic expression of MEF2 would induce expression. In vertebrates, the MEF2 family consists of 4 isoforms; A, B, C and D, each

encoded by a different gene located on different chromosomes (Pollock and Treisman, 1991; Yu et al., 1992; Breitbart et al., 1993; Hobson et al., 1995; Suzuki et al., 1996). Only one MEF2 gene is present in the genomes of *Drosophilia* and *C.elegans* (Lilly et al., 1994; Nguyen et al., 1994; Dichoso et al., 2000).

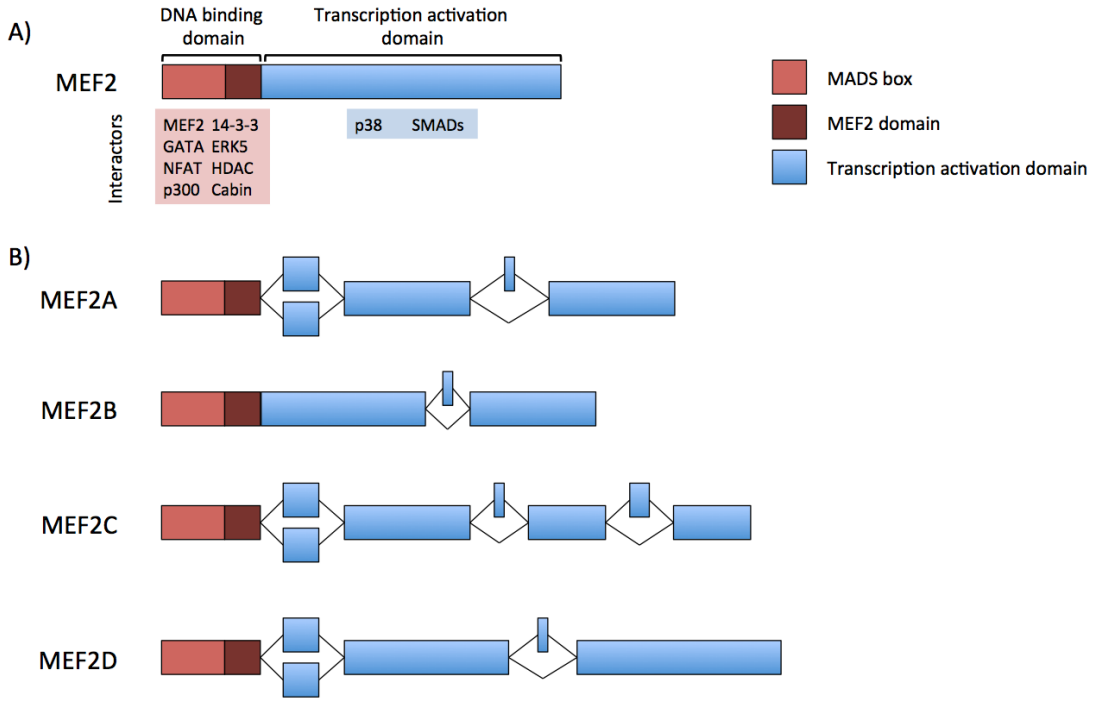

Figure 4.1: MEF2 family
A) MEF2 consists of a DNA binding domain formed from a MADS box and MEF2 domain and long C-terminal transcription activation domain. MEF2 proteins can interact with a number of proteins including kinases such as p38, transcription factors such as NFAT and GATA as well as transcription cofactors such as p300 and HDACs. B) In vertebrates, there are four MEF2 family members each encoded by a gene with several splice variants. Exons connected by lines are used alternatively.

MEF2 transcription factors consist of a N-terminal MADS box and MEF2 domain and long C-terminal transcription activation domain. The MADS box is a 57-amino acid motif that can act as a minimal DNA binding domain (DBD) (Shore and Sharrocks, 1995). Together, the MADS box and the 279-amino acid MEF2 domain form a high-affinity DBD (Molkentin et al., 1996a). The MADS box contains several invariant residues important for DNA recognition which are conserved with other MADS-box family members. The DBD is also important for dimerization and the dimer is capable of recognising DNA oligonucleotides. MEF2 transcription factors will bind their consensus sequence as homo- or heterodimers (Pollock and Treisman, 1991; Yu et al., 1992). Interaction between MEF2 proteins and other MADS-box factors do not occur, suggesting the residues

required for mediating MEF2 dimerisation are not conserved outside of the MEF2 family.

The MEF2 isoforms show around 50% amino acid homology overall, with 95% similarity within the MADS box and MEF2 domain (Black and Olson, 1998). The C-terminal regions show sequence diversity. The crystal structure of the DBD from MEF2A has been solved and shows that the MADS-box and MEF2 domain form a tightly folded structure (Wu et al., 2010). A MEF2 DBD dimer consists of a six-strand beta sheet sandwiched by a pair of α-helices above and below. The beta sheet acts as a core for protein folding, whilst the α-helices bind to DNA and promote protein-protein interactions. The α1-helix and the N-terminal extension of the MADS-box are the important regions for mediating DNA sequence recognition (Wu et al., 2010; Santelli and Richmond, 2000).

MEF2 transcription factors bind the same consensus sequence, although MEF2B has reduced affinity compared to the other family members (Pollock and Treisman, 1991; Yu et al., 1992; Molkentin et al., 1996b). Nucleotides flanking the A/T rich sequence also affect MEF2 binding to DNA sequences (Andres et al., 1995; Fickett, 1996). Whilst the MADS-box and MEF2 domain are sufficient for DNA binding, they lack transcriptional activity. The C-terminal regions of the MEF2 family members contain a transcriptional activation domain (TAD). The C-termini share little sequence homology except in certain short acidic exons and in four conserved Ser/Thr rich regions. MEF2A, C and D also possess a nuclear localisation sequence at the extreme C-terminal region. Deletion of this sequence in MEF2A renders it cytosolically located (Yu, 1996).

4.1.2 Regulation of MEF2 expression and activity

The expression of MEF2 factors is controlled at the transcriptional, translational and post-translational levels. In vertebrates, mef2 transcripts are highly expressed in muscle cell lineages (Edmondson et al., 1994). In addition to muscle cell expression, MEF2 is also enriched in the developing central nervous system (Leifer et al., 1993, 1994; Lyons et al., 1995). The expression pattern in adult tissues is still unclear with conflicting reports suggesting either limited expression of the proteins to neural and muscle tissues or ubiquitous expression of both transcripts and proteins (Pollock and Treisman, 1991; Chambers et al.,

1992; Yu et al., 1992; Martin et al., 1993; McDermott et al., 1993; Dodou et al., 1995; Ornatsky and McDermott, 1996). There is a conserved casein kinase II phosphorylation site within the MADS-box of the MEF2 family members, although this site appears to be constitutively phosphorylated *in vivo* and thus is unlikely to regulate MEF2 activity. Other post-translation modifications including phosphorylation and sumoylation occur and can alter MEF2 activity as discussed below (Molkentin et al., 1996c; Zhao et al., 1999; Grégoire et al., 2006, 2007; Kato et al., 1997; Han et al., 1997).

4.1.3 Knockout of MEF2 genes

The importance of each MEF2 factor in development is now being elucidated using knockout models. MEF2A KO mice mostly die within the first week after birth through cardiovascular defects, although skeletal muscle develops normally (Naya et al., 2002; Potthoff et al., 2007). The remaining mice suffer from cardiac defects and mitochondrial deficiency. They also suffer from the risk of sudden death. MEF2C knockout is embryonic lethal and the embryos show defects in cardiac morphogeneis at embryonic day 9 (Lin et al., 1997). The generation of conditional MEF2C knockout mice has shown the importance of MEF2C in determining myeloid cell fate, possibly by regulating expression of c-Jun (Schüler et al., 2008). MEF2B null mice are viable with no obvious muscle defects (Black and Olson, 1998). Global deletion of MEF2D has little effect on skeletal muscle development and these mice appear normal in other respects (Potthoff et al., 2007). Further study on MEF2D function using MEF2D-null mice and a transgenic model with over-expression of MEF2D demonstrated that MEF2D is important in stress-dependent cardiac growth (Kim et al., 2008b).

4.1.4 Signaling to MEF2

MEF2 proteins initiate transcription of genes and act as signaling endpoint for several pathways. It has been shown that MAPK can phosphorylate MEF2 factors in yeast and humans (Dodou and Treisman, 1997; Han et al., 1997; Kato et al., 1997). ERK5 is known to phosphorylate MEF2 leading to augmented transcriptional activity (Yang et al., 1998; Kasler et al., 2000). The C-terminal region will interact directly with the MADS-MEF2 domain of MEF2D and this interaction is required for full activation of MEF2D (Kasler

et al., 2000).

p38 is known to phosphorylate MEF2 factors (Zhao et al., 1999; Han et al., 1997). p38 was shown to phosphorylate MEF2A on two Thr residues leading to an increase in transcriptional activity, however p38 was shown to be unable to phosphorylate MEF2B or MEF2D (Zhao et al., 1999). A more recent study contradicted the work of Zhao *et al*, this study demonstrated that p38 phosphorylated MEF2D and that p38-mediated phosphorylation of MEF2D promoted interaction with Ash2L (Rampalli et al., 2007). Ash2L-containing methyltransferase complex recruitment led to trimethylation of histone H3 Lys4, epigenetically marking MEF2-regulated genes for expression in muscle progenitor cells.

Calcium signaling has also been implicated in regulating MEF2 function. Primarily, this is mediated through regulating the activity of histone deacetylases (HDACs). The Class IIa HDACs will form repressive complexes with MEF2 via the MADS-box at MEF2-dependent genes (Bertos et al., 2001; McKinsey et al., 2001a). Calcium-regulated protein kinases such as protein kinase D and calcium calmodulin-dependent protein kinases can phosphorylate class IIa HDACs on conserved serine residues (Zhang et al., 2002; McKinsey et al., 2001b). Phosphorylation of HDACs results in translocation to the cytoplasm and thus allows activation of MEF2 and transcription of MEF2-dependent genes.

MEF2D is phosphorylated at Ser444 and mutation of this site to an alanine prevents sumoylation of MEF2D at Lys439 (Grégoire et al., 2006). Mimicking phosphorylation at Ser444 with a glutamate residue induced similar levels of sumoylation as seen in wild-type MEF2D. Interestingly, Ser444 is an established CDK5 site based on *in vitro* studies (Gong et al., 2003), suggesting that post-translational modifications can be regulated by signaling events. Sumoylation at Lys439 and phosphorylation at Ser444 is known to inhibit the transcriptional activity of MEF2D (Zhu and Gulick, 2004; Grégoire and Yang, 2005; Gong et al., 2003; Zhao et al., 2005). This inhibition could be relieved through the action of calcineurin, a phosphatase (Grégoire et al., 2006). This study demonstrated that interplay between post-translational modifications affect the transcriptional activity of MEF2D. It has also been shown that HDAC4 potentiates sumoylation of MEF2D and

this may occur through recruitment of CDK5 (Grégoire et al., 2006). Similarly to calcineurin opposing CDK5, the SUMO protease SENP3 reverses sumoylation of MEF2D, enhancing transcriptional activity (Grégoire and Yang, 2005).

Protein kinase A signaling is thought to modulate MEF2 activity. Membrane depolarisation leads to cAMP-PKA pathway mediated activation of MEF2 which is required for neuronal survival (Wang et al., 2005b). PKA was shown to phosphorylate MEF2 at Thr20 *in vitro* and increase DNA binding activity. Blocking the PKA pathway or expression of a Thr20Ala mutant enhanced neuronal apoptosis upon membrane depolarisation. A later study showed PKA could phosphorylate Ser121 and Ser190 of MEF2D *in vitro* and that these phosphorylation events repressed transcriptional activity (Du et al., 2008). The overall role of PKA in modulating MEF2 activity is unclear and may be stimuli-specific and subject to modulation by other post-translational modifications.

The transcriptional activity of MEF2D can also be regulated by small molecule inhibitors that target proteins involved in modulating its activity. The CDK inhibitor, roscovitine, was shown to reduce sumoylation of MEF2D by preventing phosphorylation at Ser444 (Grégoire et al., 2006). Another study used class II HDAC inhibitors to block MEF2D transcriptional activity. This repression of activity was caused by stabilising the inhibitory HDAC3-HDAC4-MEF2D complex and reducing MEF2D gene expression (Nebbioso et al., 2009).

4.1.5 MEF2 factors in immune cells

The development and activation of T cells is a tightly regulated process mediated by many signaling pathways including calcium signaling. Calcium signaling is important in TCR-induced apoptosis (Woronicz et al., 1995). Nur77 is an important mediator of apoptosis in T cells and the Nur77 promoter contains two MEF2 sites (Youn et al., 1999). It has been reported that in resting T cells, MEF2 is complexed with HDAC7 and Cabin1, which act as transcriptional repressors and reduce Nur77 expression (Dequiedt et al., 2003; Youn et al., 2000). Within Cabin1, a C-terminal region is responsible for binding to calcineurin and MEF2. Mice expressing a mutant version of Cabin1 lacking this region had normal

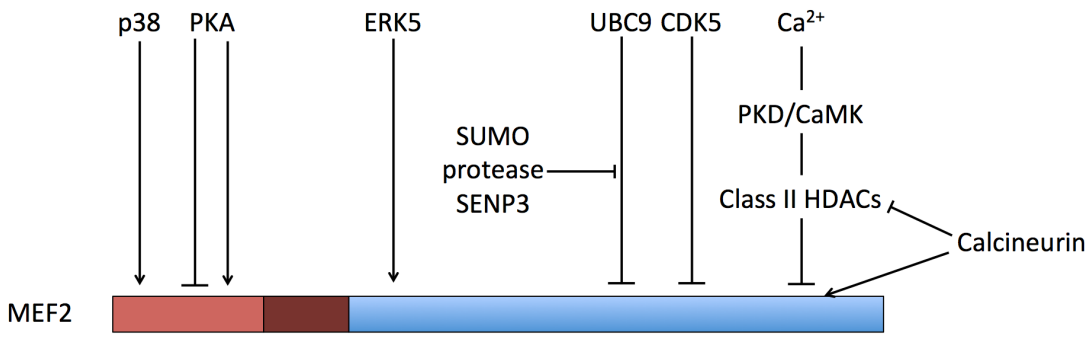

Figure 4.2: Signaling input to MEF2
MEF2 activity and stability is regulated by numerous signaling inputs. Selected inputs are illustrated here. p38 has been shown to phosphorylate and activate MEF2. PKA phosphorylates MEF2 at Thr20 and enhances DNA binding. PKA can also phosphorylate MEF2 at other sites and repress transcriptional activity. ERK5 has been shown to phosphorylate MEF2 proteins at various sites. CDK5 can phosphorylate and inhibit MEF2 and this is associated by sumoylation at a nearby residue. The sumoylation of MEF2 is reversed by the SUMO protease SENP3. Calcium signaling inhibits the action of MEF2 by promoting complex formation with Class II HDACs. This action is opposed by calcineurin

T and B cell development. Apoptosis of T cells was also unaffected, however, following TCR stimulation, mutant T cells produced higher levels of IL-2, IL-4 and IFNγ (Esau et al., 2001). This data suggests Cabin1 plays an important role in controlling cytokine production from T cells through MEF2 activity.

Furthermore, MEF2 activity is regulated following TCR activation. HDAC7 is phosphorylated by PKD1 and translocates to the nucleus with 14-3-3 proteins resulting in active MEF2 (Parra et al., 2005). MEF2 is conversely regulated by the actions of protein phosphatase 1 and myosin phosphatase targeting subunit 1, which promotes nuclear localisation of HDAC7 (Parra et al., 2007). HDAC4 and 5 were shown to interact with MEF2D and repressed transcription. CAMK phosphorylation of the DNA binding domain in MEF2D relieved HDAC4/5-mediated repression of transcription (Lu et al., 2000). HDAC4 was also shown to regulate the sumoylation of Lys424. HDAC4 was shown to interact with the E2 conjugating enzyme Ubc9 and sumoylation of Lys424 inhibited transcriptional activity. CBP was shown to be capable of acetylating MEF2 and this action was opposed by SIRT1 (Zhao et al., 2005).

In addition to calcium signaling, cAMP signaling is also engaged in response to TCR activation. It has been shown that IL-10 production in T lymphocytes can be strongly

inhibited by cAMP elevating agents. Using luciferase reporters with the IL-10 promoter, one study identified a fragment of the promoter, sensitive to cAMP-mediated inhibition, that contained a MEF2 binding site. Over-expression of MEF2 increased IL-10 promoter activity and stimulated binding to the IL-10 promoter which was sensitive to cAMP-mediated inhibition (Liopeta et al., 2009).

MEF2 proteins have also been shown to play important roles in myeloid cells. Ectopic expression of MEF2C in C57Bl/6 mice lead to a six-fold reduction in Gr-1 positive cells (a marker of granulocytic differentiation) from bone marrow (BM) cells cultured in G-CSF (Schüler et al., 2008). It was also noted that MEF2C had varying levels of expression during myelopoiesis. Interestingly, BM cells cultured in M-CSF had increased expression of MEF2C compared to those untreated or cultured in GM-CSF. Bone marrow from mice lacking MEF2C produced a lower percentage of CD11b+F4/80+ cells compared to controls, although early myeloid differentiation of common myeloid, granulocyte-monocyte, or megakaryocyte-erythroid progenitors was unaffected. As has been reported in other studies, c-Jun is a target of MEF2C in myeloid cells, where deletion of MEF2C reduced c-Jun expression. Over-expression of MEF2C enhanced c-Jun expression in response to LPS treatment in NIH3T3 cells. (Schüler et al., 2008).

MEF2D has been shown to form a heterodimer with MEF2A in macrophage differentiated HL60 cells (Aude-Garcia et al., 2010). The MEF2A/D dimer was proposed to be important in differentiation through control of c-Jun and MEF2A was shown to be bound to the c-Jun promoter by ChIP assay. MEF2A and MEF2D were also shown to be present in primary human monocytes and macrophages (Aude-Garcia et al., 2010).

Although, MEF2D is thought to regulate IL-10 in T cells, the role of MEF2D in macrophage-derived IL-10 production is unclear. LPS stimulation of macrophages activates multiple signaling pathways resulting in the activation of several transcription factors including CREB, NF-$\varkappa$B, IRF3, IRF5 and IRF7. Other transcription factors such as MEF2D may be activated and may play important roles in regulating transcription of key cytokines. Identification of novel regulating pathways may provide new therapeutic targets. In order

to identify other transcription factors activated in response to LPS, Raw264.7 cells were cultured in light- or medium-conditioned SILAC media.

4.2 MEF2D is phosphorylated in response to LPS in Raw264.7 cells

Medium-labelled Raw264.7 cells were stimulated with LPS for 30 minutes and lysates from light-labelled and medium-labelled Raw264.7 were mixed at a ratio of 1:1. Lysates were enriched for phosphopeptides and then measured by MS/MS (figure 4.3).

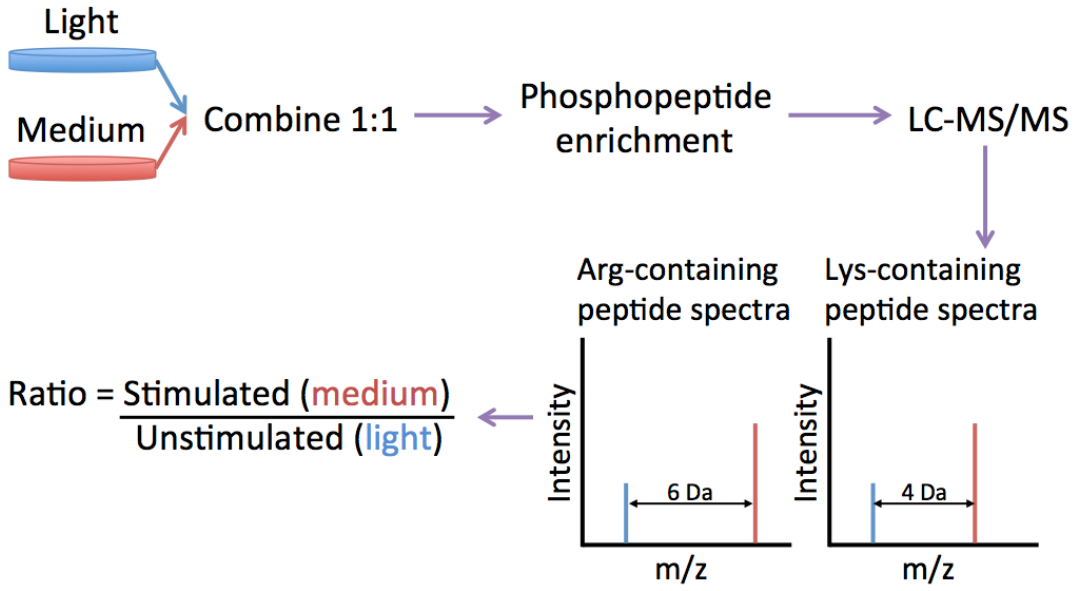

Figure 4.3: Experimental procedure for SILAC
Light (R0K0) and Medium (R6K4)-labelled Raw264.7 cells were cultured. Medium-labelled cells were stimulated with LPS and light-labelled cells unstimulated. Lysates were combined at 1:1 ratio and phosphopeptides were enriched by immobilized metal-ion (Fe^{3+}) chromatography. Phosphopeptides were analysed by MS/MS. Peptides from medium-labelled cells are distinguished from peptides from light-labelled cells and used to calculate the ratio of the peptide in medium-labelled cells compared to light-labelled cells.

Over 6000 phosphopeptides were identified and over 1000 of these peptides were more than 3 fold up-regulated in response to LPS compared to unstimulated cells. Peptides which were consistently up-regulated across 3 experiments were identified and further selected for a role in regulating transcription. Phosphopeptides from MEF2D are presented in figure 4.4 and the ratio of stimulated to unstimulated is shown. Five peptides were identified as being phosphorylated in Raw 264.7 cells, however only two of these peptides were differentially regulated in response to LPS stimulation. The RASEELDGFRR peptide (119-130 mouse) showed 8 fold enrichment in phosphorylation in stimulated compared to unstimulated Raw 264.7 cells. Phosphorylation at the Ser121 position has been

identified in previous studies, including a large-scale phosphoproteomic study which identified the S121 phosphopeptide in the brain, heart, lung, kidney and spleen. (Huttlin et al., 2010).

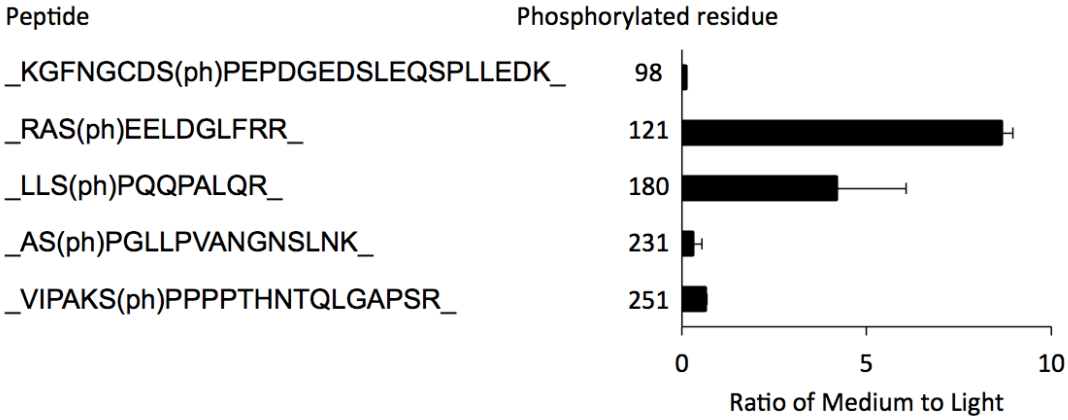

Figure 4.4: MEF2D peptides identified from LPS-stimulated SILAC Raw264.7 cells
Raw264.7 cells were unstimulated (Light condition) or stimulated with 100 ng/ml LPS for 30 mins (Medium condition) and lysed in 8M urea. Lysates were quantified and mixed at a 1:1 ratio. Phosphopeptides were enriched using immobilized metal-ion (Fe^{3+}) chromatography and analysed via MS/MS. Phosphopeptides identified from MEF2D are presented with ratio of stimulated:unstimulated (M:L) from 3 samples. Error bars represent standard deviation

4.3 Characterisation of the S121 phosphorylation site on MEF2D

Antibodies were raised against the S121 peptide and tested through dot blots to determine their specificity. When the phospho antibody was pre-incubated with S121 phosphopeptide it recognised neither the phospho- or dephosphopeptides (figure 4.5). When incubated with the dephosphopeptide, the antibody was capable of recognising 100 ng of the phosphopeptide only. The antibody alone demonstrates that it recognises predominantly the phosphopeptide with some weak binding to the dephosphopeptide. The 1st and 2nd bleeds bound the S121 peptide less strongly (data not shown) and therefore the 3rd bleed was used.

Next, the phosphorylation of MEF2D at S121 in response to LPS was validated in Raw264.7 cells through immunoprecipitation. The phospho antibody was used to pull down phosphorylated MEF2D and then immunoprecipitates were immunoblotted using a total MEF2D antibody (BD Biosciences). Stimulation with LPS does not affect protein level of MEF2D over 60 minutes (figure 4.6). Unstimulated cells do not demonstrate any phosphorylated MEF2D at S121. After 15 and 30 minutes of LPS stimulation, a band is evident sug-

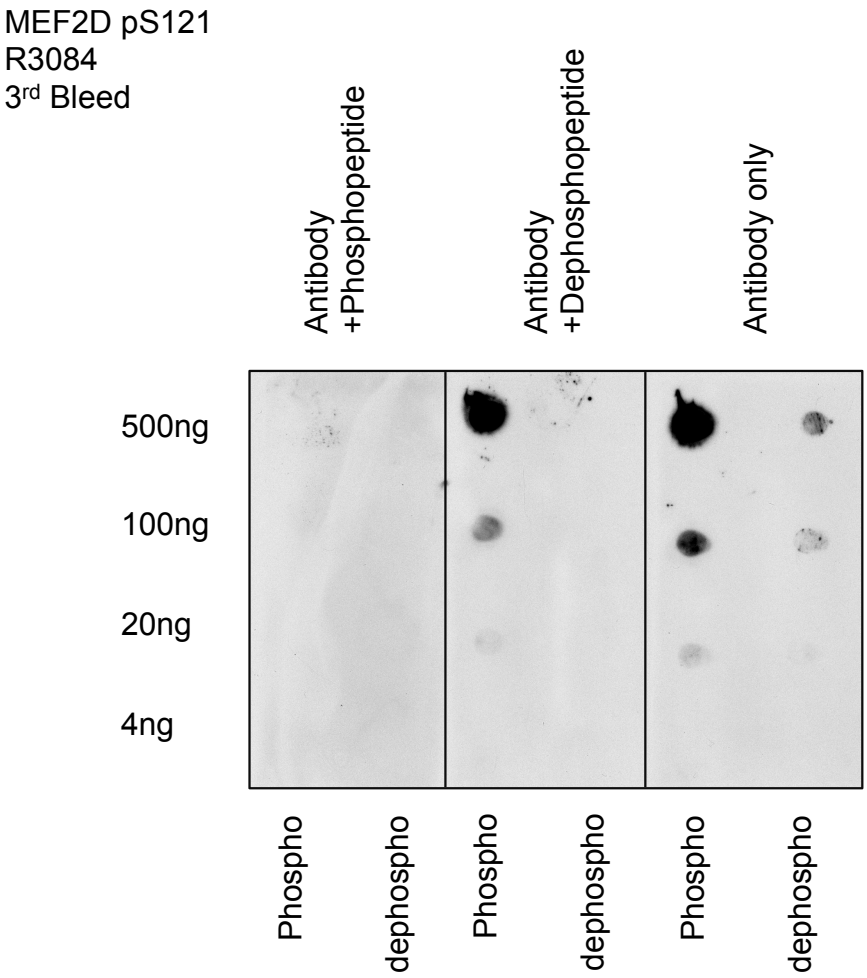

Figure 4.5: Phospho S121 MEF2D antibody dot blots
Decreasing concentrations of the phosphorylated and dephosphorylated S121 peptide were spotted onto nitrocellulose membrane before incubation with the S121 phospho antibody in the presence of 1 µg/ml phosphopeptide, in the presence of 1 µg/ml dephosphopeptide or alone. Results are representative of two independent experiments.

gesting that MEF2D is phosphorylated at S121 in response to LPS at these time points. After 60 minutes of LPS stimulation, no MEF2D band is present suggesting that the phosphorylation event has been reversed, probably through the action of a phosphatase, since MEF2D protein levels do not decrease.

4.4 Role of MEF2D in TLR signaling

In order to understand the role that MEF2D might have in TLR signaling, MEF2D KO mice were generated. Previous work in the lab had generated a MEF2D floxed allele. This was crossed with a bal1 (a constitutive cre) to generate total MEF2D knockout mice. Bone marrow from wildtype and MEF2D KO mice was isolated and BMDMs cultured. First,

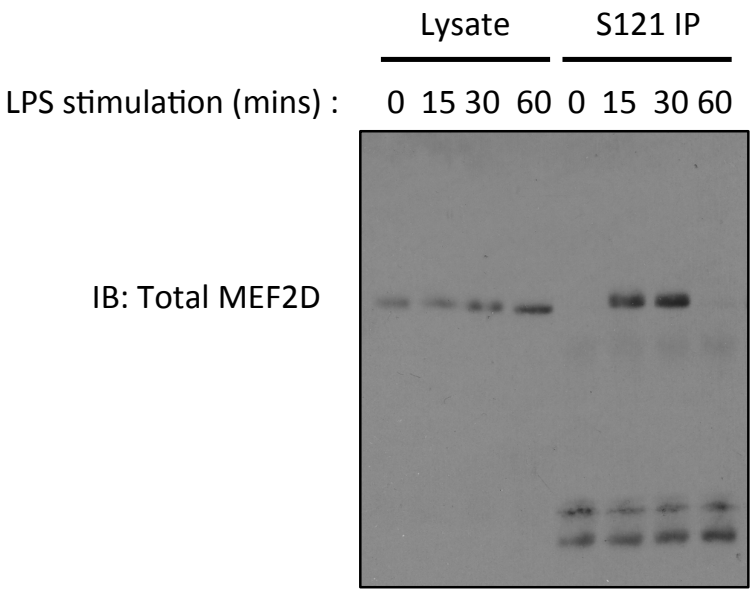

Figure 4.6: MEF2D is phosphorylated at S121 in response to LPS in Raw264.7 cells
Raw264.7 cells were stimulated with 100 ng/ml LPS for the times indicated and lysed. MEF2D was immunoprecipitated from 500 µg cell lysate using the MEF2D S121 phosphoantibody in the presence of the dephosphopeptide. Lysates and immunoprecipitate samples were seperated by SDS-PAGE and immunoblotted with a total MEF2D antibody. Results are representative of three independent experiments.

deletion of MEF2D was validated by detecting MEF2D protein. Unlike wildtype cells, MEF2D KO macrophages have no detectable MEF2D (figure 4.7). LPS stimulation leads to activation of MAPKs by 30 minutes, activation of p38 was similar in WT and MEF2D KO macrophages. The initial induction of JNK was similar between WT and MEF2D KO macrophages, however at 2 and 4 hours after stimulation there is a second wave of activation in the WT macrophages not present in MEF2D-deficient BMDMs. Activation of ERK1/2 and p105 was also comparable at 30 mins between wildtype and MEF2D-deficient macrophages, however at later time points phosphorylation of ERK1/2 and p105 appears slightly reduced. Interestingly, MEF2D KO macrophages show enhanced STAT3 phosphorylation after 2 hours of LPS stimulation suggesting an alteration in cytokine secretion. Previous work has shown that changes in IL-10 secretion from macrophages are often reflected in STAT3 phosphorylation (figure 3.5, Ananieva et al., 2008). MEF2D KO macrophages also had elevated levels of A20, a protein involved in inhibiting NFκB responses, compared to wildtype macrophages.

Changes in MAPK phosphorylation could be through alterations in the abundance of dual-specificity phosphatases. Data from a microarray experiment previously carried out in the lab was analysed. Microarray profiling identified DUSP1, 2, 4 and 5 as being over

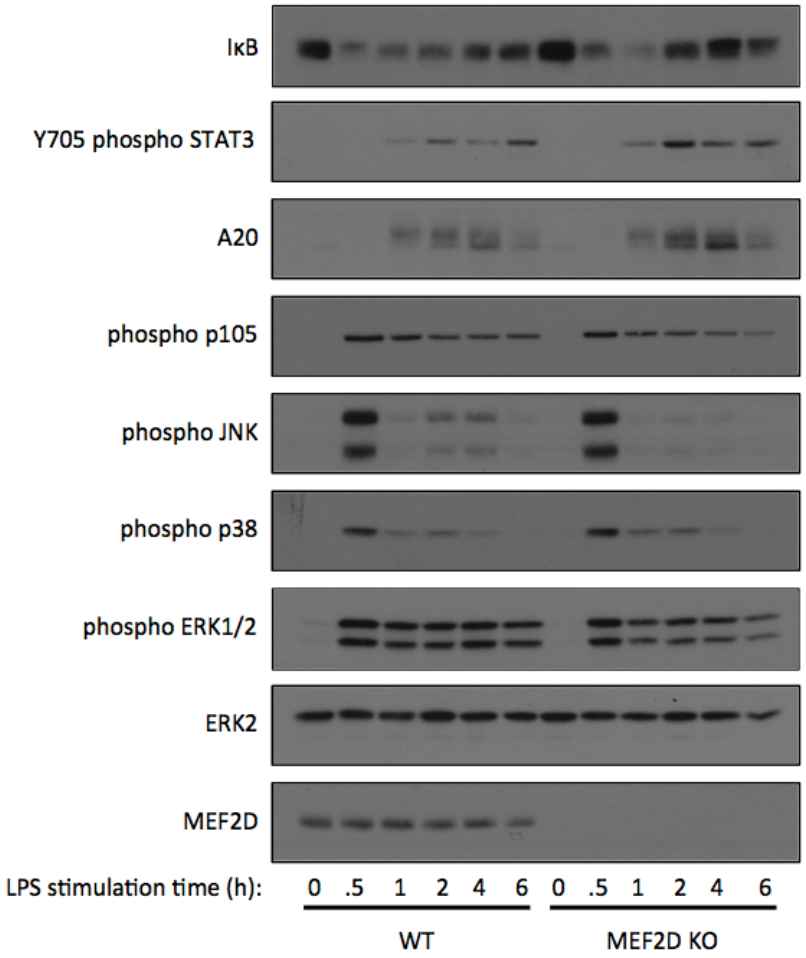

Figure 4.7: TLR signaling in MEF2D KO BMDMs
BMDMs were generated from WT and MEF2D KO bone marrow and stimulated with 100 ng/ml LPS for up to 6 hours. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to total IκB, phospho STAT3 (Y705), A20, phospho p105, phospho JNK, phospho p38, phospho ERK1/2, ERK2 and MEF2D. Results are representative of three independent experiments.

2.5 fold up-regulated in response to LPS in macrophages (see table 4.1). Wildtype and MEF2D-deficient macrophages were stimulated with LPS and DUSP1, 2 and 5 mRNA levels measured. LPS induced expression of DUSP5 at 1 hour in wildtype macrophages. Expression of DUSP5 was elevated in MEF2D KO macrophages compared to wildtype cells in response to LPS (figure 4.8). At 2 and 4 hours following stimulation with LPS, DUSP1 mRNA levels were marginally elevated in MEF2D KO cells compared to wildtype cells. Expression of DUSP2 in response to LPS was not affected by deletion of MEF2D in macrophages (figure 4.8).

Table 4.1: DUSP expression in LPS stimulated macrophages

Accession Number	Symbol	Average Expression	log(fold change)
NM_013642	Dusp1	12.10	2.80
NM_010090	Dusp2	7.51	3.81
NM_028207	Dusp3	10.22	-0.16
NM_176933	Dusp4	9.32	2.59
NM_001085390	Dusp5	9.10	4.40
NM_026268	Dusp6	8.61	-0.23
NM_153459	Dusp7	10.82	-0.14
NM_008748	Dusp8	7.02	2.22
NM_029352	Dusp9	6.61	0.25
NM_022019	Dusp10	6.67	0.30
NM_028099	Dusp11	9.84	-0.02
NM_023173	Dusp12	6.59	-0.28
NM_023173	Dusp12	5.00	-0.10
NM_001007268	Dusp13	6.40	-0.01
NM_019819	Dusp14	6.67	0.98
NM_001159376	Dusp15	3.44	-0.01
NM_130447	Dusp16	8.73	1.73
NM_173745	Dusp18	6.60	-0.14
NM_024438	Dusp19	7.62	0.06
NM_028568	Dusp21	3.33	-0.01
NM_001037955	Dusp22	9.33	-0.17
NM_026725	Dusp23	7.24	-0.06
NM_025869	Dusp26	4.69	-0.07
NM_001033344	Dusp27	3.91	-0.13
NM_175118	Dusp28	7.90	-0.25

4.5 Deletion of MEF2D increases IL-10 transcription and decreases pro-inflammatory cytokine secretion

Deletion of MEF2D from macrophages has small effects on the initial signaling events seen upon LPS stimulation. Despite this, changes in STAT3 phosphorylation suggest a modulation of cytokine levels, probably of IL-10. Therefore, WT and MEF2D KO BMDMs were stimulated with LPS and IL-10 transcription and secretion measured. LPS stimulation induced transcription of IL-10, which peaked at 2 hours. IL-10 mRNA levels were close to basal at 12 hours. MEF2D KO macrophages, in response to LPS, had higher levels of IL-10 mRNA between 1 to 4 hours with IL-10 mRNA levels returning to levels similar to that of wildtype macrophages from 8 hours onwards (figure 4.9).

MEF2D knockout increases IL-10 mRNA levels (figure 4.9), however whether this is a change in the rate of transcription or an increase in mRNA stability is not evident from this data. As MEF2D is a transcription factor, it may have a direct influence on transcription of the *il10* gene by binding to the IL-10 promoter. To investigate if MEF2D knockout influenced mRNA transcription rates, primers were designed that recognise the primary

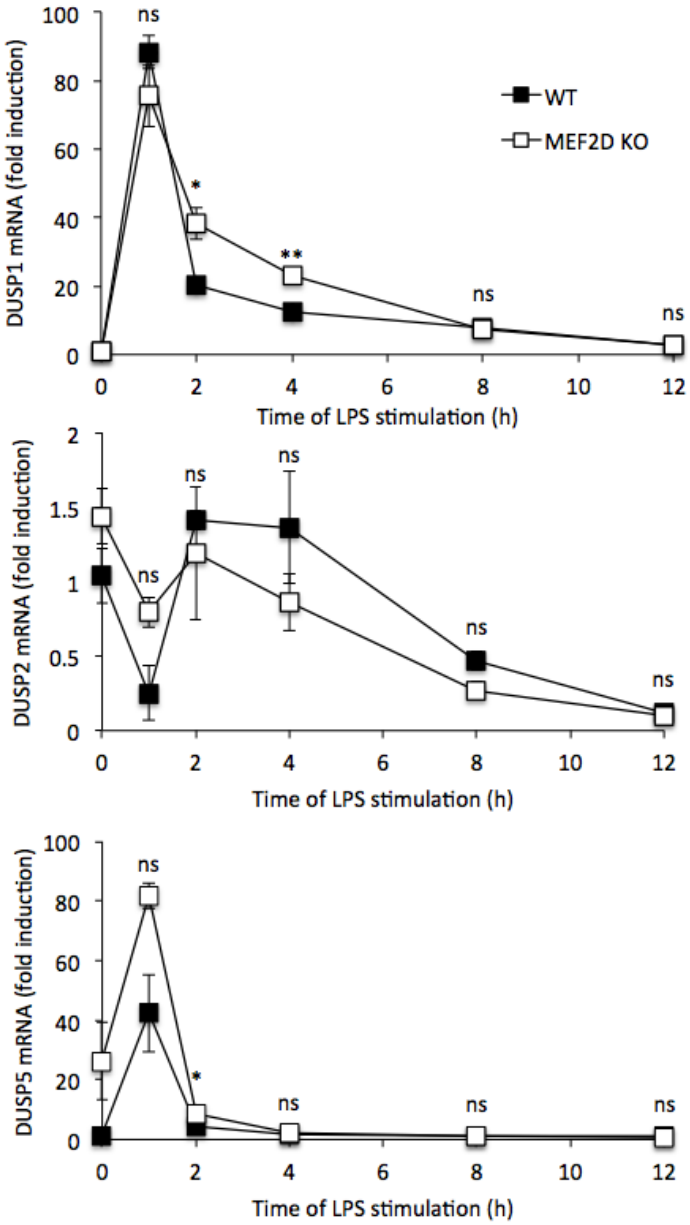

Figure 4.8: DUSP5 mRNA is elevated in MEF2D KO macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for DUSP1, DUSP2 and DUSP5 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

transcript of IL-10. By measuring levels of the IL-10 primary transcript, it would provide an indication if increases in IL-10 mRNA levels were due to changes in transcription rate or mRNA stability. Wildtype and MEF2D-deficient macrophages were stimulated with LPS over a timecourse of 16 hours and IL-10 primary transcript levels measured by qPCR. LPS induces a peak of IL-10 primary transcript at around 1 hour and this gradually de-

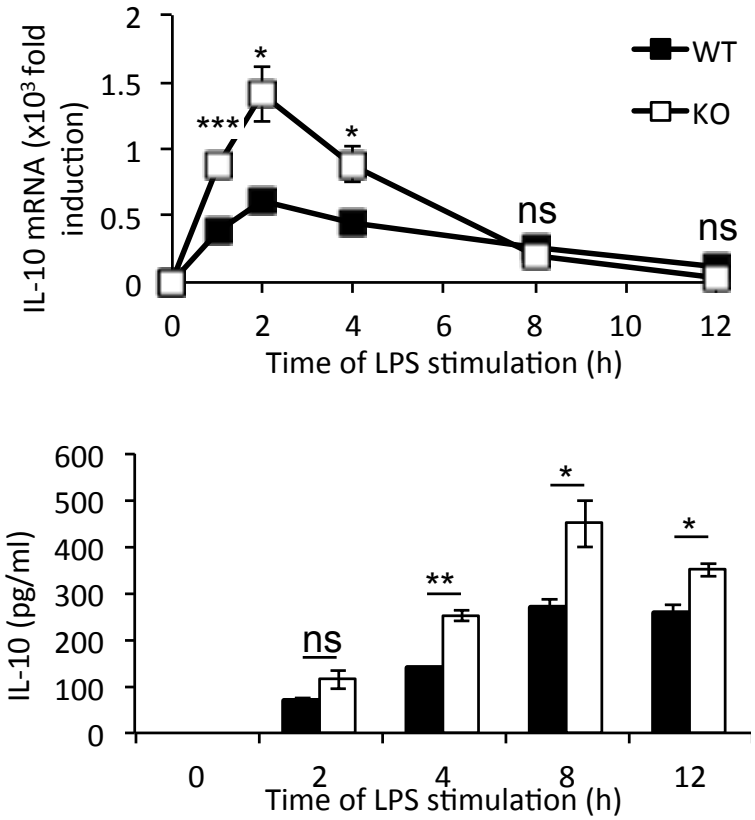

Figure 4.9: MEF2D KO macrophages have higher IL-10 transcription and secretion
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA. Culture supernatant was collected and IL-10 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

creases over the 16 hour timecourse (figure 4.10). Deletion of MEF2D from macrophages results in increased IL-10 primary transcript at 1, 4 and 8 hours of LPS stimulation. This suggests that MEF2D affects IL-10 transcription thus causing increased IL-10 mRNA levels and secretion.

IL-10 is an important anti-inflammatory cytokine and acts upon macrophages to reduce pro-inflammatory cytokine transcription and secretion. The increased secretion of IL-10 from MEF2D KO macrophages could lead to a reduction in pro-inflammatory cytokines compared to wildtype macrophages. To test this, wildtype and MEF2D-deficient macrophages were stimulated with LPS and the transcription and secretion of TNFα, IL-6 and IL-12(p40) were measured. LPS induced a peak of TNFα mRNA at 1 hour and this returned to baseline by 12 hours (figure 4.11). Whilst the initial peak was comparable, knockout of MEF2D resulted in decreased TNFα mRNA at late time points. This was

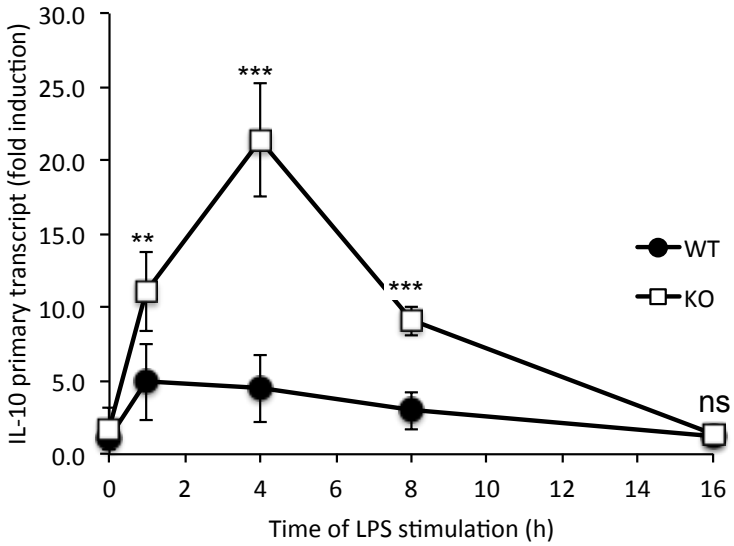

Figure 4.10: Deletion of MEF2D causes increased IL-10 primary transcript levels
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 primary transcript mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

reflected in the level of TNFα secretion which shows from 4 hours onwards that TNFα levels are decreased in MEF2D-deficient macrophages. IL-6 mRNA levels peaked later at around 4 hours in response to LPS and returned to baseline by 12 hours. Deficiency in MEF2D led to a much lower peak of IL-6 at 4 hours and this remained reduced up to 12 hours. IL-6 secretion was lower from MEF2D KO BMDMs compared to WT BMDMs at 4 and 8 hours confirming the reduction seen in mRNA levels. IL-12(p70) is a heterodimer of IL-12(p35) and IL-12(p40). IL-12(p35) is encoded by IL-12a and IL-12(p40) is encoded by IL-12b. LPS induced IL-12b mRNA levels in wildtype macrophages, which peaks around 4 hours. In MEF2D knockout macrophages IL-12 (p40) mRNA levels were decreased from 4 hours onwards. This reduced mRNA level correlated with a lower secretion of IL-12 (p40) from 4 hours onwards in MEF2D-deficient BMDMs compared to WT BMDMs (figure 4.11).

To determine if the changes in pro-inflammatory cytokine levels were solely due to the increase in IL-10 secretion, WT and MEF2D KO BMDMs were stimulated with LPS in the presence or absence of an IL-10 neutralising antibody. This antibody will block IL-10 signaling by preventing IL-10 from binding to its receptor and should therefore cause an increase in pro-inflammatory cytokine levels as the IL-10 mediated negative

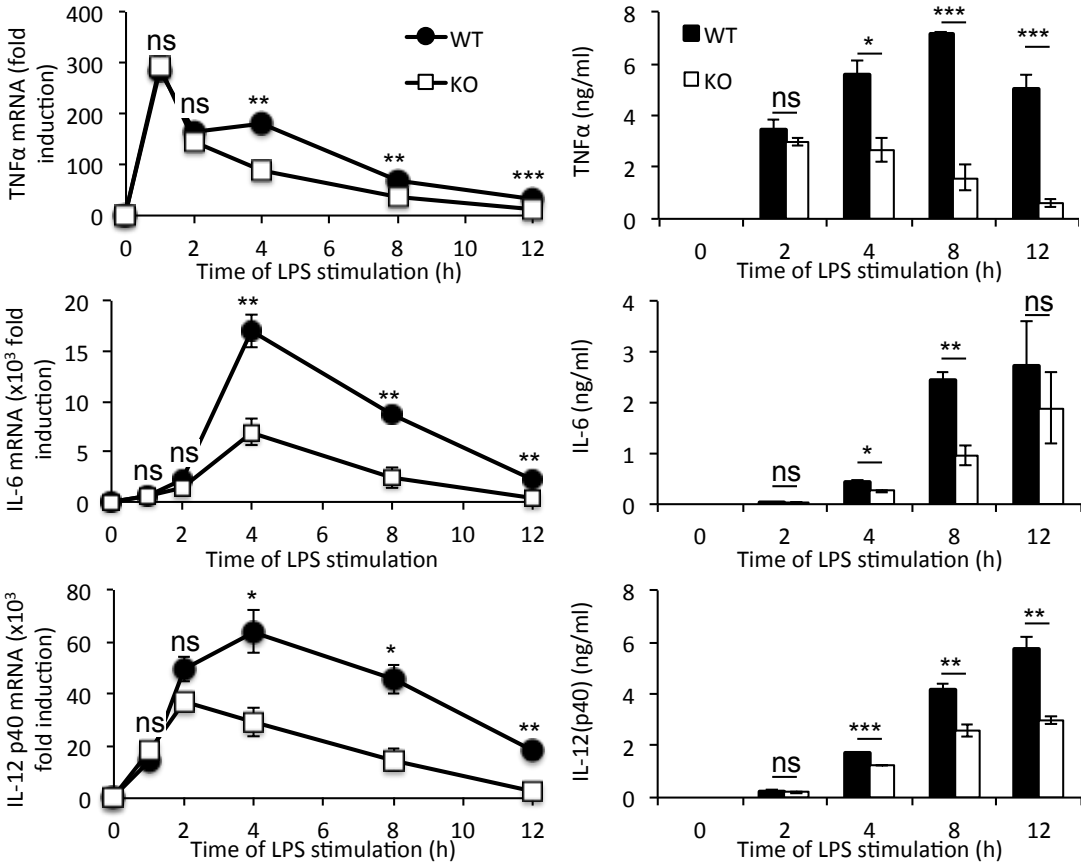

Figure 4.11: Reduced pro-inflammatory cytokines levels in MEF2D-deficient macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for TNFα, IL-6 and IL-12 (p40) mRNA. Culture supernatant was collected and TNFα, IL-6 and IL-12 (p40) secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

feedback is blocked. LPS stimulation of WT macrophages induced IL-6, TNFα, IL-12(p40) and IL-12(p70) at 8 hours and these pro-inflammatory cytokines were decreased in MEF2D-deficient macrophages in agreement with the data in figure 4.11 (figure 4.12). In the presence of the neutralising antibody, LPS induced much higher levels of these pro-inflammatory cytokines compared to LPS in the absence of the neutralising antibody. The IL-10 neutralising antibody also increased the secretion of TNFα, IL-6 and IL-12 in MEF2D knockout cells. In the case of TNFα and IL-6, secretion was comparable between wildtype and MEF2D-deficient macrophages in the presence of the IL-10 neutralising antibody. There was a slight reduction in IL-12(p40) levels in MEF2D KO BMDMs compared to WT BMDMs when pre-treated with the IL-10 neutralising antibody. Levels of IL-12(p70) were lower in MEF2D knockout macrophages compared to wildtype

macrophages in the presence of the IL-10 neutralising antibody (figure 4.12).

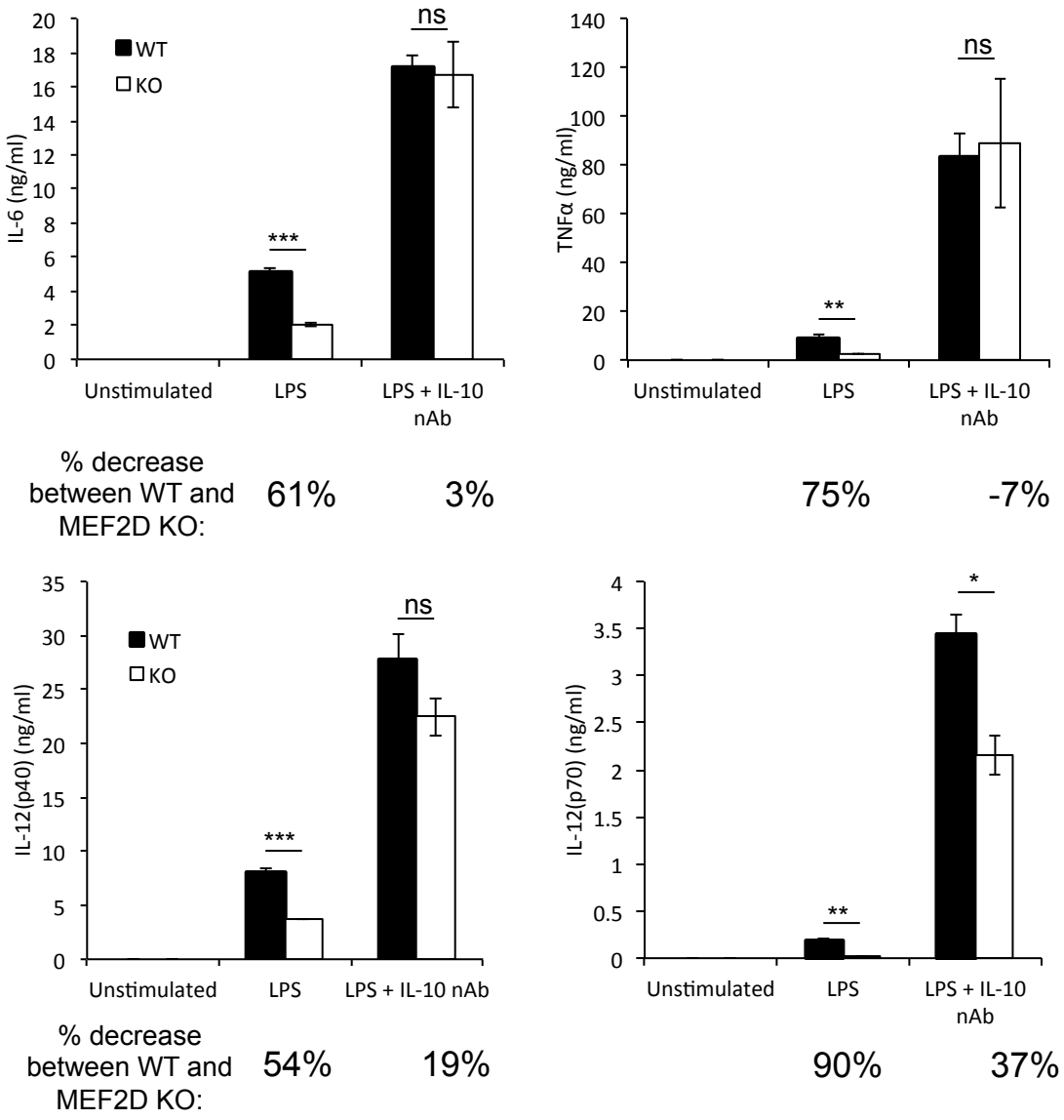

Figure 4.12: IL-10 nAb rescues pro-inflammatory cytokine levels in MEF2D-deficient macrophages Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS for 8h in the presence or absence of 2.5 µg/ml IL-10 neutralising antibody. Culture supernatant was collected and TNFα, IL-6 and IL-12 (p40) and IL-12 (p70) secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

The secretion of IL-12(p70) is reduced in MEF2D-deficient macrophages and therefore the mRNA levels of IL-12a and IL-12b were analysed following LPS stimulation of wildtype and MEF2D-deficient macrophages. LPS induced IL-12a transcription at 6 hours and IL-12a was strongly repressed in MEF2D-deficient macrophages (figure 4.13). IL-12b was induced by LPS stimulation of wildtype BMDMs and IL12b mRNA levels were decreased in MEF2D knockout BMDMs in agreement with data presented in figure 4.11.

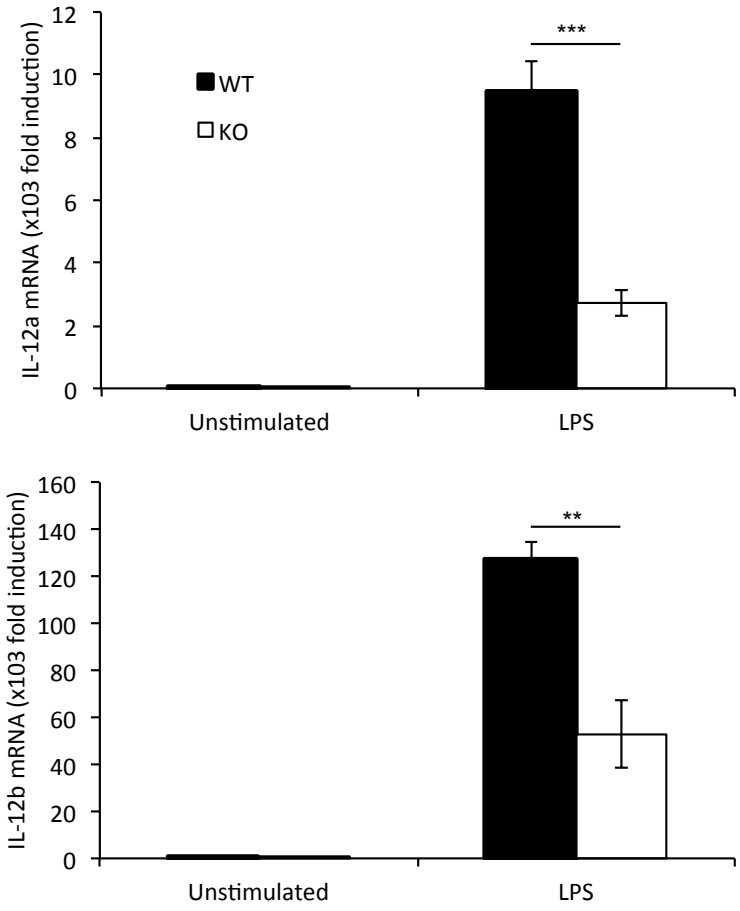

Figure 4.13: IL-12a and IL-12b mRNA levels are reduced in MEF2D KO macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-12a and IL-12b mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

4.6 Elevated IL-10 production in macrophages decreases COX2 mRNA

IL-10 has numerous effects on macrophages including reducing pro-inflammatory cytokine production. Recently, the effect of IL-10 on COX2 mRNA has been elucidated. In this study, it was shown that IL-10 led to a decrease in COX2 mRNA due to decreased p38 activation which relinquished MK2-mediated inhibition of TTP. TTP was then able to bind to and destabilise the COX2 mRNA (MacKenzie et al., 2013b). To examine if elevated IL-10 production in MEF2D-deficient macrophages affected COX2 mRNA levels, wildtype and MEF2D knockout macrophages were stimulated with LPS. LPS induced transcription of COX2, which peaked at 4 hours (figure 4.14a). COX2 mRNA levels were decreased in MEF2D KO macrophages at 4, 8 and 12 hours compared to wildtype

macrophages.

As MEF2D macrophages have elevated IL-10 production in response to LPS, the decrease seen in COX2 is probably caused by IL-10 affecting TTP activity. To demonstrate that changes in COX2 levels were dependent on IL-10 signaling, wildtype and MEF2D KO macrophages were stimulated with LPS for 8 hours in the presence or absence of the neutralising antibody. LPS induced COX2 mRNA expression in wildtype macrophages and was reduced in MEF2D KO macrophages as in figure 4.14a. Treatment with the IL-10 neutralising antibody increased COX2 expression in wild type cells in response to LPS and levels of COX2 mRNA were similar in macrophages lacking MEF2D (figure 4.14b). This suggests that decreases in COX2 mRNA in MEF2D KO macrophages are dependent on IL-10 signaling.

4.7 Knockout of MEF2D enhances IL-10 transcription downstream of TLR agonists

IL-10 is induced by activation of multiple pathways including other TLRs such as TLR1/2, TLR3, TLR7/8 and TLR9. To determine if MEF2D's effects on IL-10 were restricted to TLR4, wildtype and MEF2D-deficient macrophages were stimulated with the TLR3 agonist, poly(I:C), TLR1/2 agonist, Pam3CSK4, the TLR9 agonist, CpG and the TLR7/8 agonist, R848. Both TLR7/8 and TLR9 signal via the MyD88 adaptor and are found at the endosome. TLR3 is also present at the endosome and signals via TRIF, whilst TLR1/2 recruits MyD88 and signals from the plasma membrane. Induction of IL-10 varied between the stimuli; poly(I:C) only weakly induces IL-10 mRNA and secretion at 8 hours. Pam3CSK4, CpG and R848 all induce greater amounts of IL-10. At this time point, mRNA and protein levels of IL-10 appear enhanced in the knockout macrophages compared to wildtype in response to CpG and R848, however the affect of MEF2D deficiency may affect IL-10 induction at earlier time points (figure 4.15).

IL-10 can repress pro-inflammatory cytokine production through IL-10R-JAK-STAT signaling. The levels of IL-6, IL-12(p40) and TNFα were also measured to examine if

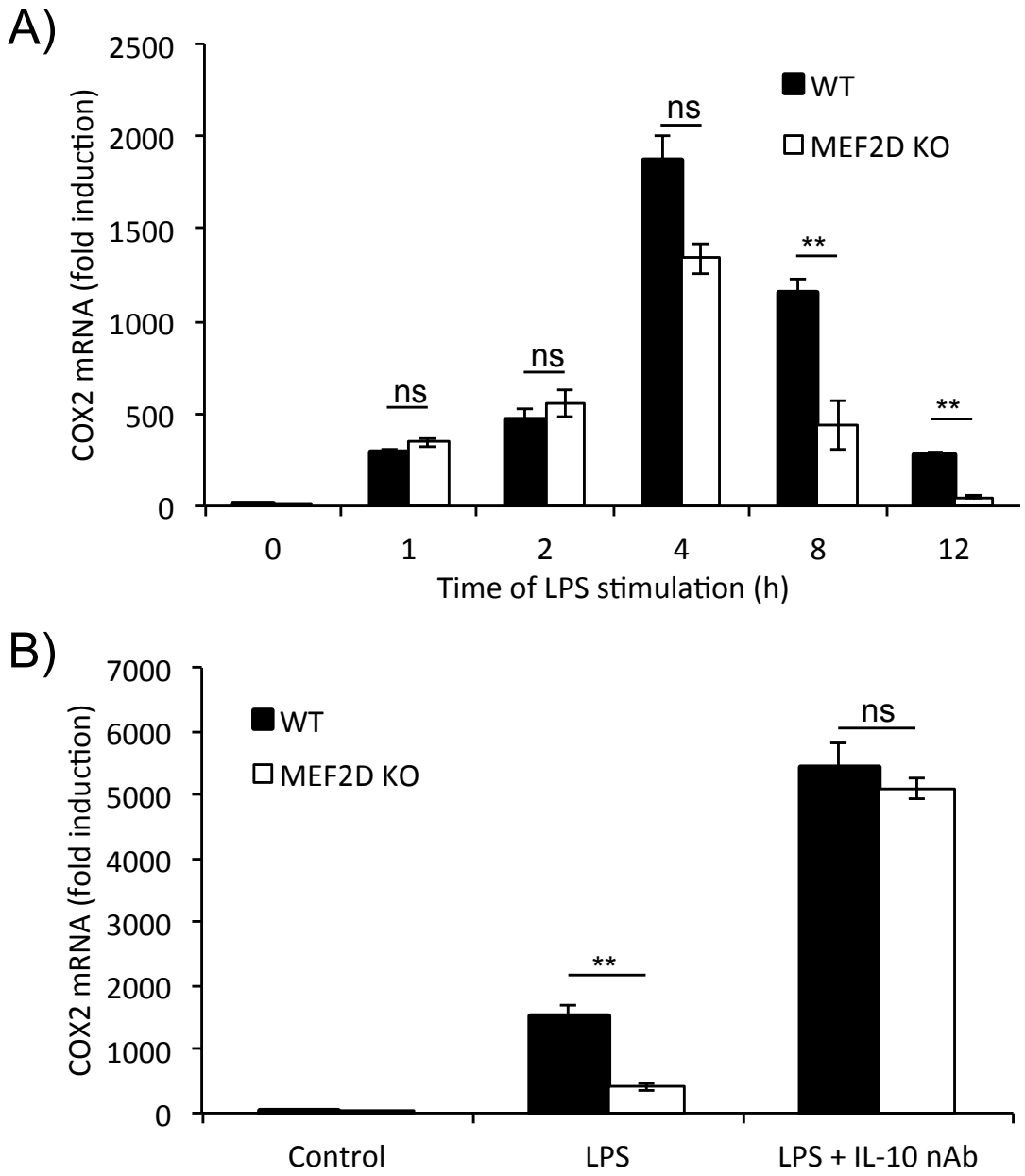

Figure 4.14: COX2 mRNA is decreased in MEF2D KO macrophages in an IL-10 dependent manner Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with A) 100 ng/mL of LPS and B) 100 ng/mL of LPS for 8h in the presence or absence of 2.5 µg/ml IL-10 neutralising antibody. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for COX2 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

MEF2D deficiency had affected production of pro-inflammatory cytokine in response to these TLR agonists. IL-6 mRNA levels were comparable in response to Pam3CSK4 and R848 and may be increased in response to CpG. poly(I:C) did not induce detectable amounts of IL-6 at the mRNA or protein level. Interestingly, IL-6 protein levels appear

decreased in the MEF2D-deficient macrophages compared to wildtype macrophages in response to CpG, Pam3CSK4 and R848.

IL-12(p40) mRNA was induced by CpG and R848 and to a lesser extent by Pam3CSK4. mRNA levels of IL-12(p40) were comparable between wildtype and MEF2D KO BMDMs. Protein levels of IL-12(p40) were slightly reduced in MEF2D-deficient macrophages compared to wildtype in response to CpG, Pam3CSK4 and R848. Poly(I:C) weakly induced IL-12(p40) mRNA and protein.

Induction of TNFα mRNA was evident in response to poly(I:C), CpG, Pam3CSK4 and R848 in wildtype macrophages. mRNA levels of TNFα were reduced in CpG-, Pam3CSK4- and R848-stimulated macrophages lacking MEF2D compared to wildtype macrophages. The changes seen in TNFα mRNA levels were reflected in protein levels with decreased TNFα secretion evident in MEF2D KO BMDMs in response to CpG, Pam3CSK4 and R848. Poly(I:C)-induced TNFα protein was not detectable 8 hours after stimulation in wildtype or MEF2D knockout cells (figure 4.15).

Both Pam3CSK4 and CpG stimulation led to reasonable induction of IL-10 and pro-inflammatory cytokines, which was altered by deletion of MEF2D in macrophages. The effects of MEF2D deficiency was examined in response to these two stimuli over a 24 hour timecourse. Stimulation of macrophages with Pam3CSK4 results in a biphasic induction of IL-10 mRNA. Initial induction of IL-10 peaks around 1-4 hours with a second higher peak evident at 16 hours (figure 4.16). MEF2D KO BMDMs demonstrated similar kinetics of IL-10 mRNA induction although with a higher initial induction up to 8 hours. These increases in IL-10 mRNA levels were translated to higher secretion of IL-10 at 8, 16 and 24 hours of Pam3CSK4 treatment. Treatment of macrophages with the TLR9 agonist, CpG, resulted in a similar induction of IL-10 mRNA with an initial peak at 1 hours and a second greater peak at 16 hours (figure 4.16). Notably, CpG stimulation of MEF2D-deficient macrophages resulted in increased IL-10 mRNA levels throughout the timecourse with increased mRNA levels. In line with the secretion data from Pam3CSK4 stimulation, increased IL-10 mRNA levels seen in CpG-stimulated MEF2D knockout macrophages translated to higher secretion of IL-10 at 8, 16 and 24 hours.

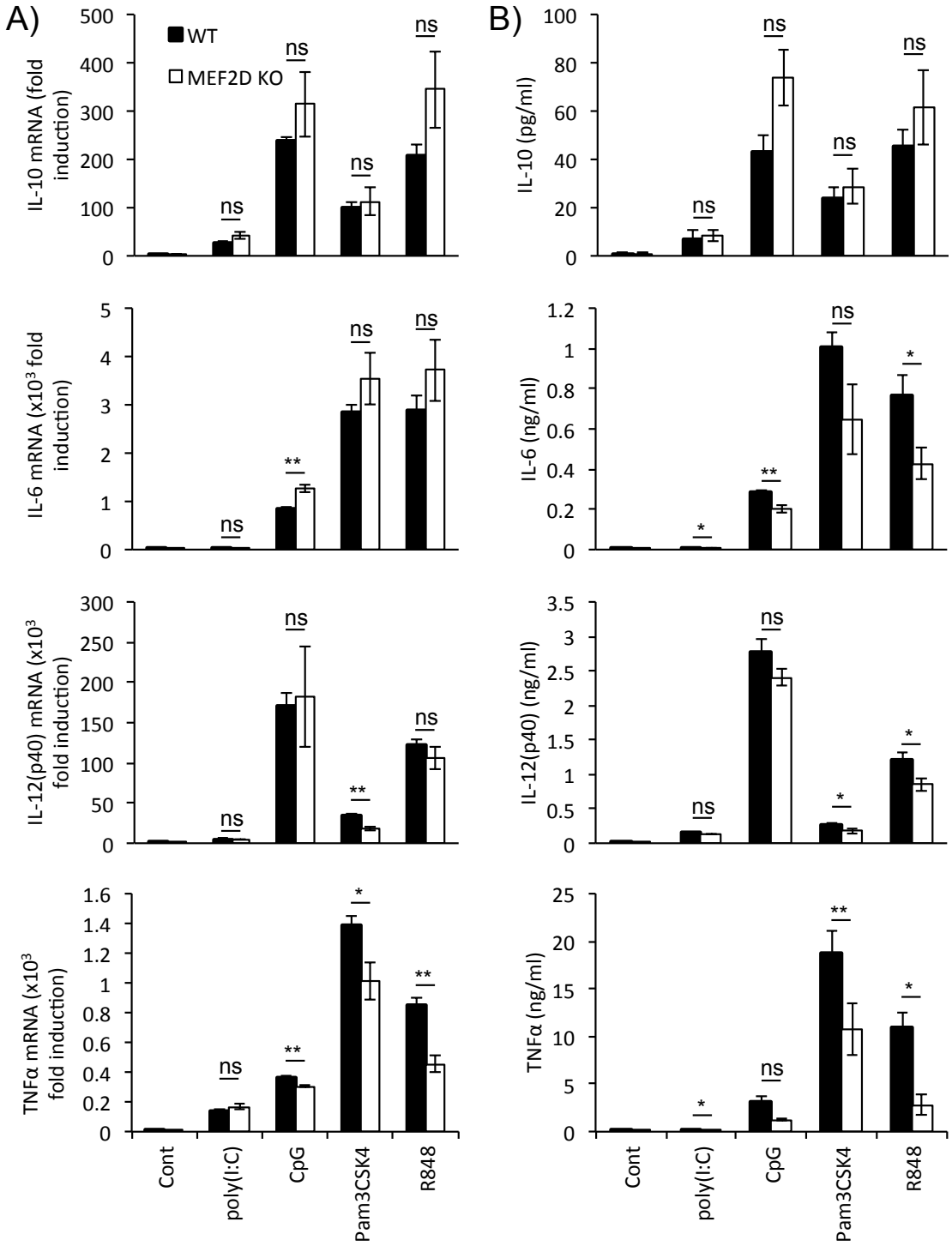

Figure 4.15: MEF2D regulates IL-10 downstream of multiple TLR agonists
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 10 μg/ml poly(I:C), 1 μg/ml Pam3CSK4, 2 μM CpG or 1 μg/ml R848. A) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10, IL-6, IL-12(p40) and TNFα mRNA. B) Culture supernatant was collected and IL-10, IL-6, IL-12(p40) and TNFα secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

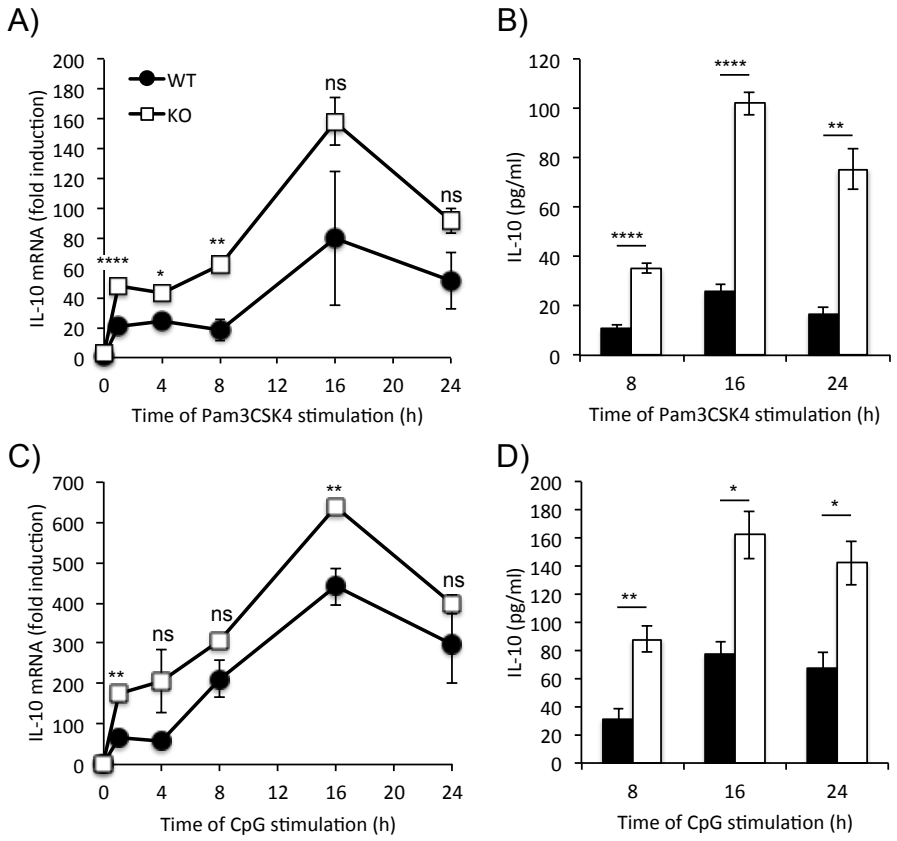

Figure 4.16: TLR1/2 and TLR9 agonists induce elevated IL-10 mRNA levels in MEF2D knockout macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with (A+B) 1 µg/ml Pam3CSK4 or (C+D) 2 µM CpG. A+C) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA. B+D) Culture supernatant was collected and IL-10 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

4.8 Dectin-1-induced IL-10 mRNA levels are not affected by MEF2D deficiency in macrophages

Other PRRs also induce the production of IL-10 in response to ligand binding. The C-type lectin receptor, Dectin-1 binds β-(1-3)-glucans and activates MAPK and NFκB signaling via the spleen tyrosine kinase (Syk). Zymosan is a crude preparation of *Saccharomyces cerevisiae* cell wall and will activate Dectin-1 and TLR2. Curdlan is a polymer of β-(1-3)-glucans, which specifically activates Dectin-1 without affecting TLR signaling. To ascertain if the effect of MEF2D on IL-10 transcription and secretion were restricted to TLR signaling, WT and MEF2D KO BMDMs were stimulated with Zymosan or Curdlan for 8 or 16 hours and IL-10 secretion measured. Zymosan strongly induced a large amount

of IL-10 at 8 hours in wildtype cells and MEF2D-deficient cells secreted similar amounts of IL-10 at both 8 and 16 hours (figure 4.17). Curdlan specifically activates Dectin-1 and stimulated lower secretion of IL-10 compared to Zymosan. However, similarly to Zymosan, treatment of MEF2D-deficient BMDMs with Curdlan led to comparable secretion of IL-10 at 8 and 16 hours compared to wildtype macrophages. This result suggests that MEF2D regulation of IL-10 transcription is limited to TLR signaling in macrophages. In line wth this, levels of pro-inflammatory cytokines were comparable between WT and MEF2D KO BMDMs at 8 and 16 hours following LPS and Zymosan stimulation, suggesting the IL-10-mediated negative feedback loop was unaffected in Zymosan- or Curdlan-stimulated macrophages.

4.9 MEF2D deficiency alters macrophage phenotype

MEF2D deficiency enhances IL-10 secretion and results in decreased pro-inflammatory cytokine secretion. The ratio of IL-10:IL-12 is often used to determine the phenotype of macrophages and a high IL-10:IL-12 ratio is indicative of a regulatory macrophage. Regulatory macrophages also express other markers including Arg1, SphK1 and LIGHT. To determine if MEF2D deficiency also affected regulatory macrophage markers, wildtype and MEF2D knockout macrophages were stimulated with LPS and marker expression measured by qPCR. LPS alone only weakly induced expression of Arg1, which encodes arginase-1, at 16 hours (figure 4.18). Deletion of MEF2D led to higher levels of Arg1 expression at 8 and 16 hours in response to LPS. Expression of SphK1 is also induced by LPS and peak mRNA levels are evident after 4 hours of stimulation. MEF2D KO macrophages expressed higher levels of SphK1 mRNA at 8 hours compared to wildtype macrophages. LIGHT is weakly induced by LPS alone, however in MEF2D-deficient cells, LIGHT is more strongly expressed at 1 and 2 hours following LPS stimulation (figure 4.18).

4.10 MEF2D does not regulate classical MEF2 target genes in macrophages

Several studies have identified MEF2 consensus sequences in the promoter of many genes, including Nur77 and c-Jun. In neurons, MEF2D is important for expression of the tran-

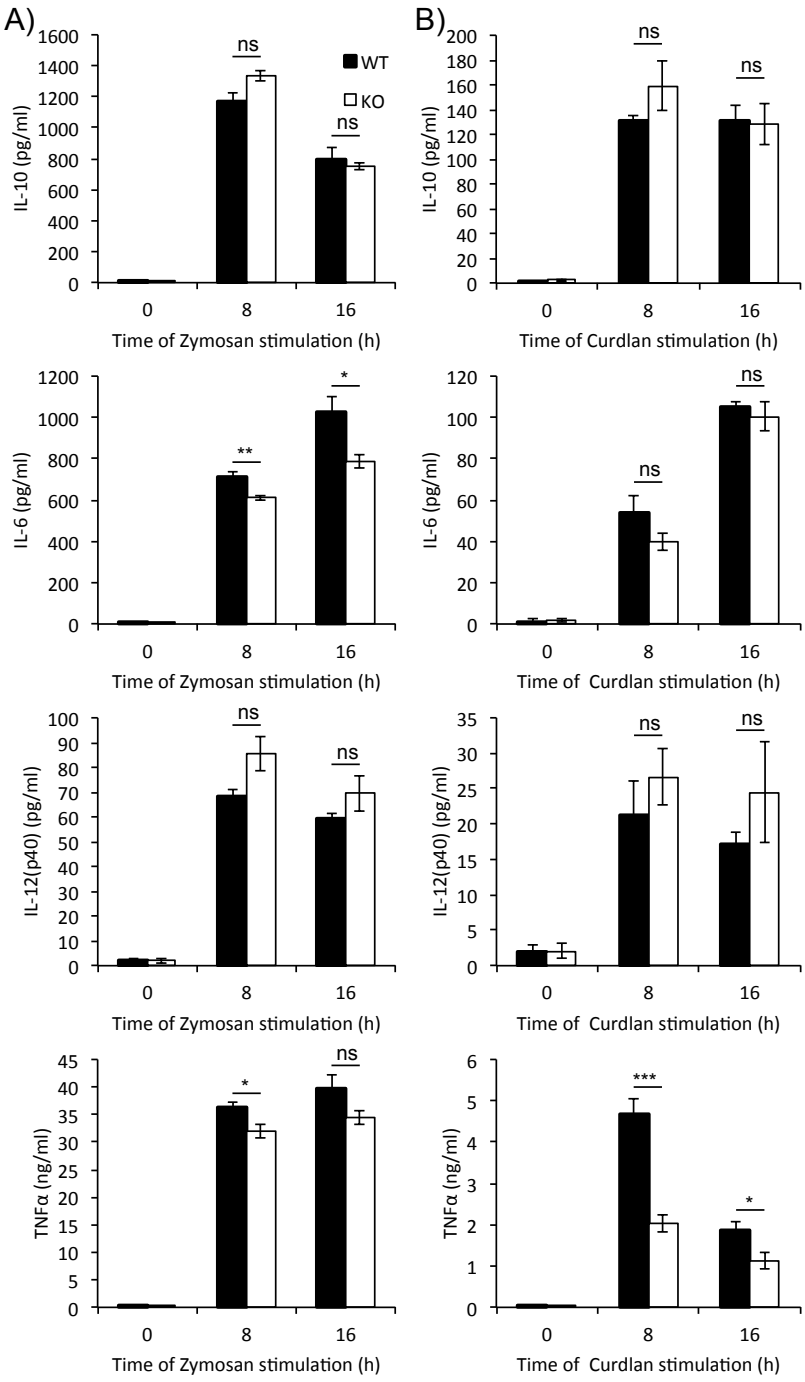

Figure 4.17: Dectin-1-mediated IL-10 secretion is not MEF2D-regulated
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with A) 200 µg/mL Zymosan or B) 10 µg/mL Curdlan. Culture supernatant was collected and IL-10, IL-6, IL-12(p40) and TNFα secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

scription factor, Nur77 (Mount et al., 2013). In the macrophage differentiated HL60 cell line, MEF2D is required for expression of c-Jun as well as for serum-induced c-Jun expression in HeLa and NIH3T3 cells (Aude-Garcia et al., 2010; Han and Prywes, 1995). To investigate whether MEF2D might be important for the expression of these immediate

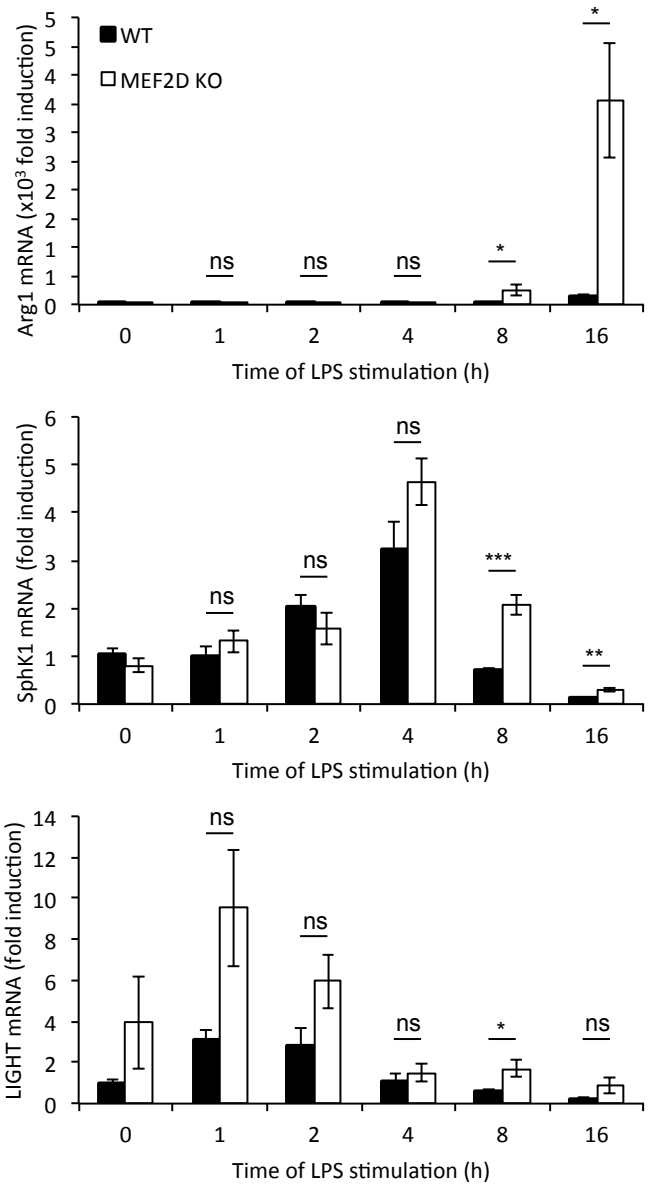

Figure 4.18: Regulatory macrophage marker expression is enhanced in MEF2D KO macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for Arg1, SphK1 and LIGHT mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

early genes (IEGs) in LPS-stimulated macrophages, BMDMs from wildtype and MEF2D KO mice were cultured and stimulated with LPS for a short timecourse over 120 minutes. LPS induced expression of c-Jun which peaked at 30 minutes and decreased to basal levels by 90 minutes (figure 4.19). Deletion of MEF2D from macrophages did not alter the induction of c-Jun by LPS. Nur77 expression was induced by LPS at 30 minutes and persisted until 60 minutes before rapidly decreasing. MEF2D-deficient macrophages

showed similar patterns of induction of Nur77 in response to LPS. These data suggest that previously identified MEF2D target genes from cell lines are not regulated by MEF2D in primary macrophages in response to TLR4 stimulation.

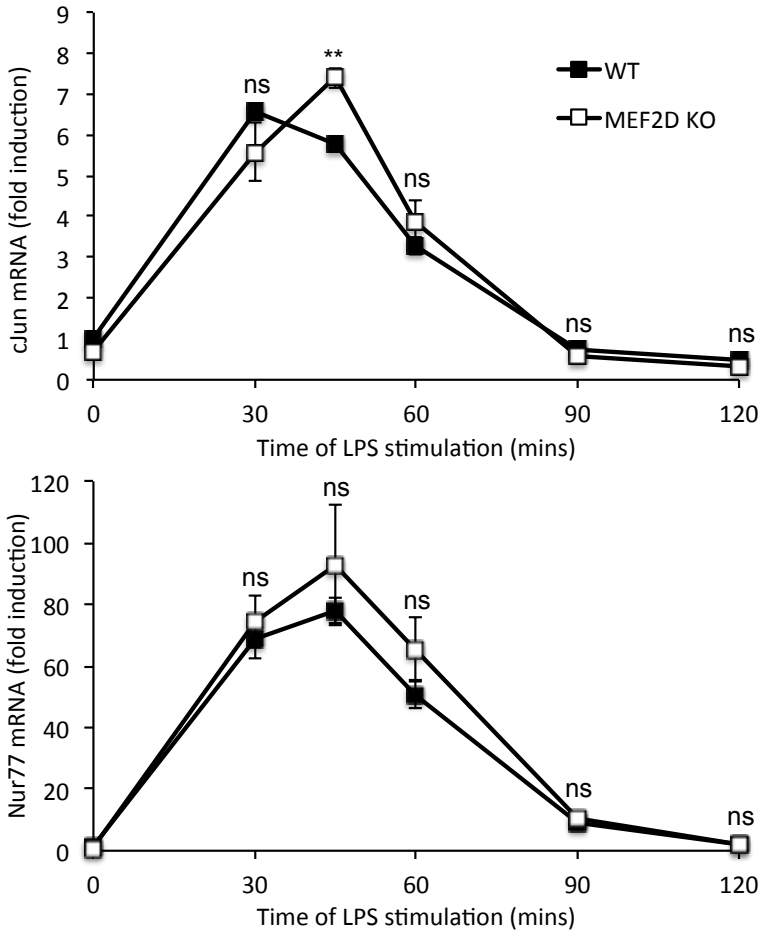

Figure 4.19: c-Jun and Nur77 are not regulated by MEF2D in primary macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for c-Jun and Nur77 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

4.11 MEF2D regulates transcription of A20

Levels of the protein A20 were increased in MEF2D knockout macrophages in response to LPS compared to wildtype cells as shown in figure 4.7. To test if MEF2D was regulating the transcription of A20/TNFAIP3, wildtype and MEF2D-deficient macrophages were stimulated with LPS and A20 mRNA levels measured. LPS induced A20 mRNA transcription after 1 hour of stimulation and mRNA levels were detectable up to 16 hours

post-stimulation. Macrophages lacking MEF2D had slightly higher levels of A20 mRNA in response to LPS 1 hour after stimulation. Levels of A20 mRNA were comparable at later time points (figure 4.20).

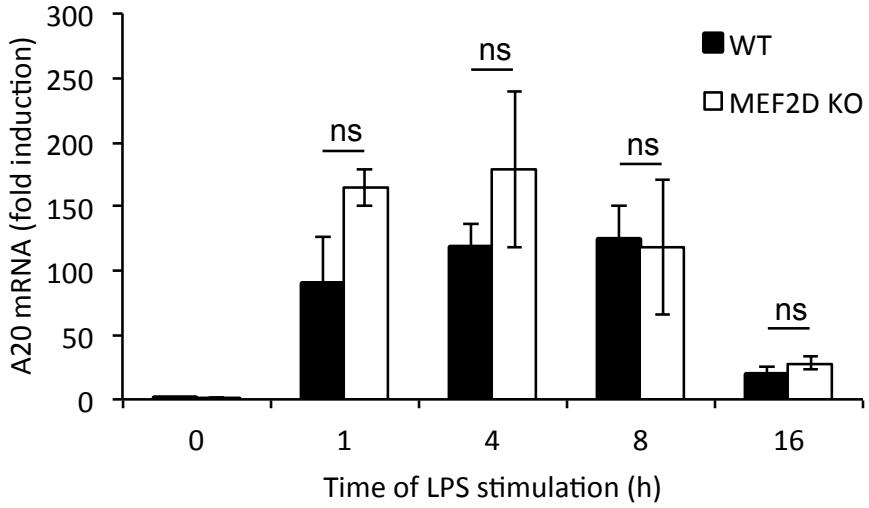

Figure 4.20: A20 mRNA is elevated in MEF2D-deficient macrophages stimulated with LPS
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 100 ng/mL of LPS. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for A20 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

MEF2D negatively regulated expression of IL-10 mRNA in response to multiple TLR agonists (figure 4.9, figure 4.16). Therefore, MEF2D may regulate A20 mRNA levels downstream of other TLRs. In order to examine if A20 expression was regulated by MEF2D downstream of other TLR agonists, macrophages were isolated from wildtype and MEF2D knockout mice and stimulated with Pam3CSK4 (TLR1/2 agonist) and CpG (TLR9 agonist). Pam3CSK4 induced A20 expression in wildtype cells from 1 hour and A20 mRNA levels were still elevated 16 hours post-stimulation (figure 4.21). MEF2D-deficient macrophages had increased A20 mRNA levels throughout the timecourse following Pam3CSK4 stimulation compared to wildtype macrophages. CpG also induced A20 mRNA expression in wildtype cells although to a lower extent than Pam3CSK4 or LPS stimulation. Notably, A20 mRNA levels in MEF2D KO cells were greatly enhanced after 1 hour and 8 hours of CpG stimulation compared to wildtype cells (figure 4.21).

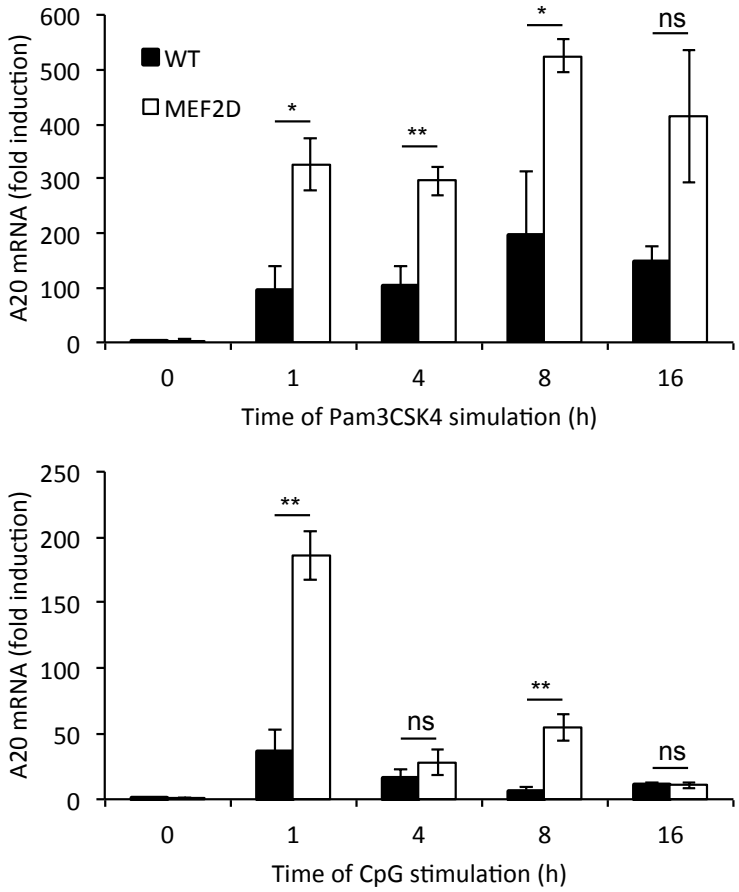

Figure 4.21: Pam3CSK4- and CpG-induced transcription of A20 is enhanced in MEF2D KO macrophages
Bone marrow-derived macrophages from WT and MEF2D KO mice were stimulated with 1 μg/ml Pam3CSK4 or 2 μM CpG. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for A20 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

4.12 Discussion

The MEF2 family of transcription factors are well known for their role in muscle development but also have emerging functions in neuronal cell survival and immune cells. The importance of MEF2D in macrophage function is poorly understood. TLR signaling activates a multitude of transcription factors responsible for controlling transcription of genes important in the inflammatory response. These transcription factors control expression of a diverse range of proteins including cytokines, chemokines, transcription factors and other signaling molecules. The involvement of CREB, Ap1, Sp1/3, NFκB and IRFs is well known within macrophages (Medzhitov and Horng, 2009; Sato et al., 1998b,a; Ping et al., 2000; Elcombe et al., 2013; Saraiva et al., 2005). However, it is likely that

other transcription factors play as yet unappreciated roles in regulating the inflammatory response.

In this chapter, the importance of MEF2D in macrophages is demonstrated. In response to LPS, MEF2D is phosphorylated on Ser121 as identified by SILAC and validated through immunoprecipitations using a phospho-specific antibody. MEF2D knockout macrophages stimulated with LPS had elevated STAT3 phosphorylation and altered activation of ERK1/2, JNK and p105. Deletion of MEF2D from macrophages led to increased transcription and secretion of IL-10, which resulted in decreased pro-inflammatory cytokine production. MEF2D also regulated IL-10 transcription in response to other TLR agonists. In addition to regulating IL-10, deletion of MEF2D resulted in increased transcription of A20 in response to LPS, Pam3CSK4 and CpG. The increase in LPS-induced A20 mRNA expression was translated to increased protein levels. Together, these results suggest that MEF2D is important in regulating the inflammatory response.

4.12.1 Phosphorylation of MEF2D

Five phosphopeptides were identified from MEF2D including the S121 peptide. This phosphorylation site has been identified in other studies (Huttlin et al., 2010; Du et al., 2008). Phospho-antibodies were raised against this peptide and the phosphorylation of S121 was confirmed through immunoprecipitations and western blotting in Raw264.7 cells. The relevance of S121 phosphorylation in response to LPS in macrophages is still unclear. It is unknown whether phosphorylation of S121 affects the localisation, DNA binding activity, transcription activation activity or other post-translational modifications (PTMs). Results show that MEF2D protein levels remain unchanged during a short stimulation of LPS suggesting that phosphorylation of MEF2D does not affect protein stability or induce its degradation. Numerous studies have identified PTMs of MEF2D including phosphorylation and sumoylation (Du et al., 2008; Grégoire et al., 2006). The influence that S121 phosphorylation has upon these reported modifications is unknown. It would be beneficial to further investigate the relevance of S121 phosphorylation with regard to other PTMs.

It would also be interesting to examine the role of this phosphorylation on MEF2D's transcription activity. ChIP assays could demonstrate the recruitment of MEF2D to specific promoter sites and through mutating the S121 site to an alanine (S121A), an understanding of its role in MEF2D DNA binding could be gained. Phosphorylation of MEF2D may also regulate the ability of MEF2D to recruit the transcription machinery and/or other co-factors and therefore may repress or promote transcription. WT and S121A MEF2D could be transfected into cells and transcription of MEF2D target genes measured to investigate the importance of S121 phosphorylation on MEF2D-mediated transcription. MEF2D is known to interact with class II HDACs, which catalyse the deacetylation of histones and therefore prevent transcription. Identification of MEF2D-HDAC interactions within macrophages would be interesting, as would the role that S121 phosphorylation might play in regulating this interaction.

LPS activates signaling through TLR4 by recruiting the MyD88 and TRIF adaptors which activates a range of signaling molecules including MAPKs and IKKs. The signaling pathway responsible for LPS-induced phosphorylation of S121 on MEF2D could be identified using small molecule inhibitors and knockout mice. By identifying the pathway responsible, another approach to understanding the relevance of the phosphorylation of MEF2D at Ser121 would be available. It would also be interesting to determine which other TLR agonists and stimuli may induce MEF2D phosphorylation at Ser121. The LPS-induced phosphorylation of MEF2D at Ser121 is transient, being detectable 15 mins after LPS stimulation, but no longer evident at 60 mins. As MEF2D protein levels remain constant throughout this time period, it suggests the action of phosphatase. It would be of further interest to identify the phosphatase that catalyses the dephosphorylation of MEF2D at Ser121.

4.12.2 Deletion of MEF2D alters TLR4 signaling

Deletion of MEF2D affects the signaling downstream of TLR4. Phosphorylation of ERK1/2, JNK and p105 was reduced in MEF2D KO macrophages compared to wild-type macrophages after the initial activation at 30 minutes. In particular, the second wave of JNK phosphorylation was not evident in MEF2D KO cells, perhaps suggesting a role

for dual-specificity phosphatases (DUSPs). Equally, the reduction of ERK1/2 phosphorylation may be DUSP-dependent. DUSPs dephosphorylate the threonine and tyrosine residues within the activation loop of MAPKs and are important in controlling MAPK signaling (Lang et al., 2006; Caunt and Keyse, 2013). DUSP5 mRNA was shown to be increased in MEF2D KO macrophages compared to wildtype macrophages. However, DUSP5 is a very selective phosphatase for ERK2 and shows no activity towards JNK or p38 (Mandl et al., 2005). DUSP1 mRNA was marginally elevated in MEF2D KO BMDMs at 2 and 4 hours following LPS stimulation. DUSP1 targets p38 and JNK *in vivo* and may explain some of the alterations to MAPK phosphorylation seen in MEF2D KO macrophages (Chen et al., 2002). The impact of MEF2D deletion on DUSPs mRNA and protein levels needs to be examined in greater detail.

Protein levels of A20 were increased in LPS-stimulated macrophages lacking MEF2D compared to wildtype LPS-stimulated macrophages. A20 is zinc-finger protein and inhibits NF$\varkappa$B signaling (Heyninck and Beyaert, 1999). A20 has been implicated in regulating immune responses and is now thought to regulate TLR signaling via interactions with poly-ubiquitin chains (Kool et al., 2011; Maelfait et al., 2012; Matmati et al., 2011; Verhelst et al., 2012). In MEF2D knockout macrophages, LPS-induced transcription of A20 was increased compared to WT macrophages. MEF2D also repressed A20 transcription downstream of TLR1/2 and TLR9 signaling. Interestingly, one study showed that silencing of A20 with siRNA caused decreased IL-10 production in response to infection with *Leishmania donovani* (Srivastav et al., 2012). Deletion of A20 in mice leads to the development of polyarthritis and high levels of pro-inflammatory cytokines in the serum (Matmati et al., 2011). The impact that increased A20 has on macrophage function needs to be further examined. Changes in A20 protein level were not evident until 4 hours post-stimulation and therefore long timecourses would be required to understand the importance of this increase.

4.12.3 TLR-induced IL-10 transcription is enhanced in MEF2D-deficient macrophages

LPS induces the anti-inflammatory cytokine, IL-10, which reduces pro-inflammatory cytokine production through IL-10R-JAK-STAT3 signaling. The data presented above

demonstrated that MEF2D deficient macrophages had enhanced transcription and secretion of IL-10 and this led to enhanced STAT3 phosphorylation following LPS stimulation. Furthermore, MEF2D knockout cells had decreased pro-inflammatory cytokine levels compared to wildtype cells. Pre-treatment of wildtype and MEF2D-deficient BMDMs with the IL-10 neutralising antibody resulted in similar levels of pro-inflammatory cytokines in response to LPS, suggesting the reduction in pro-inflammatory cytokine levels seen in MEF2D-deficient macrophages was due to the increase in IL-10-mediated signaling.

IL-12 is a heterodimer formed of p35 and p40 subunits. Whilst levels of the p40 subunit were similar in both wildtype and MEF2D-deficient macrophages in the presence of the neutralising antibody. The p70 dimer was reduced in MEF2D knockout cells compared to wildtype cells in the presence of the IL-10 neutralising antibody (figure 4.12). IL-12a encodes the p35 subunit of IL-12 and was reduced in LPS-stimulated macrophages lacking MEF2D compared to control macrophages (figure 4.13). This suggests that IL-12a may be regulated by MEF2D independently of the increased IL-10-STAT3 signaling. It would be interesting to investigate if MEF2D directly affected IL-12a transcription or if MEF2D regulates an intermediate that influences IL-12a transcription.

In addition to regulating IL-10 transcription downstream of TLR4, the data above demonstrates that MEF2D also regulates IL-10 transcription in response to Pam3CSK4 (a TLR1/2 agonist), CpG (a TLR9 agonist) and R848 (a TLR7/8 agonist). TLR1/2, 4 and 9 all signal through the MyD88 adaptor and MEF2D may possibly regulate MyD88 signaling-induced IL-10 transcription. It would be interesting to examine the role of MEF2D downstream of TLR3 which signals via TRIF in more detail. Dectin-1 activation also induces expression of IL-10 from macrophages. Dectin-1 signals via Syk and the data presented suggests that MEF2D is not important in IL-10 transcription downstream of Dectin-1. MEF2D therefore may only regulate IL-10 transcription downstream of MyD88 in macrophages.

Interestingly, the murine IL-10 promoter contains a MEF2 binding site, as does the TN-

FAIP3 (A20) promoter. The role of MEF2D in regulating transcription of these genes may be through directly binding to the promoter and regulating transcription. MEF2D may bind to the promoter and recruit class II HDACs to repress transcription or alternatively recruit the cofactor, p300, to promote transcription (Nebbioso et al., 2009; Ma et al., 2005). Through ChIP assays, the binding of MEF2D to the promoter of target genes could be examined in unstimulated and stimulated cells.

4.12.4 Role for MEF2D *in vivo*

MEF2D deletion modulates the immune response of macrophage by increasing IL-10 levels which cause decreased secretion of pro-inflammatory cytokines in response to LPS. Therefore MEF2D deficiency *in vivo* could protect mice from endotoxic shock as well as other inflammatory models such as contact hypersensitivity models which have previously been shown to be affected by macrophage and dendritic cell responses (Girard-Madoux et al., 2012; Siewe et al., 2006). However, predicting the effect of MEF2D deficiency on *in vivo* responses is complicated by potential roles within other immune cell types, particularly B cells or T cells. It would be interesting to investigate the response of MEF2D deficient mice to i.p injection of LPS, as well as to other inflammatory models such as contact hypersensitivity or irritant responses.

4.12.5 Conclusion

MEF2D is an important transcription factor during development, particularly in muscle and neural tissues. It also has roles in neuronal survival and immune cells. The results presented in this chapter demonstrate that MEF2D is phosphorylated on Ser121 in response to LPS. Deletion of MEF2D from macrophages alters the activation of key signaling molecules, such as ERK1/2, JNK and p105, in response to LPS stimulation. Notably, MEF2D controls the transcription of IL-10 and MEF2D KO macrophages produce elevated levels of IL-10 leading to a reduction in pro-inflammatory cytokine production. TLR1/2- and TLR9-induced IL-10 production is also repressed by MEF2D. Deletion of MEF2D may also promote a regulatory macrophage phenotype characterised by enhanced expression of SphK1, Arg1 and LIGHT. In addition to changes in IL-10 transcription, MEF2D deficiency in macrophages results in increased mRNA and protein levels of A20,

a negative regulator of NFκB.

Further work is required to understand the importance of MEF2D in macrophages. Firstly, the relevance of MEF2D phosphorylation at Ser121 needs to be investigated. Ser121 may alter the cellular localisation of MEF2D or regulate its ability to drive gene transcription through a number of mechanisms. Secondly, the context of MEF2D repression of IL-10 needs to be further explored to determine if these effects are specific to MyD88 signaling downstream of TLRs. It would be interesting to determine if MEF2D may regulate IL-10 transcription in other immune cell types, such as Tr1 cells and B10 cells, which have important roles in autoimmune diseases. The mechanism of MEF2D-mediated repression of IL-10 transcription also needs to be elucidated. MEF2D is capable of binding consensus sequences within the promoters of target genes and, therefore, may regulate IL-10 and A20 directly. Lastly, the role of MEF2D in macrophages independently of IL-10 should be studied. By creating a double knockout of MEF2D and IL-10, the function of MEF2D regarding macrophage phenotype, IL-12a expression and A20 expression could be further explored.

MEF2D is a novel regulator of IL-10 transcription in macrophages and also regulates expression of other genes involved in the inflammatory response including A20 and IL-12a. The full scope of MEF2D functions within macrophages and immune cells needs to be examined further.

Chapter 5

Results- Sphingosine kinase 1 and its role in cytokine production

5.1 Sphingolipids

Sphingosine kinase is involved in sphingolipid metabolism and is upregulated in regulatory macrophages indicating a possible functional role (Edwards et al., 2006). Sphingolipids are a group of lipid mediators defined by the presence of a serine headgroup with fatty acid tails (Maceyka et al., 2009; Pitson, 2011). Sphingolipid signaling has been implicated in regulating numerous processes including cell growth, survival, differentiation and migration. Among the sphingolipids, particular interest has been focused on sphingosine, sphingosine 1-phosphate (S1P), ceramide and ceramide 1-phosphate (C1P). The amount of each of these bioactive lipids is altered in response to extracellular stimuli and can regulate a diverse range of cellular processes. Sphingolipid metabolism involves multiple bidirectional pathways that can control flux through the biosynthetic or degradative pathways in both directions. Ceramide and sphingosine have pro-apoptotic and anti-proliferative effects, however phosphorylation to C1P and S1P, respectively, leads to enhanced cell growth and survival (Maceyka et al., 2009; Pitson, 2011). S1P has been implicated in several disease states including atherosclerosis, cancer and inflammatory diseases.

5.1.1 Sphingolipid metabolism

Ceramide is the backbone of all sphingolipids and is both synthesised *de novo* and generated from the turnover of sphingolipids (Strub et al., 2010). At the endoplasmic reticulum, serine and palmitoyl-CoA are condensed into 3-ketosphingaine which is quickly reduced to dihydrosphingosine (figure 5.1). Dihydrosphingosine is then N-acylated and a *trans* double bond introduced to form ceramide. Ceramide is trafficked to the Golgi body where head groups are added. Removal of these head groups and deacylation produces sphingosine. Both sphingosine and dihydrosphingosine are only produced through catabolism of other sphingolipids. Sphingosine can be either utilised to generate complex sphingolipids or phosphorylated to produce S1P (Strub et al., 2010). S1P can be degraded by dephosphorylation by phosphatases including lysosomal phosphatases and two S1P-specific phosphatases, SPP1 and 2 (Maceyka et al., 2007). Alternatively, S1P can be cleaved by S1P lyase to produce ethanolamine phosphate and hexadecenal (Bandhuvula and Saba, 2007).

5.1.2 Sphingosine 1-phosphate

S1P is a well-known pro-survival signal, but it is also important in other processes including cell trafficking, differentiation, angiogenesis and inflammation (Maceyka et al., 2002; Cyster, 2005; Payne et al., 2004). S1P is present at high nanomolar concentration in the circulation and mediates its effects via five plasma membrane-located GPCRs (Rosen and Goetzl, 2005; Spiegel and Milstien, 2000a; Hisano et al., 2012). The five receptors are termed $S1P_{1\text{-}5}$ and display differential tissue expression and are linked to various G proteins. S1P has also been shown to possess intracellular signaling activities including modulating HDAC activity and the ubiquitin ligase activity of TRAF2 (Hait et al., 2009; Alvarez et al., 2010). The phosphorylation of sphingosine to S1P is catalysed by two enzymes- SphK1 and SphK2. S1P can then be released into the extracellular fluid by S1P transporters such as SPNS2 (Mitra et al., 2006; Nishi et al., 2013; Nagahashi et al., 2012a).

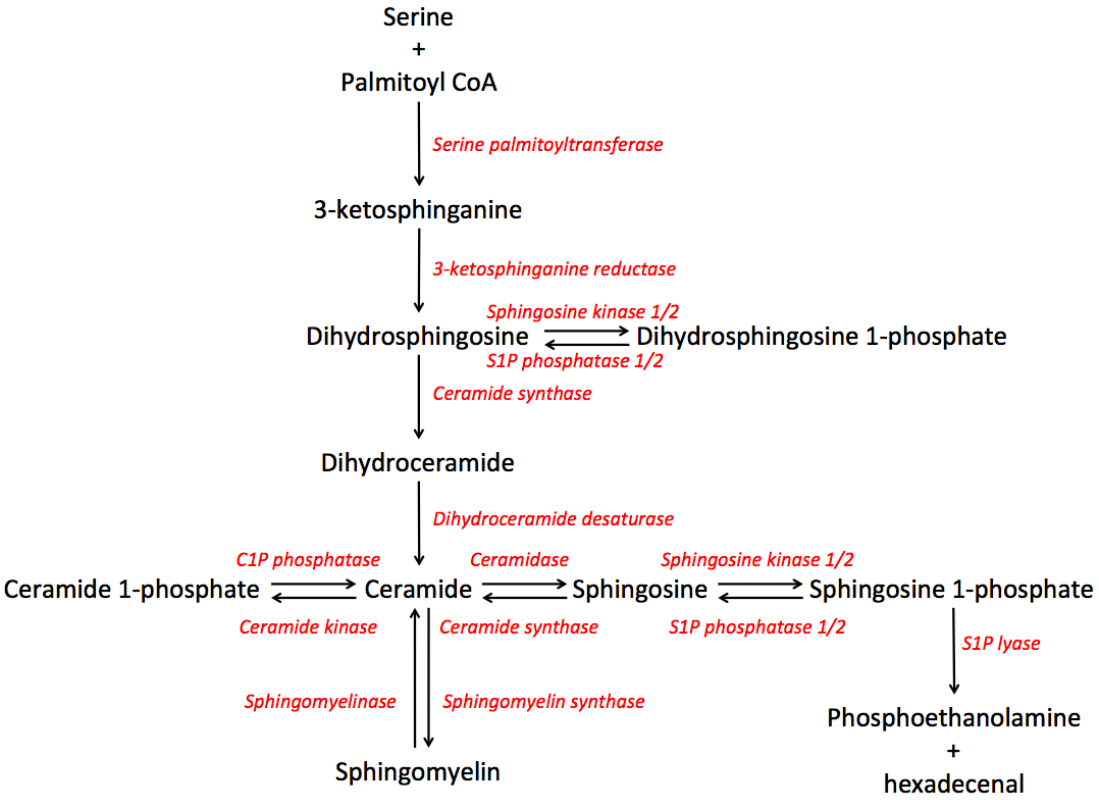

Figure 5.1: Synthesis and degradation of sphingosine 1-phosphate
Serine and palmitoyl CoA are condensed to 3-ketosphinganine, which is reduced to dihydrosphingosine. Dihydrosphingosine is then converted to dihydroceramide and then to ceramide. Ceramide can then either be phosphorylated to ceramide 1-phosphate, converted to sphingomyelin or converted to sphingosine. Sphingosine can then be phosphorylated by sphingosine kinases to form sphingosine 1-phoshate. Sphingosine 1-phosphate is a substrate for sphigonsine 1-phosphate lyase and for phosphatases including sphingosine 1-phosphate phosphatase.

5.1.3 Sphingosine kinase 1 and 2

Sphingosine kinase (SphK) 1 and 2 are lipid kinases which phosphorylate sphingosine leading to the production of S1P (Le Stunff et al., 2004). They are encoded by two genes, SPHK1 and SPHK2, although there are a number of splice variants (Taha et al., 2006; Pitson, 2011). SphK1 and 2 share a degree of sequence homology with five conserved domains, although SphK2 is a larger protein (figure 5.2). Both possess five conserved domains responsible for substrate binding and kinase activity (Liu et al., 2002). SphK2 possesses an additional N-terminal domain with a proline-rich polypeptide insert that is not present in SphK1. SphK1 and 2 have different expression patterns during development as well as different tissue distribution in adults (Taha et al., 2006). Differential tissue expression and subcellular localisation suggests that SphK1 and SphK2 may play different physiological roles. Deletion of either SphK1 or SphK2 results in mice that are viable and fertile (Allende et al., 2004; Mizugishi et al., 2005). Deletion of both SphK1 and

2 produces mice which die *in utero* due to problems in angiogenesis and neurogenesis (Mizugishi et al., 2005). Specific targeting of SphK1 reduces the severity and incidence in a murine model of arthritis, whilst targeting of SphK2 caused a more severe disease progression (Lai et al., 2009). In mast cells, only SphK1 is important in antigen-induced degranulation and migration, although both isoforms are required for efficient cytokine production (Oskeritzian et al., 2008). This suggests that SphK1 and SphK2 may have different physiological roles. SphK1 and 2 can phosphorylate both dihydrosphingosine and sphingosine.

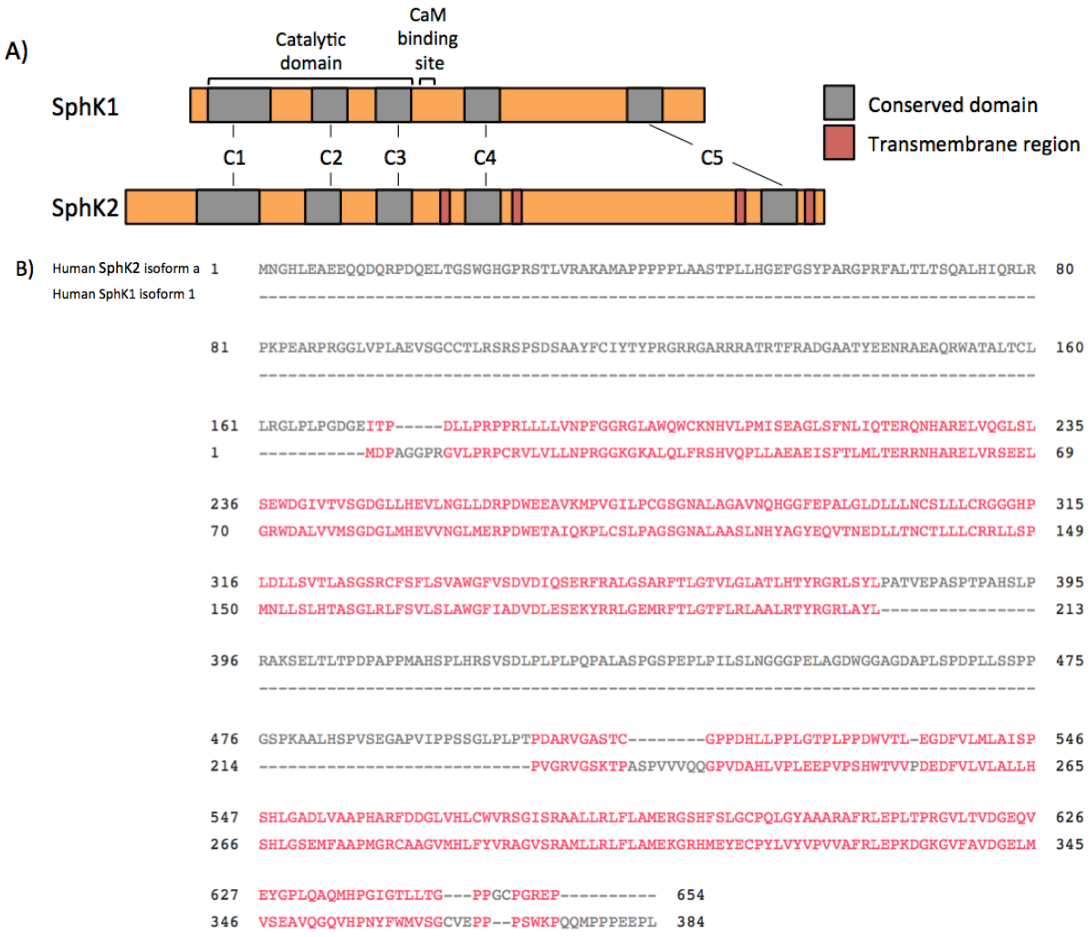

Figure 5.2: SphK domain structure
A) Schematic representation of SphK1 and SphK2 showing the five conserved domains within the overall structure. C1-3 form the catalytic domain of SphK. Transmembrane regions are present in SphK2 but not SphK1. The calmodulin binding site is found at residues 191-206 in human SphK1. B) Alignment of human SphK2 isoform a (NP_064511.2) and human SphK1 isoform 1 (NP_068807.2) using protein BLAST (http://blast.ncbi.nlm.nih.gov/). Sequences in red demonstrate homology.

5.1.4 Sphingosine kinase 1 activation and localisation

SphK1 is ubiquitously expressed and activated by many diverse stimuli including growth

factors and cytokines (Leclercq and Pitson, 2006). These stimuli lead to a rapid and transient increase in cellular SphK1 activity that was shown to be mediated by phosphorylation on Ser225 by ERK1/2 (Pitson et al., 2003). This phosphorylation event resulted in a 14-fold increase in catalytic activity although ATP and sphingosine affinities were unaffected. This activation is transient due to dephosphorylation of Ser225 by PP2A (Barr et al., 2008). Another function of phosphorylation on Ser225 is to change the cellular localisation of SphK1 (Pitson et al., 2003). Normally located in the cytoplasm, phosphorylation at Ser225 is essential for the translocation of SphK1 to the plasma membrane. The plasma membrane localisation is thought to be important as it allows release of S1P or interactions with effector proteins.

The mechanism by which phosphorylation at Ser225 mediates plasma membrane localisation is not fully understood. Associations with phosphatidylserine, phosphatidic acid and filamin have all been suggested (Stahelin et al., 2005; Delon et al., 2004; Maceyka et al., 2008). Further work demonstrated a requirement for the calmodulin-binding site in SphK1 for translocation to the plasma membrane (Jarman et al., 2010; Sutherland et al., 2006). SphK1 was shown to interact with calcium and integrin-binding protein 1 (CIB1) via its calmodulin binding site in a calcium-dependent manner. CIB1 is a calcium-myristoyl switch protein that translocates to the nucleus following calcium fluxes, which have also been associated with SphK1 activation. Calcium flux leads to the translocation and extrusion of the myristoyl tag on CIB1 allowing insertion into the plasma membrane (Jarman et al., 2010; Spiegel and Milstien, 2000b). It has also been suggested that SphK1 is activated by PKCδ downstream of EGF signaling, although whether this is a direct phosphorylation event or via activation of ERK1/2 is unclear (Paugh et al., 2008a).

Phosphorylated SphK1 is found within membrane microdomains or lipid rafts in the plasma membrane rather than having a diffuse distribution. This accumulation to lipid rafts is shown to be required for its effects on cell growth (Hengst et al., 2009). Constitutive localisation to lipid rafts by introducing the Lck tyrosine kinase myristoylation-dual palmitoylation motif enhanced growth and survival in serum-deprived conditions (Pitson et al., 2005). Diffuse plasma membrane localisation of SphK1 by adding the single

myristoylation site of c-Src led to inhibition of cell proliferation whilst retaining the anti-apoptotic effects of SphK1 (Safadi-Chamberlain et al., 2005). Both human and murine SphK1 splice variants have been shown to be constitutively located at the plasma membrane due to additional sequences at the N-terminus, although the N-terminal sequences differ between the human and murine variants (Venkataraman et al., 2006; Kihara et al., 2006). The human SphK1b isoform has a potential palmitoylation site, which is known to promote localisation to lipid rafts.

5.1.5 Sphingosine kinase 2 activation and localisation

SphK2 activity is also increased by certain stimuli, including EGF, IL-1 and TNFα, although the Ser225 site present in SphK1 is not conserved (Alemany et al., 2007; Mastrandrea et al., 2005; Olivera et al., 2006; Hait et al., 2005). Instead, it has been proposed that ERK1/2 might phosphorylate other residues such as Ser351 or Thr578 to activate SphK2 (Hait et al., 2007). SphK2 is located in the nucleus and cytoplasm although its distribution can change under certain conditions. Serum starvation appears to induce an accumulation of SphK2 in the endoplasmic reticulum, whilst PKC activation results in a decrease of nuclear SphK2 (Maceyka et al., 2005; Ding et al., 2007). SphK2 possesses nuclear localisation and export signals (Igarashi et al., 2003). Phosphorylation of Ser383 or 385 by protein kinase D activates the NES (Ding et al., 2007). Nuclear SphK2 can form a complex with histone H3 and HDAC1/2 (Hait et al., 2009). HDAC1 and 2 are direct intracellular targets of S1P. S1P inhibits their HDAC activity and promotes transcription of certain genes including c-fos. Phorbol esters strongly activate SphK2 resulting in increased nuclear S1P and complexing with histone H3 and HDACs.

5.1.6 Extracellular sphingosine kinases

Interestingly, sphingosine kinases can be secreted from the cell and generate S1P in the extracellular environment (Tani et al., 2007). SphK1 is released from cells via a non-classical secretory pathway and this occurs in response to stress from fibroblasts or in response to oxidised low-density lipoprotein immune complexes in macrophages (Waters et al., 2003; Venkataraman et al., 2006; Soldi et al., 2007; Hammad et al., 2006). Another study demonstrated release of SphK2 from cells undergoing apoptosis following caspase-

mediated cleavage of the N-terminus (Weigert et al., 2010). Extracellular SphK can use ATP released by cells and sphingosine present due to degradation of sphingomyelin (Tani et al., 2007; Elliott et al., 2009). S1P is subject to dephosphorylation by S1P phosphatases and degradation by S1P lyases, however, when produced extracellularly, exposure to S1P phosphatases or lyases will be limited. The role of extracellular SphKs is still unclear. Recent studies have shown that secreted vesicles from human tumour cells contain SphK1 and sphingosine and they may be utilised to activate $S1P_{1\text{-}5}$ signaling (Rigogliuso et al., 2010). In apoptotic cells, the function of extracellular SphK activity may be to influence the chemoattraction of phagocytic cells (Gude et al., 2008).

5.1.7 Therapeutic targeting of sphingosine 1-phosphate levels and function

S1P has many important roles and influences a range of processes including cell survival, angiogenesis and immune responses. S1P can be controlled at numerous levels; regulating its synthesis, its degradation, its export and its signaling via receptors (Kunkel et al., 2013). As a result, S1P levels can be affected by drugs targeting a range of enzymes involved in S1P metabolism (figure 5.3). Due to the effects of SphK1 in regulating cell survival over apoptosis, targeting SphK1 has mainly been considered for cancer treatment. Several drugs are now in clinical trials that regulate the S1P axis. Fingolimod or FTY720 is a modulator of $S1P_1$ function, in the short term it acts as an agonist but can promote receptor internalisation (Brinkmann et al., 2010). Fingolimod has been approved for treatment of multiple sclerosis (Ingwersen et al., 2012). It is also being trialled in schizophrenia and kidney transplants (Budde et al., 2002; Kunkel et al., 2013). The anti-S1P monoclonal antibody, Sonepcizumab is being trialled in age-related macular degeneration and solid tumours (Sabbadini, 2011). S1P receptor agonists have been investigated for effectiveness against ulcerative colitis, multiple sclerosis and plaque psoriasis (Kunkel et al., 2013). A S1P lyase inhibitor, LX3305, has undergone phase I and II trials against rheumatoid arthritis (Gräler, 2010; Fleischmann, 2012). The selective SphK2 inhibitor, ABC294640, is in phase I clinical trials for pancreatic cancer (French et al., 2010). The range of targets within the S1P axis should provide the ability to treat a range of diseases in which S1P is implicated with some degree of tissue specificity and reduced side effects.

5.1.7.1 SphK inhibitors

Several SphK inhibitors have now been described, including SK1 I, SK1 II and, most recently, PF-543 (Edmonds et al., 2011; French et al., 2003; Schnute et al., 2012; Paugh et al., 2008b). SphK inhibitors should block the phosphorylation of sphingosine and thus reduce the pool of S1P and thereby reduce or block S1P signaling both intracellularly and extracellularly. SKI I targets SphK1 with a Ki of 10 μM *in vitro* and has been shown to decrease cancer progression, angiogenesis and airway hyper responsiveness (Nagahashi et al., 2012b; Paugh et al., 2008b; Kapitonov et al., 2009; Price et al., 2013). SKI II targets both SphK1 and 2 (16 and 8 μM IC50 respectively *in vitro*) and limits cancer progression (Gao et al., 2012). PF-543 was described more recently and is a sphingosine competitive inhibitor with a nanomolar K_i (3.6 nM) for SphK1 *in vitro*. Notably, PF-543 is 100 fold selective for SphK1 over the SphK2 isoform. PF-543 was shown to block S1P production in the 1483 head neck carcinoma cells and in whole blood, although cell growth was unaffected (Schnute et al., 2012).

5.1.8 S1P signaling

S1P binds to five different GPCRs and can then initiate downstream signaling through various G proteins. S1P receptors have differential expression and are coupled to different G proteins. S1P receptors are activated in an autocrine or paracrine manner. Signaling from the five S1P receptors is described below.

5.1.8.1 $S1P_1$ signaling

$S1P_1$ is ubiquitously expressed with highest expression in brain, lung, spleen and the cardiovascular system. $S1P_1$ deletion is embryonically lethal due to haemorrhage. Lack of $S1P_1$ results in incomplete vascular maturation due to a failure of smooth muscle cell and pericyte migration (Liu et al., 2000). Endothelial-specific deletion of $S1P_1$ displays the same vascular deficiencies as total deletion suggesting that $S1P_1$ located on endothelial cells is responsible for vascular maturation (Allende et al., 2003). $S1P_1$, in conjunction with $S1P_2$ and $S1P_3$, can increase vascular integrity to maintain endothelial and epithelial barrier integrity (McVerry and Garcia, 2004; Singleton et al., 2006). Disruption of endothelial barriers results in greater vascular permeability which is often evident in tumours

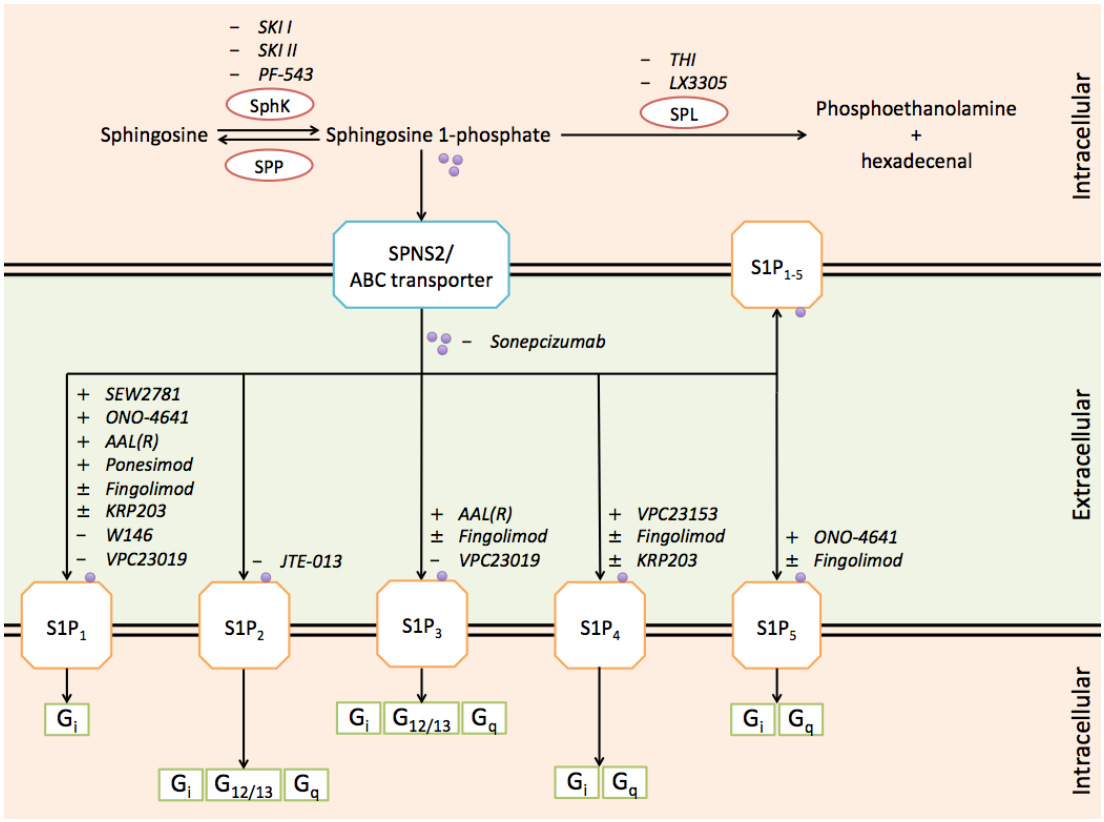

Figure 5.3: Therapeutically targeting the sphingosine 1-phosphate axis
The sphingosine 1-phosphate axis can be therapeutically targeted at several points. Sphingosine is phosphorylated by sphingosine kinases which can be targeted to prevent the generation of S1P. Degradation of S1P is catalysed by S1P lyase (SPL) and can be targeted to promote an accumulation of S1P. S1P can have intracellular and extracellular effects. S1P is transported by SPNS2 or ABC transporters. Once in the extracellular space, it can act in an autocrine or paracrine manner to initiate G protein-mediated signaling. Sonepcizumab is a monoclonal antibody against S1P that prevents S1P signaling through its receptors. Additionally, S1P receptors can be targeted with a range of agonists, antagonists or functional modulators including fingolimod and ponesimod.

and during inflammation. siRNA-mediated silencing of $S1P_1$ blocks barrier enhancement and the same study identified Akt and Rac as effectors of $S1P_1$ (Singleton et al., 2006). A $S1P_1$ selective antagonist showed that $S1P_1$ is required for preserving vascular tone, a role that has also been shown *in vivo* (Sanna et al., 2006; Finigan et al., 2005; McVerry and Garcia, 2005). Interestingly, conditional SphK1/SphK2 double knockout mice have normal vascular integrity, despite low levels of circulating S1P, which suggests that normal expression of $S1P_1$ is more important to vascular integrity than the circulating level of S1P (Pappu et al., 2007).

$S1P_1$ is important in lymphocyte egress from lymph nodes. Resting T and B cells express $S1P_1$ and silencing or deletion causes lymphopenia (Matloubian et al., 2004; Allende et al., 2004). Transplantation of $S1P_1$-deficient thymocytes and lymphocytes into

wild-type mice also demonstrates the importance of $S1P_1$ in controlling lymphocyte circulation as the $S1P_1$-deficient cells are sequestered in lymph nodes and Peyer's patches (Matloubian et al., 2004; Chi and Flavell, 2005).

5.1.8.2 $S1P_2$ signaling

$S1P_2$ is widely expressed among different cell types. Deletion of $S1P_2$ does not affect development, although $S1P_2$-deficient mice develop sporadic seizures around 3-7 weeks (MacLennan et al., 2001). It was shown in the same study that neocortical pyramidal cells from $S1P_2$-deficient mice display increased excitability. $S1P_2$-deficient mice are deaf, showing a requirement for $S1P_2$ in auditory system development (Kono et al., 2007). $S1P_2$ is considered to oppose the actions of $S1P_1$ and $S1P_3$, which promote cell migration, as $S1P_2$ inhibits cell migration (Lepley et al., 2005). Interestingly, $S1P_2$ activates ROCK/Rho to increase vascular permeability (Sanchez et al., 2007). In mast cells, $S1P_2$ is necessary for degranulation, which correlates with the reliance seen on SphK1/2 expression for mast cell function (Jolly et al., 2004; Olivera et al., 2007; Oskeritzian et al., 2008).

5.1.8.3 $S1P_3$ signaling

$S1P_3$ is expressed in lungs, kidney, spleen, intestines and the cardiovascular system. Deletion of $S1P_3$ does not cause any evident phenotypic effects (Ishii et al., 2001). Knockout of $S1P_2$ and $S1P_3$ moderately increases perinatal lethality (Ishii et al., 2002). Triple knockout of $S1P_{1\text{-}3}$ is embryonic lethal with severe vascular deficiencies (Kono et al., 2004). The defects in vasculature seen in the $S1P_{1\text{-}3}$ KO may be more severe than those seen in the $S1P_1$ deficient mice, suggesting roles for $S1P_2$ and $S1P_3$. Similarly to $S1P_2$, $S1P_3$ activates ROCK/Rho to regulate vascular permeability (Sanchez et al., 2007). $S1P_3$ is expressed in myocytes and perivascular smooth muscle cells and activation of $S1P_3$ in these cells results in bradycardia and hypertension (Forrest et al., 2004).

5.1.8.4 $S1P_4$ signaling

$S1P_4$ is less widely expressed than $S1P_{1\text{-}3}$, primarily being expressed in lymphoid tissue including thymus, spleen, bone marrow and peripheral lymphocytes (Gräler et al., 1998;

Kluk and Hla, 2002). $S1P_4$ activates G_i and $G\alpha_q$ and over-expression of $S1P_4$, in Jurkat cells, increased cell motility in the absence of S1P (Gräler et al., 2003). $S1P_4$ activation leads to activation of ERK1/2 and phospholipase C (Van Brocklyn et al., 2000). In addition, $S1P_4$ stimulation modulates calcium release from intracellular stores (Yamazaki et al., 2000). In mouse T cells lacking endogenous S1P receptors, expression of $S1P_4$ failed to initiate a chemotactic response when stimulated. However, $S1P_4$ expression did lead to increased secretion of IL-10 and reduced proliferation when activated with S1P (Wang et al., 2005a).

5.1.8.5 $S1P_5$ signaling

$S1P_5$ is highly expressed in oligodendrocytes and natural killer (NK) cells (Im et al., 2000; Terai et al., 2003; Walzer et al., 2007). Silencing of $S1P_5$ expression does not affect oligodendrocyte function (Jaillard et al., 2005). Stimulation of rat oligodendrocytes with platelet-derived growth factor increased $S1P_1$ expression whilst decreasing $S1P_5$ expression (Jung et al., 2007). Activation of $S1P_5$ results in the inhibition of ERK1/2 activity due to phosphatase activity, thus $S1P_5$ signaling inhibits cell proliferation (Gonda et al., 1999; Im et al., 2000). The importance of $S1P_5$ in NK cells has been described and this study showed that NK cells lacking $S1P_5$ are unable to properly home to inflamed organs (Walzer et al., 2007).

5.1.9 S1P intracellular signaling

In addition to activating its receptors, S1P can also act in an intracellular manner (Spiegel and Milstien, 2011). Deletion of S1P phosphatase and lyase from yeast cells results in slow growth and a large accumulation in S1P. Deletion of the major SphK in yeast allows normal cell growth again (Kim et al., 2000; Zhang et al., 2001). The inhibition of cell growth may be caused by S1P-induced increases in intracellular calcium levels (Birchwood et al., 2001). Heat shock induces cell cycle arrest in yeast but it also increases SphK activity and S1P accumulation suggesting a possible role for S1P in responses to heat shock (Lanterman and Saba, 1998; Skrzypek et al., 1999). Heat shock tolerance can be increased by deleting the S1P phosphatase or lyase (Skrzypek et al., 1999; Mao et al., 1999; Mandala et al., 2000). Interestingly, no apparent cell surface receptor for S1P is

encoded by the yeast genome and addition of exogenous S1P does not affect cell growth suggesting an intracellular role for S1P (Lanterman and Saba, 1998).

Intracellular effects of S1P have also been identified in mammalian cells. Expression of SphK1 leads to decreases in ceramide levels and increases in dihydrosphingosine levels (Maceyka et al., 2005). The conversion of ceramide to dihydrosphingosine is catalysed by a group of ceramide synthases (CerS). The changes in lipid levels caused by SphK1 expression suggests that S1P may inhibit a CerS. S1P was shown to be an *in vitro* non-competitive inhibitor of CerS2 and further experiments identified two domains with homology to S1P receptors. A point mutation within each of the domains relieved S1P inhibition on CerS2 (Laviad et al., 2008). However, CerS2 does not use C16 or C18 acyl CoAs and ceramides produced from these substrates are the most affected by SphK1 expression (Maceyka et al., 2005). Further work is required to understand the role that S1P has in modulating ceramide synthesis.

Whilst SphK1 normally translocates to the plasma membrane, both SphK1 and SphK2 can be targeted to other internal locations. Both Acy1 and RPK118 have been shown to target SphK1 to internal membranes (Hayashi et al., 2002; Maceyka et al., 2004). SphK1 has also been identified at nascent phagosomes where it can then promote maturation into phagolysosomes (Thompson et al., 2005). SphK1 over-expression has been shown to promote G1/S transition (Olivera et al., 1999). This regulation of cell cycle progression may be explained by the finding that SphK1 is located at the nuclear envelope during S phase (Kleuser et al., 2001). SphK2 is found in the nucleoplasm and contains a NES activated by PKD (Ding et al., 2007). Whether translocation to the cytoplasm acts to reduce nuclear S1P signaling or increase cytosolic S1P signaling is unclear. Notably, SphK1 or SphK2 targeted to the ER promotes apoptosis (Maceyka et al., 2005; Liu et al., 2003a).

The target of ER-localised S1P remains unknown. S1P can induce calcium release from permeabilised cells in an inositol triphosphate receptor-independent manner (Mattie et al., 1994). S1P also induced calcium release from cell fractions rich in rough, but not smooth, ER (Ghosh et al., 1994). In line with this, over-expression of SphK2, that partially lo-

calises to ER, also raised intracellular calcium (Maceyka et al., 2005). Mast cells derived from SphK2-/- mice have defective calcium mobilisation in response to crosslinking of the IgE receptor (Olivera et al., 2007). In HEK293 cells, microinjection of S1P can elevate calcium levels in the presence of pertussis toxin, a GPCR inhibitor (Meyer zu Heringdorf et al., 1998). Likewise, caged S1P, that forms S1P on exposure to light, induced increased calcium levels in cells that do not respond to exogenous S1P (Meyer Zu Heringdorf et al., 2003). These studies all propose a role for S1P as a second messenger responsible for calcium mobilisation.

In addition to modulating calcium release, S1P has other effects that are S1P receptor-independent. Over-expression of SphK1 leads to the survival of endothelial cells, possibly due to expression of PECAM-1 and activation of Akt. These effects are not reproduced by the addition of exogenous S1P (Limaye et al., 2005). SphKs can also phosphorylate dihydrosphingosine to produce dihydrosphingosine 1-phosphate (DHS1P). Both DHS1P and S1P bind and activate S1P receptors with similar affinities, however DHS1P does not mimic all of the effects of S1P (Van Brocklyn et al., 1998; Suomalainen et al., 2005; Morita et al., 2000). S1P protects male germ cells from apoptosis and similar effects were seen in HL-60 and PC-12 cells (Suomalainen et al., 2005; Van Brocklyn et al., 1998). DHS1P could not protect male germ cells from apoptosis (Suomalainen et al., 2005). Interestingly, S1P-phosphonate protected HL-60 and PC-12 cells from apoptosis despite failing to bind S1P receptors (Van Brocklyn et al., 1998). SphK1 over-expression in embryonic fibroblasts from S1P-receptor knockout mice enhanced growth and survival (Olivera et al., 2003). These results suggest that S1P has important intracellular targets that protect cells from apoptosis. The FTY720 analog AAL(R) is phosphorylated by SphK2 and only poorly by SphK1 and induces apoptosis in Jurkat cells and primary splenocytes. AAL(R) did not induce apoptosis in cells from SphK2-/- mice (Don et al., 2007). This suggests an intracellular action for S1P generated by SphK2 in promoting apoptosis.

TNFα is known to induce SphK1 activity and the S1P produced may function intracellularly (Xia et al., 1998). It was shown that S1P activates NF$\varkappa$B independently of S1P-

receptors (Alvarez et al., 2010). Previously, it had been shown that SphK1 binds to TRAF2 which is recruited to the TNFα receptor upon stimulation (Xia et al., 2002). It has now been demonstrated that active SphK1 is important for the activation of NFκB as S1P is a cofactor for TRAF2. TRAF2 is necessary for the ubiquitination of RIP1 (Alvarez et al., 2010), which then recruits other proteins required for NFκB activation (Bhoj and Chen, 2009; Ea et al., 2006). Interestingly, DHS1P is unable to mimic the actions of S1P with TRAF2. Poly-ubiquitinated RIP1 is also prevented from interacting with pro-caspase 8 and thus this prevents apoptosis (Alvarez et al., 2010).

5.1.10 S1P in macrophages

Macrophages undergo apoptosis during microbial infection and the role of S1P in protecting from apoptosis in other cells is well known. S1P generated by apoptotic cells was able to induce phosphoinositide 3-kinase (PI3K), ERK1/2 and Ca^{2+} signaling in primary macrophages and this protected macrophages from TNFα-induced cell death (Weigert et al., 2006). The activation of PI3K signaling resulted in the inhibition of acid sphingomyelinase and therefore prevented ceramide accumulation. These changes in ceramide levels were accompanied by increased expression of Bcl-XL and decreased caspase-3 cleavage (Gómez-Muñoz et al., 2003). S1P has also been shown to prevent cytochrome c and Smac/DIABLO translocation from the mitochondria to the cytosol following apoptosis-inducing stimuli and therefore prevents caspase-3 activation (Cuvillier et al., 1996). In Raw 264.7 cells, LPS treatment induced activation of SphK1 and translocation to the plasma membrane. Inhibition of SphK1 sensitised cells to LPS-induced cell death, although it is unclear whether this is due to an intra- or extracellular signaling event (Wu et al., 2004; Hammad et al., 2008).

Macrophages are recruited to sites of inflammation by chemokines and S1P acts as a chemoattractant for primary human monocytes and macrophages *in vitro* as well as for the monocytic cell lines U937 and THP-1 (Schwab and Cyster, 2007; Gude et al., 2008). FTY-720, the S1P-receptor ligand, has been shown to reduce macrophage infiltration to the CNS *in vivo* in a model of EAE (Fujino et al., 2003). FTY720 also reduced the number of macrophages in atherosclerotic lesions (Theilmeier et al., 2006). The use of a $S1P_1$-

specific agonist decreased macrophage infiltration in a model of experimental autoimmune myocarditis (Ogawa et al., 2007). These studies suggest an important role for S1P-$S1P_1$ in macrophage trafficking. The effects of FTY720 on monocyte-endothelium interactions may partly explain reduced recruitment of macrophages to inflamed tissues. S1P has been shown to prevent adhesion of monocytes to endothelium and this could be explained by $S1P_3$-dependent production of nitric oxide inhibiting MCP-1, a chemokine required for monocyte adhesion (Bolick et al., 2005; Theilmeier et al., 2006). Interestingly, intraocular injection of a monoclonal antibody, Sonepcizumab, reduced macrophage infiltration into the ischemic retina following induced ischemic retinopathy (Xie et al., 2009). Although, scavenging S1P may alter macrophage infiltration by increasing apoptosis within macrophages.

Mycobacterium tuberculosis inhibits phagosome maturation and is though to prevent maturation by inhibiting SphK1 translocation to nascent phagosomes. Dead *M.tuberculosis*, however, activates SphK1 resulting in a calcium-dependent translocation to the phagosome (Malik et al., 2003; Thompson et al., 2005). Addition of S1P to *M.tuberculosis* infected human macrophages reduced pathogen viability by phospholipase D activation and phagolysosome generation (Garg et al., 2004). Injection of S1P into mice infected with *Mycobacteria* reduced pathogen growth and tissue damage. Treatment with sphingosine had similar effects on *Mycobacteria,* although no receptors are currently identified for sphingosine, therefore sphingosine may enter the cell to enhance pathogen killing (Garg et al., 2004; Gutierrez et al., 2009). Whether it then becomes phosphorylated or whether S1P is dephosphorylated to sphingosine remains unclear. S1P may also contribute to phagosome maturation by influencing actin assembly (Kuehnel et al., 2009a,b). Furthermore, $SphK2^{-/-}$ mice have higher susceptibility to bacterial lung infection (Zemann et al., 2007). These reports demonstrate a clear role for SphK-S1P in mediating clearance of bacterial infections.

Extracellular effects of S1P are often considered to be anti-inflammatory. S1P stimulation of alveolar macrophages led to production of reactive oxygen species, although weakly compared to LPS (Hornuss et al., 2001). In fact, S1P was shown to block LPS-

dependent NO production (Hughes et al., 2008). S1P treatment did promote vascular endothelial growth factor (VEGF) production in human macrophages (Weis et al., 2009). Macrophages pre-incubated with S1P or a $S1P_1$-specific agonist, had reduced levels of TNFα, MCP-1 and IL-12 following treatment with LPS (Hughes et al., 2008).

S1P was shown to increase COX-2 expression by regulating the localisation of the RNA binding protein, HuR. Elimination of S1P from the supernatant reduced PGE_2 production in apoptotic cells (Johann et al., 2008). A micromolar dose of S1P may be able to induce PGE_2 secretion (Hammad et al., 2008). In PGE_2-stimulated Raw 264.7 cells, S1P increased cAMP levels through $S1P_2$-G_{13} signaling (Jiang et al., 2007). PGE_2 production is an important constituent for resolution of inflammation (Bystrom et al., 2008). SphK1 expression is also evident in regulatory macrophages although a function has yet to be described (Edwards et al., 2006). Interestingly, in Raw 264.7 cells, LPS induced SphK1 expression after 16 hours and was shown to be required for TNFα-induced PGE_2 release using SphK1 siRNA (Hammad et al., 2008).

Signaling from TLR4 has been shown to activate and increase expression of SphK1 (Wu et al., 2004). S1P binding to $S1P_3$ has been shown to affect induction of MCP-1, whereas signaling via $S1P_2$ contributes to IL-1 and IL-18 production (Keul et al., 2011; Skoura et al., 2011). Deletion of SphK1 can protect mice from sepsis (Niessen et al., 2008). It has been suggested that S1P is required for DC activation, which is required for systemic inflammation during sepsis. In this proposed mechanism, S1P signals via $S1P_3$ to regulate the amplification of the inflammatory response as part of PAR1 signaling (Niessen et al., 2008). These studies suggest a complex regulation of cytokine and chemokine production by S1P.

Further examples of the anti-inflammatory aspects of S1P have been described in various disease models. S1P is protective before and during atherosclerosis (Argraves and Argraves, 2007). This may be partially explained by negative crosstalk between $S1P_1$ and $S1P_2$ and TLR2 pathways (Dueñas et al., 2008). In a model of acute necrotising pancreatitis, S1P and FTY720 reduced NFκB activation resulting in reduced production of IL-1,

IL-6 and TNFα (Liu et al., 2008a). Notably, S1P may promote Th2 responses. Knock-down of SphK1 in a model of allergic arthritis led to reduced macrophage numbers and reduced IL-4 and IL-5 secretion (Lai et al., 2008). This was further underlined by treatment of murine fibrosis with the monoclonal antibody, Sonepcizumab, which neutralises S1P. Sonepcizumab treatment reduced disease parameters in murine fibrosis (Caballero et al., 2009; Meneghin and Hogaboam, 2007).

Immature DCs have low expression of CCR7 which makes them unresponsive to CCL19 preventing RAC activation and migration (König et al., 2010). In addition, $S1P_2$ signaling activates RHO leading to FLH2-mediated repression of $S1P_1$ transcription. Maturation of DCs results in CCR7 up-regulation and a down-regulation of $S1P_2$. Together, this results in RAC activation and migration. There is also less RHO-mediated repression of $S1P_1$ transcription. Positioning of immature DCs within the spleen has been shown to be $S1P_1$-dependent (König et al., 2010). These results demonstrate how the expression profile of the S1P receptors is carefully regulated by cells during their maturation.

5.2 Expression of Sphk1 in Zymosan-stimulated macrophages

Zymosan activates Dectin-1 and TLR2 signaling and in macrophages leads to high levels of IL-10 and low levels of IL-12, which is often characteristic of regulatory macrophages. Regulatory macrophages are thought to be induced by TLR agonists and a secondary stimulus, such as PGE_2, and are important in dampening the inflammatory response. Studies from other labs have identified markers, which are induced as part of the regulatory macrophage phenotype (Edwards et al., 2006). Markers for wound-healing macrophages have also been identified in various studies (Raes et al., 2002, 2005; Stein et al., 1992). To examine which of these markers were induced by Zymosan, data from a microarray experiment carried out by a previous PhD student in the lab was analysed. In this experiment, wildtype bone marrow-derived macrophages were stimulated with LPS or Zymosan for 1 or 8 hours and then expression of mRNA quantified. LPS and Zymosan both strongly induced expression of IL-10 at 1 and 8 hours (figure 5.4), consistent with mRNA analysis through qPCR. Markers of wound-healing (M2a) macrophages, Clec10a, MRC1 and Ym1, showed moderate down-regulation with LPS and Zymosan at 8 hours.

Arg1 was modestly induced by Zymosan at 8 hours, whilst remaining around basal levels with LPS stimulation. SphK1 and LIGHT are considered markers of regulatory markers (Edwards et al., 2006). LPS induced both LIGHT and Sphk1 at 1 hour before it returned to basal expression. Notably, Zymosan induced sustained expression of both LIGHT and SphK1. LIGHT is a member of the TNFα ligand family and may trigger signaling via TNFRSF14. As SphK1 is important for the generation of S1P and as S1P has roles in the immune system I investigated the role of SphK1 might play in Zymosan-induced cytokine production.

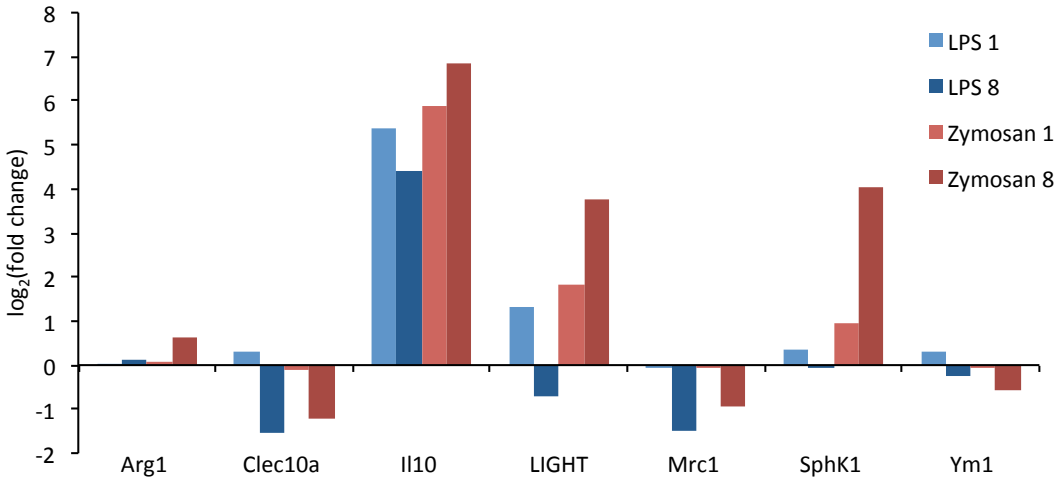

Figure 5.4: Expression of macrophage phenotype markers
BMDMs were stimulated with 100 ng/ml LPS or 200 µg/ml Zymosan for 1 or 8 hours. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen miniRNeasy kit. The arrays were generated by the Finnish Microarray and Sequencing Centre (Turku, Finland), according to the manufacturer's protocols. Four biological replicates were analyzed per group. The data analysis was carried out in the High Performance Computing facility of the College of Life Sciences using Affymetrix Power Tools, R (version 2.13.1) – Bioconductor and Partek GS 6.5 (version 6.11.0321) software.

Firstly, the data from the microarray was validated. BMDMs were stimulated with LPS and Zymosan over a 8 hour timecourse and SphK1 mRNA levels measured by qPCR. As seen in the array data, SphK1 mRNA is induced by Zymosan treatment peaking at 6 hours (figure 5.5). LPS only weakly induces SphK1 expression with a peak expression level at 2 hours. The induction of SphK1 by Zymosan suggests it may play a role in maintaining or generating the phenotype observed in Zymosan-stimulated macrophages. One of these features is high levels of IL-10 compared to LPS-stimulated macrophages. It is possible that SphK1 may be important in driving the high levels of IL-10 secretion or that IL-10 may drive SphK1 expression in order to generate the regulatory phenotype observed in Zymosan-stimulated macrophages. To address the possible function of SphK1, I used

inhibitors of SphK1 to block its function in macrophages.

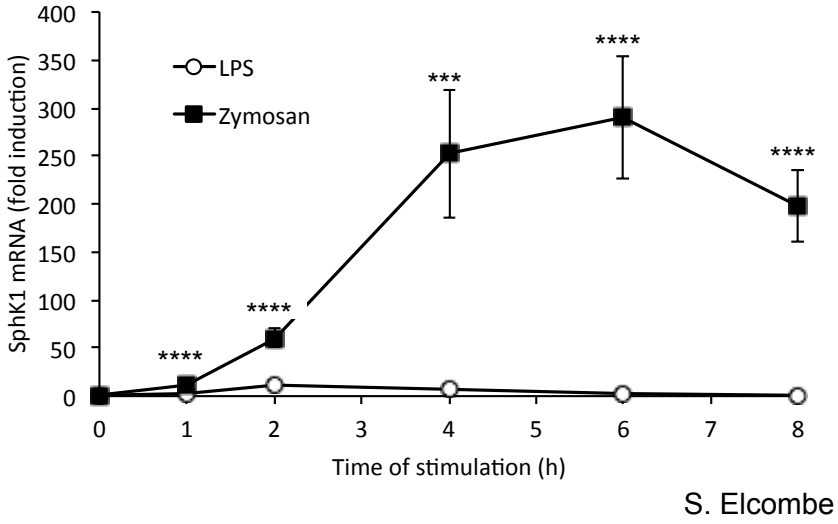

Figure 5.5: Comparison of SphK1 expression in LPS- and Zymosan-stimulated macrophages
Bone marrow-derived macrophages from WT mice were stimulated with 100 ng/mL of LPS or 200 µg/ml Zymosan for the times indicated. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for SphK1 mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

5.3 Characterisation of SphK inhibitors

Several SphK1 inhibitors have been described (Paugh et al., 2008b; French et al., 2003; Schnute et al., 2012; Kunkel et al., 2013; Gräler, 2010). Paugh *et al* describe SK1 I as an ATP-competitive inhibitor of SphK1, with minimal effects on SphK2 or PKC. In addition to the ATP-competitive inhibitor, SK1 I, other inhibitors of SphK1 have been described. Both SK1 II and PF-543 are substrate-competitive inhibitors of SphK1. However, SK1 II shows a similar IC50 for SphK2 (8 µM) as for SphK1 (16 µM) (French et al., 2003). PF-543 is highly selective for SphK1 with a nanomolar K_i and only a small amount of inhibition of SphK2 evident at 10 µM (Schnute et al., 2012).

In order to determine the selectivity of these structurally distinct inhibitors against protein kinases, SK1 I, SK1 II and PF-543 were profiled against a panel of protein kinases. SK1 I and PF-543 demonstrated no specific inhibition of any protein kinase (figure 5.7A+C). SK1 II caused considerable inhibition of several protein kinases including CAMK1, GCK, MLK1, MLK3, PIM1 and PIM3 (all inhibited to below 40% remaining activity at 10 µM) (figure 5.7+B). SK1 I is an ATP-competitive inhibitor and may have been expected to

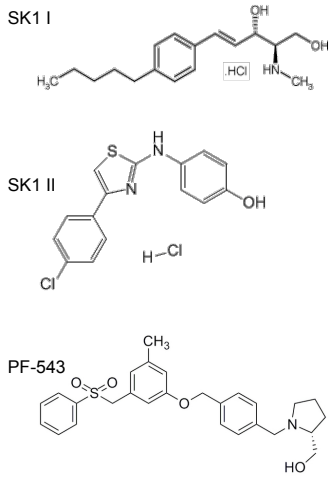

Figure 5.6: SphK inhibitor structures
Structures of three SphK inhibitors (SK1 I, SK1 II and PF-543) used in this study are presented here.

inhibit protein kinases rather than the substrate-competitive SK1 II.

Treatment of macrophages with Zymosan will lead to the activation of ERK1/2 which can phosphorylate and activate SphK1 (Pitson et al., 2003). Active SphK1 should cause an increase in S1P levels within the cell and this increase should be blocked by SphK inhibitors. In addition, all three inhibitors were found to have acute effects in macrophages as discussed in section §5.4. In order to determine if Zymosan activated SphK1 and induced S1P accumulation and if the SphK inhibitors prevented this S1P accumulation in cells, macrophages were stimulated with Zymosan for 15 minutes and S1P levels were calculated via mass spectrometry. Zymosan treatment of BMDMs only weakly stimulated an increase in S1P levels, although S1P levels were still decreased by the addition of SphK1 inhibitors (figure 5.8). Another product of SphK1 was also measured by mass spectrometry. SphK1 can also catalyse the phosphorylation of dihydrosphingosine (DHS) resulting in dihydrosphingosine-1-phosphate (DHS1P). Interestingly, Zymosan stimulated a 2-fold increase in DHS1P levels in macrophages and this increase was partially or completely blocked by pre-treatment with SphK inhibitors. Levels of dihydrosphingosine were increased in response to Zymosan treatment and this increase was inhibited by SK1 I and SK1 II. Other sphingolipids and ceramides were also quantified and remained largely unchanged (table 5.1). This data suggests that the three inhibitors used can block Zymosan-stimulated SphK1 activity and particularly affect the accumulation of DHS1P.

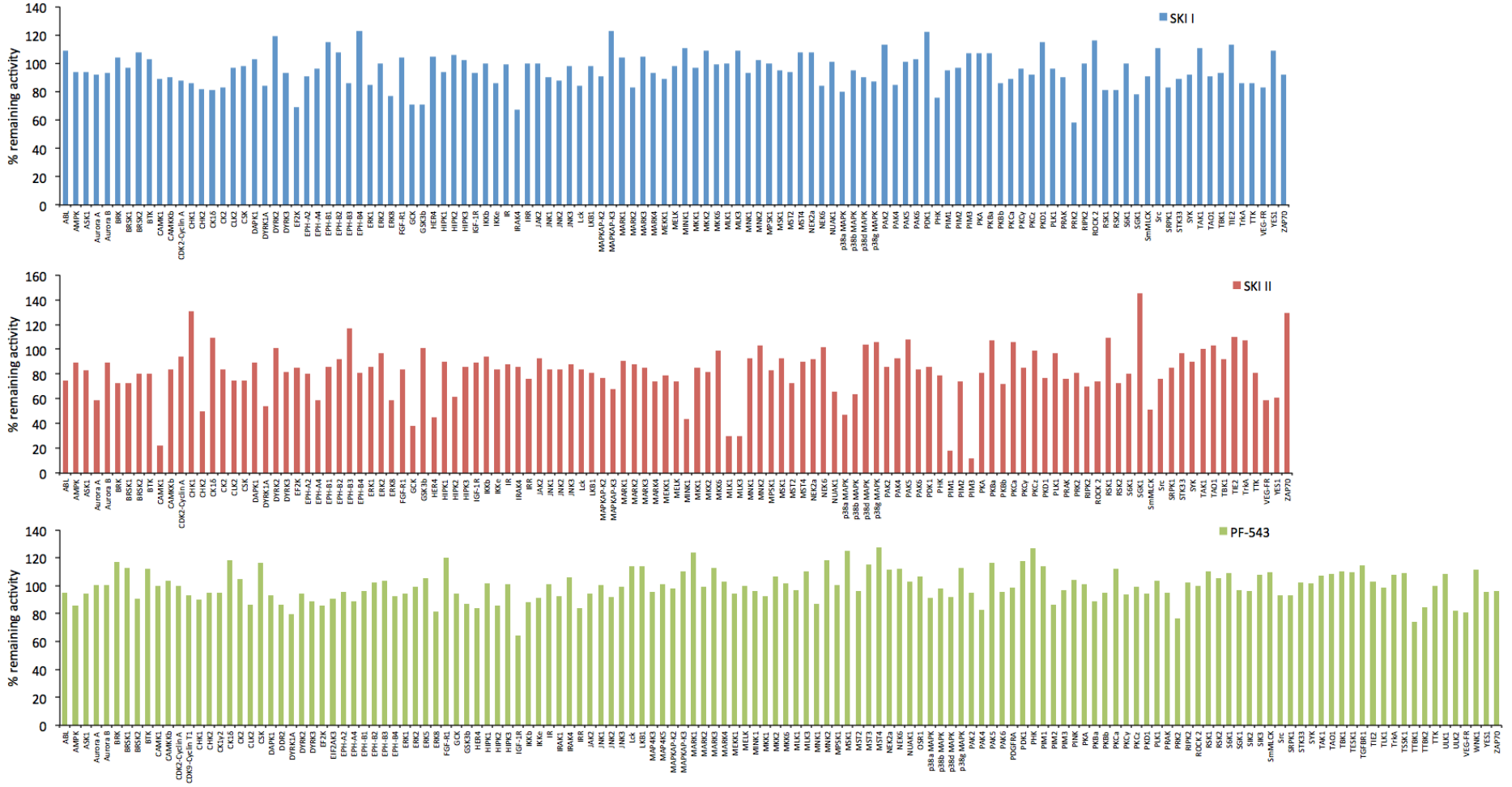

Figure 5.7: Protein kinase profiling of SphK inhibitors

SK1 I, SK1 II and PF-543 were screened against a panel of protein kinases *in vitro* at 10 μM by the International Center for Kinase Profiling using an ATP competitive assay. Kinases were ordered alphabetically.

Table 5.1: Sphingolipid levels in Zymosan-stimulated macrophages
BMDMs were pre-incubated with 10 μM SK1 I, 10 μM SK1 II and 5 μM PF-543 for 1 hour. BMDMs were then stimulated with 200 μg/ml Zymosan for 15 minutes. Cells were lysed and the lysate quantified. Spingholipids were extracted and then analysed by LC-MS/MS - (J Allegood, Virginia Commonwealth University). values represent average concentration (pmol/mg total protein) ± standard deviation, n=3.

	Unstimulated	**Zymosan**	**Zymosan + SK1 I**	**Zymosan + SK1 II**	**Zymosan + PF-543**
Sphingosine	30.56±0.51	31.50±0.61	8.70±0.98	21.47±1.53	34.98±0.83
Dihydrosphingosine	0.59±0.03	5.39±0.18	2.95±0.15	2.85±0.09	5.12±0.25
Sphingosine-1-phosphate	0.87±0.02	1.00±0.02	0.77±0.01	0.36±0.03	0.54±0.02
Dihydrosphingosine-1-phosphate	0.28±0.01	0.61±0.02	0.47±0.03	0.28±0.02	0.34±0.02
Ceramide					
C14:0	11.30±1.37	10.46±1.95	9.65±0.67	8.12±0.76	9.51±0.29
C16:0	191.83±8.02	177.30±6.50	176.77±11.48	131.37±9.34	167.70±3.11
C18:1	30.43±1.08	28.61±2.61	31.10±0.07	22.90±2.05	22.13±4.57
C18:0	9.21±1.38	7.55±1.56	7.83±0.39	4.85±1.02	4.89±0.62
C20:0	6.99±1.11	5.13±.067	4.24±0.49	2.63±0.49	3.03±0.54
C22:0	57.64±9.09	43.64±7.11	41.80±3.28	27.85±4.74	29.36±3.04
C24:1	132.95±8.47	116.03±10.40	109.75±5.88	74.01±13.15	71.18±5.96
C24:0	115.83±15.48	87.47±6.81	89.91±1.38	63.01±13.35	65.15±7.14
C26:1	43.00±8.37	25.60±4.14	26.56±3.55	17.74±3.60	19.06±0.87
C26:0	0.02±0.02	0.09±0.05	0.05±0.04	0.09±0.05	0.04±0.03
Sphingomyelin					
C14:0	158.53±15.08	158.26±12.74	123.71±24.85	107.75±26.74	125.54±15.16
C16:0	1877.46±36.68	2093.56±26.63	2029.79±159.38	1934.30±157.85	2147.64±73.01
C18:1	63.47±12.46	63.33±5.94	42.46±10.06	35.85±8.72	40.71±4.11
C18:0	493.10±7.46	543.20±0.18	533.30±33.05	487.63±39.96	556.28±16.40
C20:0	322.27±19.47	312.57±18.75	227.85±35.88	199.61±42.71	200.02±5.59
C22:0	1101.03±41.96	1131.11±28.86	1028.00±27.61	935.58±54.36	1018.14±69.43
C24:1	1469.75±92.52	1445.09±36.51	1287.85±56.81	1201.71±74.02	1274.46±61.84
C24:0	1392.37±114.13	1414.76±30.01	1244.63±65.15	1126.51±96.74	1207.25±52.52
C26:1	46.48±8.49	33.20±4.75	23.56±3.39	17.55±2.87	17.00±1.50
C26:0	22.49±6.36	16.42±3.71	10.54±1.99	8.21±2.47	7.96±0.27

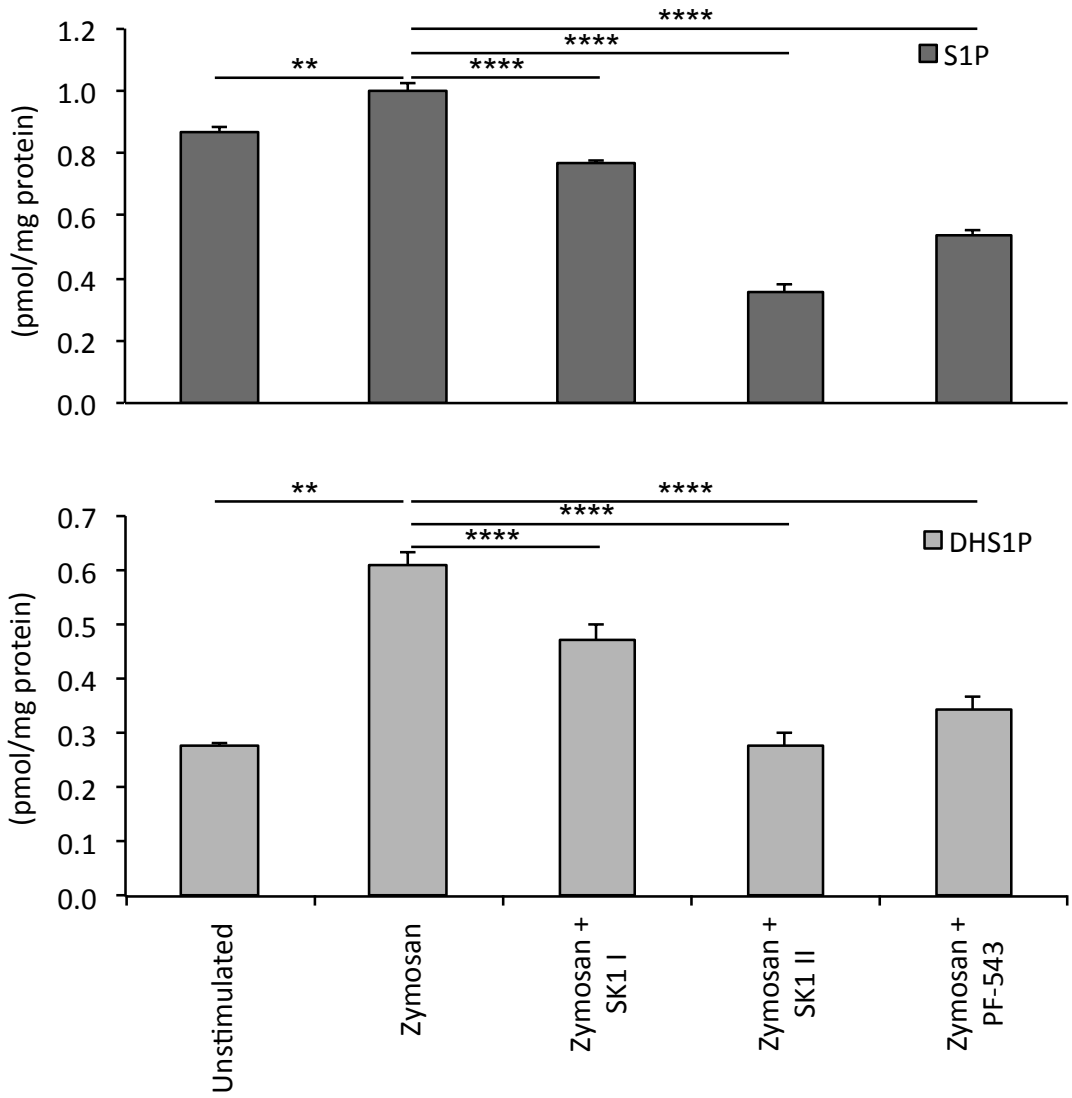

Figure 5.8: Zymosan induces DHS1P accumulation
BMDMs were pre-incubated with 10 μM SK1 I, 10 μM SK1 II and 5 μM PF-543 for 1 hour. BMDMs were then stimulated with 200 μg/ml Zymosan for 15 minutes. Cells were lysed and the lysate quantified. Spingholipids were extracted and then analysed by LC-MS/MS - (J Allegood, Virginia Commonwealth University). n=3, error bars represent the standard deviation

5.4 SphK inhibitors reduce IL-10 transcription and secretion

In order to understand what effects SphK1 might have in Zymosan-induced cytokine production, wildtype macrophages were pre-treated with SK1 I for 1 hour prior to treatment with Zymosan and IL-10 mRNA measured by qPCR. Zymosan treatment of macrophages led to a strong induction of IL-10 that peaked at 8 hours and returned to basal by 24 hours (figure 5.9). Treatment of macrophages with SK1 I reduced IL-10 mRNA levels across the 24 hour timecourse compared to Zymosan stimulation alone. Culture supernatant was also collected and IL-10 secretion measured to determine if changes in mRNA levels seen

with SK1 I treatment translated to changes in protein level. Zymosan stimulation led to IL-10 secretion at 8 and 16 hours and this was reduced in macrophages pre-treated with SK1 I (figure 5.9). This suggests that SphK1 activity is required for maximal production of IL-10 from Zymosan-stimulated macrophages.

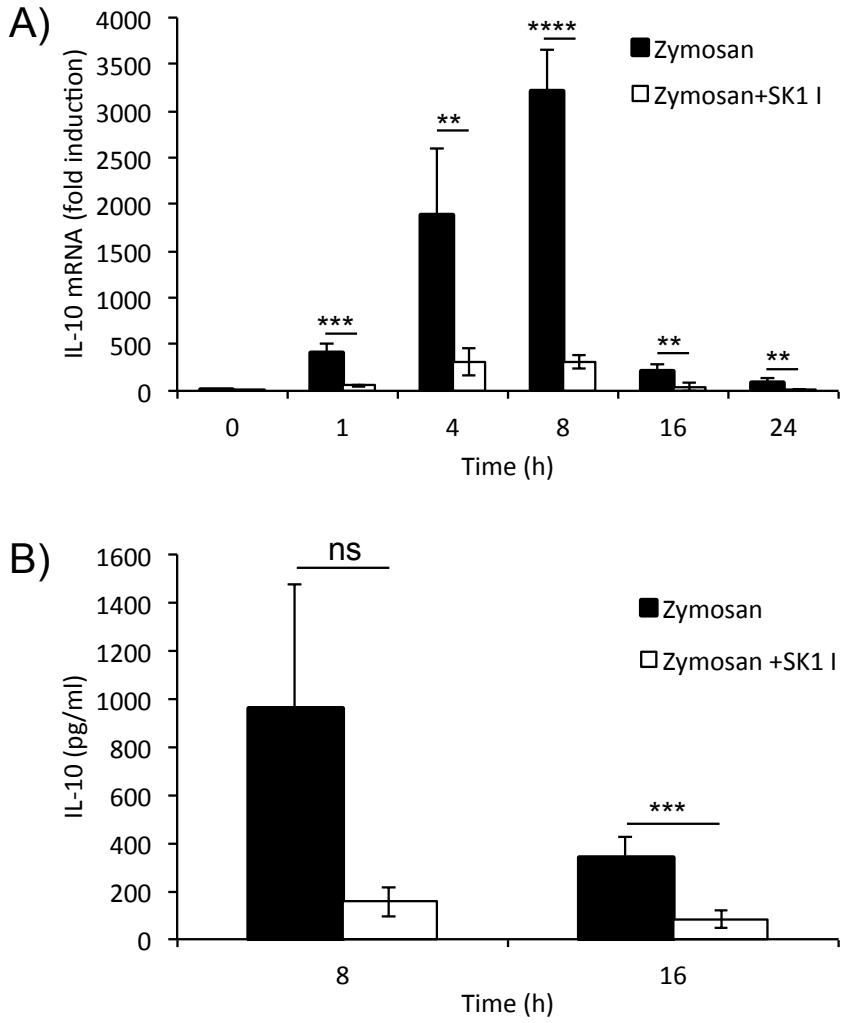

Figure 5.9: Inhibition of SphK results in reduced IL-10 mRNA and secretion
Bone marrow-derived macrophages from WT mice were pretreated with 10 μM SK1 I for 1 hour before stimulation with 200 μg/ml Zymosan for the times indicated. A) Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-10 mRNA mRNA. B) Culture supernatant was collected and IL-10 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

Each SphK inhibitor is structurally distinct (see figure 5.6) and therefore SK1 II and PF-543 were used to confirm the findings seen with SK1 I in figure 5.9. BMDMs were pretreated with SK1 I, SK1 II and PF-543 for 1 hour before stimulation with Zymosan for 8 hours. The culture supernatant was collected and IL-10 secretion assayed by Bioplex.

Zymosan stimulation of wild-type macrophages for 8 hours led to the secretion of around 1400 pg/ml of IL-10 similar to that seen in previous experiments. Pre-treatment with SK1 I again strongly repressed secretion of IL-10 (figure 5.10). Treatment with the structurally distinct inhibitors, SK1 II and PF-543, also led to the marked reduction of IL-10 secretion from Zymosan-stimulated macrophages. This corroborates the effect of SK1 I and suggests that SphK1 does indeed play a role in maximal production of IL-10 downstream of Zymosan.

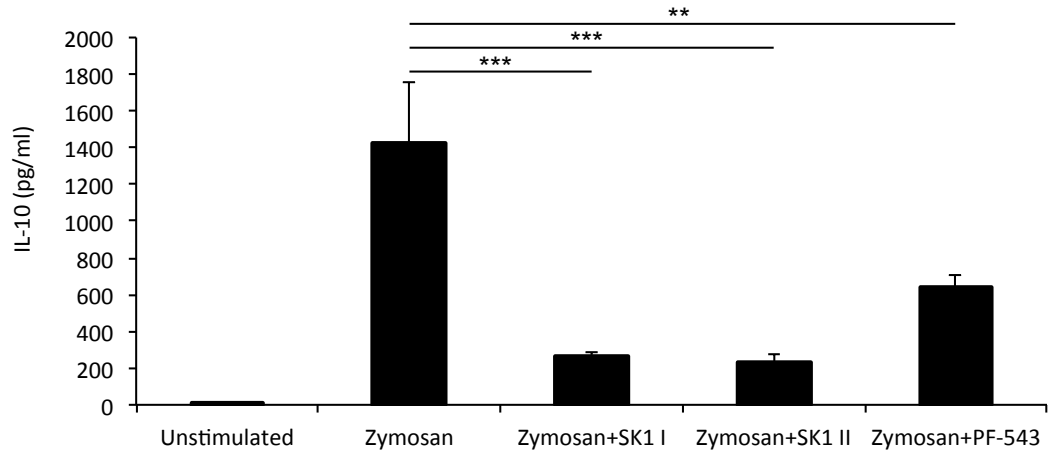

Figure 5.10: Structurally distinct SphK inhibitors reduce IL-10 secretion
Bone marrow-derived macrophages from WT mice were pretreated with SK1 I (10μM), SK1 II (10 μM) or PF-543 (5 μM) for 1 hour before stimulation with 200 μg/ml Zymosan for 8 hours. Culture supernatant was collected and IL-10 secretion measured by Bioplex (Bio-Rad) according to the manufacturers protocol. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

5.5 SphK inhibitors reduce Akt and STAT3 phosphorylation

Treatment of macrophages with SphK inhibitors leads to reduced IL-10 mRNA levels and secretion. Both ERK1/2 and p38 regulate IL-10 transcription and therefore perturbations in ERK or p38 activation may result in decreased IL-10 mRNA levels. In order to understand if this reduction in IL-10 was due to deregulated signaling downstream of Zymosan, macrophages were pretreated with SK1 I before stimulation with Zymosan. Cells were then lysed and signaling events analysed by western blotting. Zymosan treatment led to phosphorylation of the MAPKs, ERK1/2 and p38 after 30 minutes (figure 5.11). It also led to phosphorylation of Akt at Ser473 30 minutes after stimulation. By two hours, Zymosan treatment induced phosphorylation of STAT3 at Tyr705 which acts as a readout for

IL-10 production. Treatment of macrophages with 10 μM SK1 I before stimulation with Zymosan led to similar phosphorylation of ERK1/2 and p38. Phosphorylation of Akt at S473 was reduced for the duration of the timecourse. In line with the changes seen in IL-10 secretion, phosphorylation of Y705 of STAT3 was reduced in macrophages pre-treated with SK1 I.

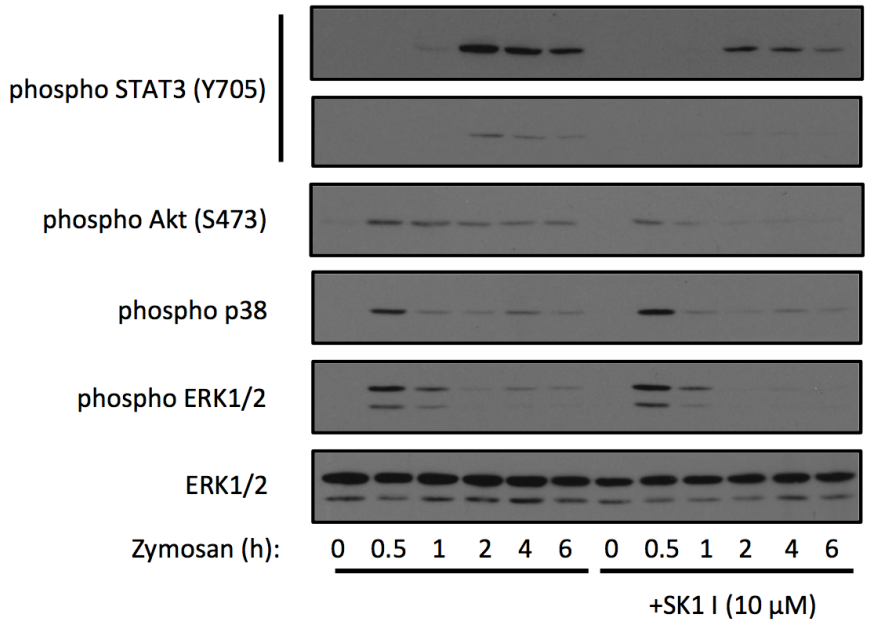

Figure 5.11: SK1 I reduces Akt and STAT3 phosphorylation in response to Zymosan
BMDMs were generated from WT bone marrow and pretreated with 10 μM SK1 I where indicated. Cells were stimulated with 200 μg/ml Zymosan for up to 6 hours. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho STAT3 (Y705), phospho Akt (S473), phospho p38, phospho ERK1/2, ERK1/2. Results are representative of two independent experiments.

The reduction in phosphorylation of Akt seen with SK1 I pre-treatment may be a result of blocking S1P signaling or an off-target effect of the inhibitor. To validate the changes in phosphorylation seen with SK1 I, the two structurally distinct SphK inhibitors, SK1 II and PF-543 were also used to block SphK activity in Zymosan-stimulated macrophages. BMDMs were stimulated with Zymosan alone for a 6 hour timecourse or pretreated with SK1 II for 1 hour prior to Zymosan treatment. Treatment of macrophages with SK1 II reduced the initial induction of phosphorylation of Akt at both T308 and S473 and the phosphorylation at these sites was not sustained throughout the timecourse (figure 5.12). Treatment with SK1 II also blocked phosphorylation of STAT3 at Y705. Treatment with SK1 II reduced the sustained phosphorylation of ERK1/2 and p38 in response to Zymosan. The loss of signaling of events seen with SK1 II correlate with those seen with

SK1 I and furthermore loss of STAT3 phosphorylation corroborates the reductions seen in IL-10 secretion associated with SK1 II pre-treatment (figure 5.10).

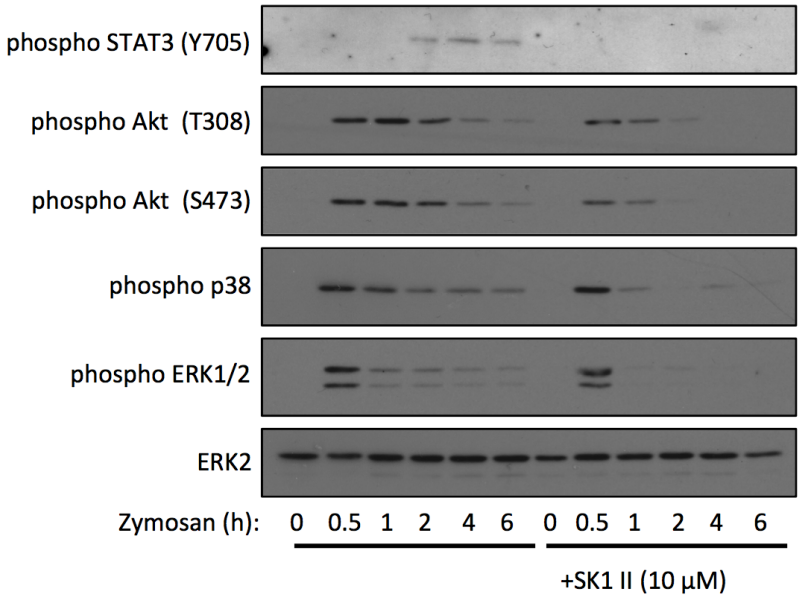

Figure 5.12: SK1 II blocks Akt and STAT3 phosphorylation in response to Zymosan
BMDMs were generated from WT bone marrow and pretreated with 10 µM SK1 II where indicated. Cells were stimulated with 200 µg/ml Zymosan for up to 6 hours. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho STAT3 (Y705), phospho Akt (S473), phospho Akt (T308), phospho p38, phospho ERK1/2 and ERK1/2. Results are representative of two independent experiments.

A third SphK1 inhibitor, PF-543, was also used to determine if the changes to signaling observed with SK1 I and SK1 II were consistent. Pre-treatment with PF-543 caused reduced phosphorylation of Akt at 30 minutes at both T308 and S473 (figure 5.13). Phosphorylation of STAT3 at Y705 was also reduced from 2 to 6 hours. Treatment with PF-543 reduced the sustained phosphorylation of ERK1/2 and p38 in response to Zymosan. The effects of PF-543 were comparable to those of SK1 I and SK1 II. Along with the IL-10 secretion data, the consistent reduction of Akt and STAT3 phosphorylation caused by three structurally distinct SphK1 inhibitors suggests that SphK1 activity is important in sustaining Akt activity and for maximal IL-10 secretion.

5.6 SphK inhibitors block LPS-stimulated STAT3 phosphorylation

Treatment of macrophages with SphK inhibitors leads to acute effects on cytokine production prior to the induction of SphK1 mRNA (see figure 5.9 and figure 5.5). This suggests that SphK1 may be expressed at a protein level in unstimulated macrophages and may

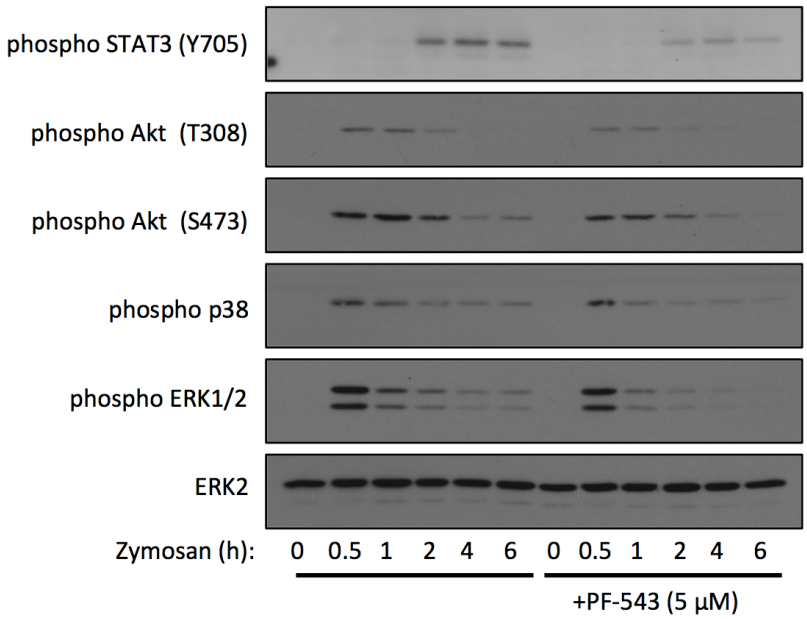

Figure 5.13: PF-543 reduces Akt and STAT3 phosphorylation in response to Zymosan
BMDMs were generated from WT bone marrow and pretreated with 5 µM PF-543 where indicated. Cells were stimulated with 200 µg/ml Zymosan for up to 6 hours. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho STAT3 (Y705), phospho Akt (S473), phospho Akt (T308), phospho p38, phospho ERK1/2 and ERK1/2. Results are representative of two independent experiments.

play a role downstream of other PRRs. IL-10 secretion is also induced downstream of TLR4. It would be interesting if SphK1 activity was also important in regulating IL-10 production in response to LPS, a TLR4 agonist. It was investigated whether SphK1 activity was important in LPS stimulated macrophages by examining phosphorylation of key signaling molecules. Macrophages were treated with LPS alone for 1 or 8 hours or pre-treated with SphK1 inhibitors prior to LPS stimulation. LPS stimulation leads to activation of ERK1/2 and p38 at 1 hour with some weak phosphorylation still detectable at 8 hours (figure 5.14). LPS also induced the degradation of IκB at 1 hour which indicates activation of NFκB. IκB was detectable again at 8 hours. Akt was phosphorylated weakly at 1 hour and showed stronger phosphorylation after 8 hours of LPS treatment. STAT3 was also strongly phosphorylated at 8 hours of LPS treatment. Treatment with SphK inhibitors strongly reduced Akt and STAT3 phosphorylation at 8 hours. Initial activation of ERK1/2 and p38 was unaffected. Treatment of macrophages with SphK inhibitors did not affect the initial degradation of IκB at 1 hour, however, at 8 hours IκB was undetectable suggesting a failure to reexpress IκB or continued degradation of IκB.

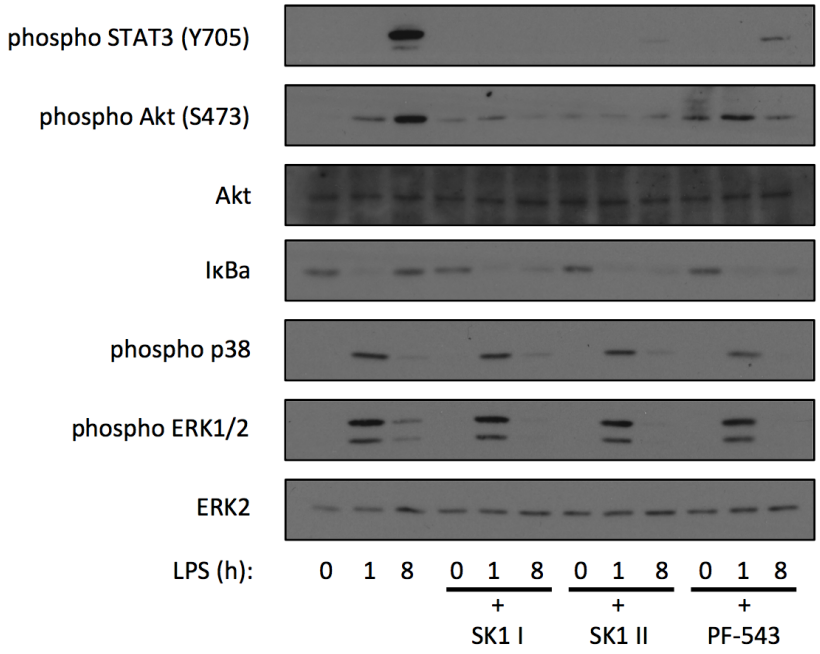

Figure 5.14: SphK1 inhibitors block LPS stimulated phosphorylation of Akt and STAT3
BMDMs were generated from WT bone marrow and pretreated with 10 μM SK1 I, 10 μM SK1 II or 5 μM PF-543 where indicated. Cells were stimulated with 100 ng/ml LPS for 1 or 8 hours. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho STAT3 (Y705), phospho Akt (S473), Akt, IκBα, phospho p38, phospho ERK1/2, ERK1/2. Results are representative of two independent experiments.

5.7 Exogenous DHS1P does not rescue the effects of SK1 I

SphK inhibitors block the phosphorylation of sphingosine resulting in a decrease in available DHS1P (figure 5.8). DHS1P can signal extracellularly via five GPCRs ($S1P_{1\text{-}5}$) or intracellularly. The effect of SphK inhibitors on STAT3 phosphorylation should therefore be overcome by the introduction of exogenous DHS1P. Initially, the concentration of DHS1P required to stimulate cells was determined through titration of DHS1P. DHS1P stimulation induced phosphorylation of ERK1/2 after 15 minutes. 10 μM of DHS1P induced similar levels of ERK activation to that seen with EGF stimulation.

10 μM of DHS1P induced strong phosphorylation of ERK1/2 and was therefore selected as an appropriate concentration to stimulate BMDMs. BMDMs were stimulated with DHS1P alone which failed to induce STAT3 phosphorylation at Tyr705. Zymosan stimulation of BMDMs led to a strong induction of phosphorylation at Tyr705 of STAT3. Pretreatment with SK1 I blocked the Zymosan-induced phosphorylation of STAT3 at Tyr705. Interestingly, co-stimulation of macrophages with DHS1P and Zymosan lead to stronger phosphorylation of STAT3 at Tyr705 compared to Zymosan stimulation alone. However,

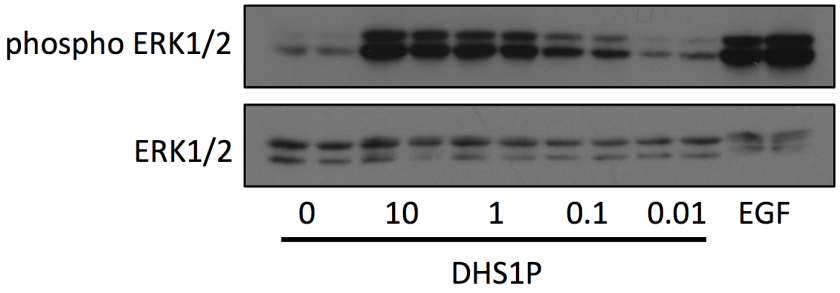

Figure 5.15: DHS1P titration in HeLa cells
Cells were stimulated with indicated concentrations of DHS1P or 100 ng/ml EGF for 15 mins. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho ERK1/2 and ERK1/2. Results are representative of three independent experiments.

pre-treatment of cells with SK1 I prevented phosphorylation of STAT3 by co-stimulation with DHS1P and Zymosan (figure 5.16).

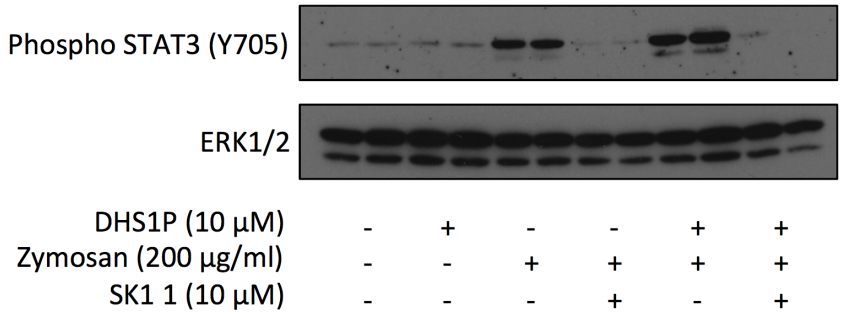

Figure 5.16: Exogenous DHS1P does not rescue STAT3 phosphorylation in SK1 I pre-treated macrophages
BMDMs were pre-treated with 10 µM SK1 I where indicated. Cells were stimulated with 10 µM DHS1P, 200 µg/ml Zymosan or a combination of both for 4 hours where indicated. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho STAT3 (Y705) and ERK1/2. Results are representative of two independent experiments.

Whilst Zymosan stimulation did not lead to accumulation of S1P, treatment with SphK inhibitors did reduce levels of S1P in macrophages compared to basal levels. To investigate if changes in S1P levels were responsible for the effects seen on STAT3 phosphorylation in macrophages, exogenous S1P was added to Zymosan-stimulated macrophages in the presence or absence of PF-543. Treatment with PF-543 reduced the Zymosan-induced phosphorylation at Tyr705 on STAT3, at both phosphorylation sites on Akt and on ERK1/2. Costimulation of PF-543 pre-treated macrophages with S1P and Zymosan did not rescue the reduction in phosphorylation nor did costimulation of macrophages with S1P and Zymosan lead to greater phosphorylation of STAT3 or Akt compared to

Zymosan alone (figure 5.17). Pre-treatment of macrophages with THI (a S1P lyase inhibitor, which should elevate S1P levels) did not increase Zymosan-induced STAT3 or Akt phosphorylation compared to Zymosan alone.

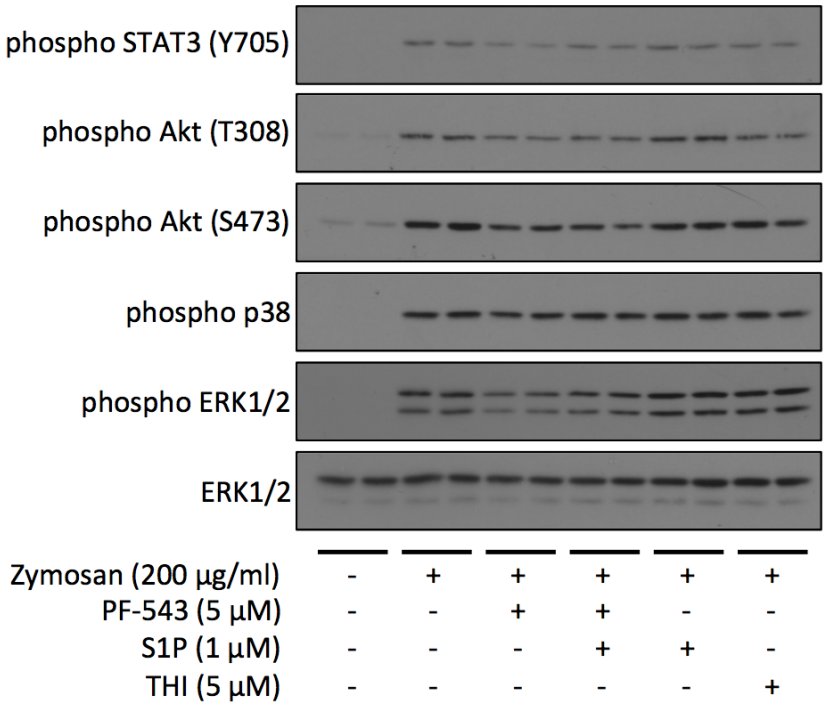

Figure 5.17: S1P addition does not rescue STAT3 phosphorylation in PF-543-treated macrophages BMDMs were pre-treated with 5 µM PF-543 or 5 µM THI where indicated for 1 hour. Cells were stimulated with 1 µM S1P, 200 µg/ml Zymosan or a combination of both for 4 hours where indicated. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to phospho STAT3 (Y705), phospho Akt (T308), phospho Akt (S473), phospho p38 and phospho ERK1/2 and ERK1/2. Results are representative of two independent experiments.

5.8 Discussion

S1P signaling is important in many processes and in particular is required for appropriate immune cell trafficking. The role of S1P, DHS1P and the sphingosine kinases 1 and 2 in regulating immune cell function is less well understood. Macrophages are part of the innate immune response and are involved in the resolution of inflammation. Macrophages can become polarised into different phenotypes of which the regulatory phenotype is characterised by high levels of IL-10 production as well as expression of several markers including SphK1. Zymosan-stimulated macrophages share several features with regulatory macrophages including the high levels of IL-10 and expression of SphK1. The aim of this work was to investigate a role for SphK1 in Zymosan-stimulated macrophages.

The results presented in this chapter show that SphK1 expression is strongly up-regulated in response to treatment with Zymosan after 8 hours and that LPS stimulation of macrophages does not induce SphK1 expression. Interestingly, Zymosan treatment of macrophages leads to an acute accumulation of DHS1P not S1P. This increase in DHS1P levels is blocked by pre-treatment with each of three structurally distinct SphK inhibitors. Pre-treatment of Zymosan-stimulated macrophages also affected the transcription and secretion of the anti-inflammatory cytokine, IL-10. SphK inhibitors block the phosphorylation of STAT3 at Tyr705, which is in line with decreased IL-10 secretion. Phosphorylation of Akt at Thr308 and Ser473 is also reduced in SphK inhibitor-treated macrophages. In addition to blocking Zymosan-induced phosphorylation of STAT3 and Akt, SphK inhibitor treatment reduced STAT3 and Akt phosphorylation in LPS-stimulated BMDMs. DHS1P and S1P can signal via five plasma membrane-located GPCRs or via intracellular mechanisms. Macrophages were treated with Zymosan and DHS1P or Zymosan and S1P in the presence of a SphK inhibitor and neither DHS1P or S1P were able to rescue Zymosan-induced phosphorylation of STAT3 in the presence of the SphK inhibitor.

5.8.1 Zymosan-induced expression and activation of SphK1

Zymosan stimulation of macrophages led to a strong induction of SphK1 mRNA expression by 8 hours. LPS stimulation does not result in a similar induction of SphK1 expression. The reasons behind the induction of SphK1 expression in response to Zymosan signaling are unclear. It would be beneficial to measure the protein level of SphK at basal conditions and following TLR4 and Dectin-1 stimulation as it would demonstrate whether increased mRNA levels seen with Zymosan stimulation translated to an increased pool of SphK1 which would be available to generate to DHS1P and S1P. However, there must be a sufficient pool of SphK1 or SphK2 at basal conditions which allows for the rapid production of DHS1P following 15 minutes of Zymosan stimulation (figure 5.8). A SphK1 antibody was tried, but failed to detect SphK1 in Zymosan-stimulated Raw264.7 cells. Interestingly, intraplantar injection of Zymosan into wild type mice caused a decrease in S1P levels at 1 hour and S1P levels were reduced by about 60% at 6 hours, although levels increased back to basal at 48 hours (Linke et al., 2012). Unfortunately, measurements

of DHS1P were not conducted in this study. In order to fully understand the importance of S1P/DHS1P signaling in macrophages, the kinetics of sphingolipid levels need to be measured over a timecourse following Zymosan stimulation. It has been reported that SphK1 is activated via phosphorylation of Ser225 by ERK1/2 (Pitson et al., 2003), the use of small molecule inhibitors could confirm this occurs in macrophage downstream of Dectin-1 activation. Both Dectin-1 and TLR4 signaling activates ERK1/2. It would also be interesting to see if TLR signaling also induced the accumulation of DHS1P and whether the activation of SphK in macrophages is dependent on ERK1/2 activity.

5.8.2 SphK inhibitors block Zymosan-induced accumulation of DHS1P

In this study, three structurally distinct SphK inhibitors were used to determine the role of SphK in Zymosan-stimulated macrophages. Each inhibitor was relatively selective against the 120 or more protein kinases screened, however, more extensive screening would be required to rule out any off-target protein kinases. Furthermore, each inhibitor blocked the Zymosan-induced accumulation of DHS1P in macrophages. Treatment with these SphK inhibitors also reduced levels of S1P in macrophages despite its production not being induced by Zymosan. This suggests a turnover rate of S1P that is sensitive to the inhibition of SphK for 1 hour.

5.8.3 Effects of SphK inhibitors on signaling and cytokine production

Zymosan-induced cytokine production was affected by pre-treatment of macrophages with the SphK inhibitors. The results in this chapter show decreased mRNA and protein levels of IL-10. In addition, signaling downstream of Zymosan was affected by the SphK inhibitors. Phosphorylation of Akt at Thr308 and Ser473 was reduced in the presence of the inhibitors. Also, STAT3 phosphorylation at Tyr705 was reduced. The reduced levels of IL-10 could explain the reduced STAT3 phosphorylation as IL-10 leads to STAT3 phosphorylation via IL-10R-JAK. S1P and DHS1P can signal through five GPCRs or intracellularly. Akt activation has been linked to signaling from GPCRs and this may explain the reduced activation of Akt evident in SphK inhibitor-treated macrophages (Murga et al., 1998). However, if the effects seen on Akt activation and STAT3 phosphorylation were caused by decreased S1P/DHS1P levels then the addition of exogenous lipid would

be predicted to restore activation of Akt and STAT3 to levels seen in Zymosan-stimulated macrophages. Addition of DHS1P did not restore STAT3 phosphorylation in Zymosan-stimulated macrophages in the presence of SK1 I nor did S1P restore Akt or STAT3 phosphorylation in Zymosan-stimulated macrophages in the presence of PF-543. This would suggest that the reduction in IL-10 transcription is the result of off-target effects of the SphK inhibitors. Another explanation is that the addition of exogenous S1P/DHS1P may not restore changes caused by reductions in intracellular sphingolipid levels as it may only act on the five GPCRs located at the plasma membrane.

In order to validate the off-target effects of the SphK inhibitors, macrophages need to be isolated from mice lacking SphK1 or SphK2. SphK1- or SphK2-deficient macrophages would then be stimulated with Zymosan in the presence or absence of the SphK inhibitors and cytokine production and signaling would be assessed. It would be predicted that the inhibitors would still reduce STAT3 and Akt phosphorylation in SphK1/2-deficient cells as seen in wildtype cells, although redundancy between SphK1 and SphK2 may complicate the understanding of results obtained from single knockout macrophages. In support of this, deletion of SphK1 and SphK2 from macrophages did not alter macrophage responses or the *in vivo* response to LPS injection. Furthermore, IL-10 serum levels following LPS injection were comparable between SphK1/2-deficient mice and control mice (Xiong et al., 2013).

Interestingly, SphK inhibitors had similar effects on both LPS- and Zymosan-induced signaling and cytokine production. LPS and Zymosan activate similar pathways and drive transcription of many of the same cytokines and this suggests the SphK inhibitors inhibit a shared component of LPS and Zymosan signaling.

5.8.4 Importance of SphK1 in the regulatory macrophage phenotype

Regulatory macrophages are characterised by high levels of IL-10 production and the expression of certain markers including SphK1. Interestingly, in IL-10 knockout macrophages, SphK1 induction in response to Zymosan is reduced compared to wildtype macrophages and LIGHT expression is similarly reduced (Elcombe et al., 2013). This suggests that the

expression of IL-10 is required for maximal SphK1 expression. Notably, IL-10 knockout macrophages fail to induce expression of SphK1 and LIGHT to similar levels as seen in wildtype cells in response to LPS and PGE_2 (MacKenzie et al., 2013a), suggesting the importance of IL-10 in generating the regulatory phenotype. The importance of SphK1 in the induction or maintenance of the regulatory macrophage phenotype is unknown. It would be interesting to determine if SphK1-deficient mice were able to generate regulatory macrophages as recognised by high levels of IL-10 and expression of LIGHT.

Other approaches could be used to further explore the role of SphK and S1P/DHS1P in macrophage function. S1P/DHS1P is degraded by several enzymes including the sphingosine 1-phosphate lyase. Inhibitors are available that block the action of the S1P lyase. Primarily, it would be beneficial to see if these inhibitors increased intracellular concentrations of S1P and DHS1P in unstimulated and Zymosan-stimulated macrophages. Once their effectiveness was established, then their effects on cytokine production and signaling could be investigated. Another approach would be to characterise macrophage responses from SphK1- and SphK2-deficient mice, however the complications of redundancy between the two isoforms may complicate the interpretation of the results. Both single knockouts are viable but the double knockout is embryonic lethal (Mizugishi et al., 2005; Allende et al., 2004). Recently, it was shown that conditional knockout of SphK1 and SphK2 from macrophages did not alter the inflammatory response following LPS stimulation suggesting SphK1 is not important in cytokine production in BMDMs (Xiong et al., 2013).

SphK1 expression may not be intrinsically important to regulatory macrophage function but may be important for controlling aspects of the immune response. The importance of S1P in regulating immune cell function and trafficking is well established (Chi and Flavell, 2005; Walzer et al., 2007; Niessen et al., 2008). Therefore SphK1 expression may be important in regulatory macrophages by promoting recruitment of certain immune cells or modulating the response of these cells. In particular, S1P is important for dendritic cell and T cell localisation as well as being linked to NK cell function (Chi and Flavell, 2005; König et al., 2010; Pappu et al., 2007; Walzer et al., 2007). SphK function is also

associated with appropriate mast cell function (Olivera et al., 2007; Oskeritzian et al., 2008; Jolly et al., 2004). S1P is also important in regulating monocyte adhesion and recruitment and therefore may be important in regulating macrophage migration (Bolick et al., 2005; Keul et al., 2011; Maceyka et al., 2008). The complex range of processes that can be influenced by the SphK/S1P axis may therefore be partially regulated by SphK1 expression in macrophages. Macrophage-specific deletion of SphK1 would allow the dissection of the importance of the cellular source of S1P and DHS1P in determining the immune response.

5.8.5 Control of SphK1 expression

Zymosan induces expression of Sphk1 by activating signaling downstream of Dectin-1 and TLRs. Dectin-1 signaling leads to the activation of NF$\varkappa$B and MAPKs, however LPS signaling activates similar pathways and yet fails to induce SphK1 expression to the same degree. Expression of SphK1 in response to Zymosan has been shown to be blocked by a Syk inhibitor (Elcombe et al., 2013). The transcription factor Sp1 has been shown to be important for SphK1 expression in response to nerve growth factor and was shown to bind to the promoter region (Sobue et al., 2005). It has also been shown that PGE_2 in combination with LPS enhances SphK1 expression in a PKA-dependent manner (MacKenzie et al., 2013a). It would be interesting to investigate the signaling pathways and transcription factors that control expression of SphK1 in response to Zymosan.

5.8.6 Conclusion

S1P signaling has been implicated in regulating many aspect of immune cells. The results in this chapter demonstrate that Zymosan strongly induces SphK1 expression and furthermore activates SphK in macrophages. Zymosan stimulation of macrophages led to the accumulation of DHS1P, which was blocked by three structurally distinct SphK inhibitors. SphK1 is a marker of regulatory macrophages which are also characterised by high levels of IL-10 production.

Zymosan stimulated macrophages produce high amounts of IL-10 and pre-treatment of macrophages with SphK inhibitors reduced transcription of IL-10 in response to Zy-

mosan. The reduced transcription of IL-10 translated to lower levels of IL-10 protein. In line with this, phosphorylation of STAT3 at Tyr705 which is mediated by IL-10R-JAK was decreased in Zymosan-stimulated macrophages pre-treated with SphK inhibitors. Inhibitor pre-treatment also reduced phosphorylation of Akt at two sites, Thr308 and Ser473. Similar reductions in STAT3 and Akt phosphorylation were also evident in LPS-stimulated macrophages pre-treated with SphK inhibitors. Addition of exogenous DHS1P or S1P failed to restore phosphorylation of Akt or STAT3 to levels seen in Zymosan-stimulated macrophages in the presence of a SphK inhibitor. This would suggest that the SphK inhibitors target a shared component of TLR4 and Dectin-1 pathways which is important for driving transcription of IL-10 and the same or an additional target may also influence Akt activation. SphKs are activated in Zymosan-stimulated macrophages leading to the accumulation of DHS1P. The relevance of this accumulation or of the Zymosan-induced expression of SphK1 is unclear.

Conclusions and future perspectives

The results presented in this thesis describe several mechanisms that regulate the transcription of IL-10 in macrophages. The importance of IFNβ signaling in sustaining transcription of IL-10 following LPS stimulation was demonstrated. The JAK inhibitor, Ruxolitinib, prevented maximal induction of IL-10, as did deletion of the type I IFN receptor. Treatment with Ruxolitnib also increased pro-inflammatory cytokine production from macrophages due to blocking the signaling of IL-10. Tofacitinib, another JAK inhibitor, was shown to have a similar effect on cytokine production although a higher concentration was required to block IL-10 signaling than that required for IFNβ signaling. Both Ruxolitinib treatment and deletion of the type I IFN receptor resulted in a loss of phosphorylated STAT1 in response to LPS. The direct binding of STAT1 to the IL-10 promoter was not shown and therefore it is unclear whether STAT1 directly regulates IL-10 or whether an intermediate is required to drive IL-10 transcription downstream of IFNβ signaling.

MCP-1 was also identified as requiring IFNβ signaling for maximal transcription. MCP-1 transcription was directly stimulated by IFNβ and the binding of STAT1 to a STAT binding site within the MCP-1 promoter was demonstrated in response to IFNβ. MCP-1 is an important chemokine and is required for appropriate responses to viral infections. Unfortunately, due to the changes in cytokine production caused by Ruxolitinib treatment or deletion of the type I IFN receptor, it would not have been possible to demonstrate that the reduced MCP-1 affected the immune response to viral or bacterial infection.

This thesis also describes the role of MEF2D in regulating IL-10 transcription in macrophages. MEF2D was phosphorylated in response to LPS at Ser121. The relevance of this phosphorylation event was not determined, however a specific phospho-antibody to this site

was characterised. The function of Ser121 phosphorylation will need to be investigated further. Phosphorylation of MEF2D at Ser121 may alter its DNA binding activity, cellular localisation or interactions with co-activators/co-repressors. Stable expression of MEF2D protein with the phosphorylation site mutated may help to determine the function of this phosphorylation event. MEF2D negatively influences IL-10 transcription in response to TLR agonists. Deletion of MEF2D increases IL-10 production and results in decreased pro-inflammatory cytokine secretion. The mechanism through which MEF2D negatively regulates IL-10 was not determined in this study. However, MEF2D KO macrophages had elevated primary transcript levels of IL-10 suggesting that MEF2D affects transcription of IL-10 rather than IL-10 mRNA stability. Analysis of MEF2D binding to the IL-10 promoter using chromatin immunoprecipitation may demonstrate whether this is a direct effect of MEF2D at the IL-10 promoter or via an intermediate.

The control of IL-10 transcription is important due to its potent anti-inflammatory effects. Loss of IL-10 production leads to the development of colitis (Kühn et al., 1993), therefore understanding pathways that regulate IL-10 production is important in a clinical context. Understanding the pathways that regulate MEF2D activity may provide a novel approach to influence IL-10 levels. In addition to its role in regulating IL-10 production from macrophages, it would be important to determine if MEF2D regulates IL-10 production in other cell types such as B10 cells and Tr1 cells, which produce high levels of IL-10.

MEF2D also influences A20 mRNA levels and this regulation needs to be studied in greater detail. MEF2D may also bind to the A20 promoter which contains MEF2 consensus sequences and therefore chromatin immunoprecipitations may demonstrate the direct binding of MEF2D to the A20 promoter. A20 is a negative regulator of NFκB signaling and is important in the termination of TLR signaling (Heyninck and Beyaert, 1999; Verhelst et al., 2012; Boone et al., 2004). Interestingly, A20 deficiency has been linked to protection against influenza infection, however it has also been linked to the development of polyarthritis (Maelfait et al., 2012; Matmati et al., 2011). The increased levels of A20 protein seen in MEF2D-deficient macrophages may therefore affect immune responses to viral infection. The generation of MEF2D and IL-10 double knockout mice would allow

the investigation of changes to A20 levels without the complicating factors of increased IL-10 and decreased TNFα, IL-6 and IL-12 from macrophages.

The role of SphK1 in macrophages was investigated in the final part of this thesis. Zymosan strongly induces the expression of SphK1, a marker of regulatory macrophages. Zymosan stimulation activates SphK leading to the accumulation of DHS1P. Three structurally distinct inhibitors blocked the accumulation of DHS1P in response to Zymosan as well as reducing S1P levels. The effect of these inhibitors on macrophage cytokine production and signaling was then examined. Each inhibitor reduced production of IL-10 and reduced phosphorylation of Akt at Thr308 and Ser473 and STAT3 at Tyr705 in response to Zymosan. The addition of exogenous DHS1P or S1P did not restore the phosphorylation of Akt or STAT3 in response to Zymosan in the presence of a SphK inhibitor. The SphK inhibitors may therefore have off-target effects within macrophages that reduce IL-10 production and reduce Akt activation.

The functional relevance of SphK1 expression in macrophages was not established. Zymosan stimulation of SphK1- and SphK2-deficient macrophages would be necessary to confirm that the effects of the inhibitors seen on IL-10 production and Akt activation were not a result of SphK inhibition. Interestingly, LPS-stimulated macrophages were similary sensitive to the SphK inhibitors despite not leading to induction of SphK1 expression. However SphK1 is known to be activated by ERK1/2 which is activated by both Zymosan and LPS stimulation. SphK1 expression in macrophages may not regulate macrophage function but may influence the function or recruitment of immune cells in a paracine manner.

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Appendices

Appendix A- Kinase selectivity screens for Ruxolitinib and Tofacitinib

The percentage *in vitro* kinase activity remaining relative to no inhibitor controls was determined using *in vitro* kinase assays as described in the methods. Data represents the average and range of duplicate measurements for each condition. n.d. = not determined

	0.1 μM Ruxolitinib		1 μM Ruxolitinib		0.1 μM Tofacitinib		1 μM Tofacitinib	
kinase	% activity	range	% activity	range	% activity	range	% activity	range
ABL	100	5	101	2	103	13	116	10
AMPK	107	6	89	10	83	1	95	5
ASK1	107	3	77	8	105	7	130	11
Aurora A	86	18	26	0	93	8	119	16
Aurora B	112	10	95	7	69	6	92	8
BRK	101	4	84	11	80	18	92	7
BRSK1	92	6	78	17	73	2	92	7
BRSK2	93	3	92	3	82	5	101	3
BTK	76	12	58	2	96	6	94	11
CAMK1	91	10	58	0	93	1	111	2
CAMKKb	100	17	104	2	139	15	116	10
CDK2	96	11	57	6	69	4	116	37
CHK1	99	0	106	7	86	13	88	1
CHK2	97	10	65	14	106	7	95	9
CK1	94	11	74	6	105	4	109	9
CK2	90	25	77	6	92	1	97	7
CLK2	73	2	21	2	106	10	108	1
CSK	91	23	63	6	84	7	78	3
DAPK1	88	39	61	64	145	5	123	11
DYRK1A	101	3	67	1	102	1	92	7
DYRK2	95	17	86	12	99	14	103	5
DYRK3	103	17	107	3	110	18	116	2
EF2K	82	15	83	12	103	1	100	2
EIF2AK3	89	2	94	2	114	8	97	9
EPH-A2	98	29	62	1	106	6	114	19
EPH-A4	85	4	58	5	121	2	109	3
EPH-B1	71	2	48	4	93	4	93	4
EPH-B2	132	38	79	7	91	10	121	12
EPH-B3	96	9	86	9	103	8	85	5
EPH-B4	78	9	59	0	131	46	120	4
ERK1	101	16	92	17	108	5	97	4
ERK2	115	15	102	3	99	17	97	10
ERK8	86	29	47	4	121	1	121	6
FGF-R1	79	9	34	4	42	4	85	3
GCK	86	9	44	2	87	0	109	7
GSK3β	100	8	62	4	110	2	108	9
HER4	106	6	90	5	116	8	121	19
HIPK1	102	4	95	6	90	4	112	4
HIPK2	94	3	69	2	128	21	117	6
HIPK3	116	3	114	7	109	14	108	3
IGF-1R	94	11	100	5	109	9	104	11
IKKβ	101	18	76	15	126	3	105	2
IKKε	105	33	46	5	90	7	87	8
IR	95	9	89	1	87	8	105	9
IRAK1	63	12	19	4	111	4	106	3
IRAK4	89	27	97	7	80	1	87	0

kinase	0.1 μM Ruxolitinib		1 μM Ruxolitinib		0.1 μM Tofacitinib		1 μM Tofacitinib	
	% activity	range	% activity	range	% activity	range	% activity	range
IRR	97	22	65	22	102	3	103	5
JAK2	3	1	2	1	3	1	4	0
JNK1	84	26	79	15	81	5	80	3
JNK2	105	37	89	13	88	9	94	4
JNK3	101	27	94	1	107	10	119	12
Lck	95	1	69	8	30	0	73	9
LKB1	106	36	87	13	95	5	101	9
MK2	102	13	102	9	91	11	82	7
MK3	89	1	101	2	118	7	91	4
MARK1	96	7	55	3	63	1	82	10
MARK2	97	6	42	7	85	4	98	1
MARK3	56	22	13	6	33	4	94	16
MARK4	94	20	88	1	86	1	93	2
MEKK1	89	31	89	6	83	80	97	7
MELK	81	11	42	7	74	5	115	12
MINK1	122	27	94	5	90	9	96	7
MKK1	108	10	73	6	67	6	105	6
MKK2	99	4	93	4	112	20	110	3
MKK6	95	5	101	8	89	2	97	8
MLK1	82	13	34	2	90	8	86	5
MLK3	65	20	27	5	75	1	83	12
MNK1	81	9	66	5	115	0	100	11
MNK2	88	11	39	1	64	10	97	2
MPSK1	104	4	101	5	83	14	94	10
MSK1	84	0	29	1	76	15	83	10
MST2	97	28	68	8	64	12	79	7
MST4	140	31	105	24	109	0	87	8
NEK2a	108	22	82	1	107	32	102	16
NEK6	84	10	83	22	90	5	87	3
NUAK1	89	24	26	1	30	2	74	5
OSR1	80	30	40	3	94	12	112	9
p38α MAPK	85	15	83	1	103	4	100	1
p38β MAPK	97	16	79	18	115	3	106	12
p38γ MAPK	67	22	66	3	108	17	101	7
p38δ MAPK	94	15	86	5	130	9	115	6
PAK2	88	24	84	9	114	2	99	2
PAK4	96	3	57	0	116	16	104	10
PAK5	94	15	72	4	94	4	86	7
PAK6	96	10	70	1	116	10	100	3
PDK1	101	37	66	8	123	3	107	14
PHK	60	3	15	0	85	11	96	2
PIM1	101	16	85	3	101	8	112	5
PIM2	87	5	90	12	121	21	102	5
PIM3	90	13	85	10	114	9	124	12
PKA	95	11	53	2	89	1	94	4
PKBα	93	24	77	15	125	4	115	1
PKBβ	86	15	87	4	92	6	85	9
PKCα	88	14	67	5	71	0	96	0
PKCζ	100	21	95	1	106	20	100	21
PKCγ	93	8	80	2	102	5	99	11
PKD1	101	6	67	16	106	27	92	4
PLK1	108	1	95	6	121	18	128	7
PRAK	83	16	84	3	123	7	113	10
PRK2	84	5	61	7	22	0	50	2
RIPK2	108	17	78	4	92	3	91	9
ROCK 2	60	9	59	59	46	9	95	15
RSK1	82	6	68	9	76	23	96	6
RSK2	92	4	81	3	86	2	91	3
S6K1	79	23	53	2	77	16	88	0

	0.1 μM Ruxolitinib		1 μM Ruxolitinib		0.1 μM Tofacitinib		1 μM Tofacitinib	
kinase	% activity	range	% activity	range	% activity	range	% activity	range
SGK1	100	13	75	10	106	19	92	2
SmMLCK	93	22	67	6	104	3	105	3
Src	67	14	62	4	90	7	92	5
SRPK1	85	13	67	4	89	8	84	6
STK33	108	1	84	7	103	19	98	4
SYK	96	18	83	30	152	16	131	7
TAK1	85	2	60	1	113	34	115	7
TAO1	104	1	85	15	103	4	104	12
TBK1	82	16	46	0	83	19	99	6
TIE2	98	4	78	3	106	19	99	1
TLK1	82	3	80	8	104	8	100	3
TrkA	32	1	18	3	87	11	106	13
TTK	95	7	44	2	115	7	99	3
VEG-FR	102	32	56	10	58	10	99	5
YES1	75	10	59	5	65	4	104	3
ZAP70	95	7	92	16	125	3	133	0

Appendix B- Kinase selectivity screens for sphingosine kinase inhibitors

The percentage *in vitro* kinase activity remaining relative to no inhibitor controls was determined using *in vitro* kinase assays as described in the methods. Data represents the average and standard deviation of duplicate measurements for each condition. n.d. = not determined

	10 μM SKI I		10 μM SKI II		10 μM PF-543	
protein kinase	% activity	st. dev.	% activity	st. dev.	% activity	st. dev.
ABL	109	10	75	5	94	3
AMPK	94	6	89	7	85	5
ASK1	94	0	83	1	94	5
Aurora A	92	15	59	5	100	2
Aurora B	93	0	89	4	100	6
BRK	104	2	73	3	116	14
BRSK1	97	1	73	11	112	4
BRSK2	108	7	80	4	90	7
BTK	103	15	80	12	112	4
CAMK1	89	5	22	1	99	11
CAMKKb	90	2	84	6	103	5
CDK2-Cyclin A	88	3	94	12	99	11
CDK9-Cyclin T1	n.d.	n.d.	n.d.	n.d.	93	2
CHK1	86	9	131	24	89	6
CHK2	82	8	50	11	95	5
CK1γ2	n.d.	n.d.	n.d.	n.d.	94	11
CK1δ	81	4	109	10	118	27
CK2	83	2	84	13	104	17
CLK2	97	11	75	0	86	6
CSK	98	5	75	11	116	1
DAPK1	103	18	89	16	93	10
DDR2	n.d.	n.d.	n.d.	n.d.	86	2
DYRK1A	84	6	54	11	79	4
DYRK2	119	7	101	0	94	23
DYRK3	93	9	82	2	88	6
EF2K	69	1	85	6	85	2
EIF2AK3	n.d.	n.d.	n.d.	n.d.	90	4
EPH-A2	91	6	80	6	95	12
EPH-A4	96	7	59	2	88	7
EPH-B1	115	18	86	14	96	15
EPH-B2	108	5	92	5	102	9
EPH-B3	86	0	117	4	103	6
EPH-B4	123	13	81	8	92	7
ERK1	85	4	86	23	94	15
ERK2	100	5	97	1	99	15
ERK5	n.d.	n.d.	n.d.	n.d.	105	4
ERK8	77	0	59	1	81	14
FGF-R1	104	1	84	2	119	34
GCK	71	9	38	8	94	2
GSK3b	71	2	101	4	86	1
HER4	105	6	45	13	84	12
HIPK1	94	18	90	16	101	1
HIPK2	106	5	62	2	85	1
HIPK3	102	3	86	20	100	5
IGF-1R	93	14	89	10	64	11
IKKb	100	6	94	0	87	10
IKKe	86	4	84	3	91	5
IR	99	2	88	18	101	29

	10 μM SKI I		10 μM SKI II		10 μM PF-543	
protein kinase	% activity	st. dev.	% activity	st. dev.	% activity	st. dev.
IRAK1	n.d.	n.d.	n.d.	n.d.	92	3
IRAK4	67	1	86	3	105	3
IRR	100	5	76	22	83	3
JAK2	100	10	93	8	94	7
JNK1	90	21	84	4	100	3
JNK2	88	5	84	17	91	6
JNK3	98	8	88	3	99	6
Lck	84	11	84	3	113	9
LKB1	98	4	81	4	113	2
MAP4K3	n.d.	n.d.	n.d.	n.d.	95	5
MAP4K5	n.d.	n.d.	n.d.	n.d.	100	14
MAPKAP-K2	91	4	77	4	97	3
MAPKAP-K3	123	7	68	5	110	1
MARK1	104	6	91	14	123	25
MARK2	83	6	88	1	99	1
MARK3	105	0	85	10	112	0
MARK4	93	10	74	3	102	1
MEKK1	89	0	79	7	94	1
MELK	98	14	74	4	99	6
MINK1	111	13	44	1	96	2
MKK1	97	9	85	5	92	3
MKK2	109	19	82	4	106	26
MKK6	99	1	99	26	101	8
MLK1	100	3	30	3	96	2
MLK3	109	12	30	0	109	28
MNK1	93	1	93	4	86	3
MNK2	102	14	103	3	118	4
MPSK1	100	4	83	9	100	16
MSK1	95	1	93	7	124	1
MST2	94	7	73	5	96	5
MST3	n.d.	n.d.	n.d.	n.d.	115	6
MST4	108	13	90	8	127	23
NEK2a	108	7	92	3	111	10
NEK6	84	9	102	19	111	13
NUAK1	101	5	66	8	102	10
OSR1	n.d.	n.d.	n.d.	n.d.	106	0
p38a MAPK	80	9	47	9	91	7
p38b MAPK	95	2	64	6	97	5
p38d MAPK	90	1	104	5	92	3
p38g MAPK	87	5	106	16	112	13
PAK2	113	8	86	18	95	12
PAK4	85	5	93	2	82	7
PAK5	101	4	108	10	116	5
PAK6	103	5	84	6	95	7
PDGFRA	n.d.	n.d.	n.d.	n.d.	98	26
PDK1	122	9	86	6	117	22
PHK	76	0	79	1	126	12
PIM1	95	10	18	1	113	17
PIM2	97	2	74	0	86	12
PIM3	107	2	12	0	96	1
PINK	n.d.	n.d.	n.d.	n.d.	104	3
PKA	107	12	81	4	100	5
PKBa	107	24	107	7	89	11
PKBb	86	2	72	10	94	1
PKCa	89	5	106	12	111	6
PKCγ	96	1	85	6	93	11
PKCz	92	5	99	6	99	4
PKD1	115	15	77	4	94	7
PLK1	96	3	97	5	103	1

	10 µM SKI I		10 µM SKI II		10 µM PF-543	
protein kinase	% activity	st. dev.	% activity	st. dev.	% activity	st. dev.
PRAK	90	3	76	6	94	5
PRK2	58	1	81	8	76	0
RIPK2	100	4	70	3	102	8
ROCK 2	116	3	74	8	99	9
RSK1	81	8	109	3	110	13
RSK2	81	11	73	6	105	1
S6K1	100	0	80	2	109	4
SGK1	78	4	145	17	96	1
SIK2	n.d.	n.d.	n.d.	n.d.	95	3
SIK3	n.d.	n.d.	n.d.	n.d.	107	7
SmMLCK	91	15	51	3	109	3
Src	111	13	76	1	93	2
SRPK1	83	9	85	3	92	3
STK33	89	16	97	1	102	1
SYK	92	13	90	8	101	6
TAK1	111	2	100	8	106	1
TAO1	91	6	103	10	108	7
TBK1	93	1	92	4	110	7
TESK1	n.d.	n.d.	n.d.	n.d.	109	10
TGFBR1	n.d.	n.d.	n.d.	n.d.	114	19
TIE2	113	4	110	8	102	2
TLK1	n.d.	n.d.	n.d.	n.d.	98	6
TrkA	86	0	107	20	107	25
TSSK1	n.d.	n.d.	n.d.	n.d.	108	2
TTBK1	n.d.	n.d.	n.d.	n.d.	74	7
TTBK2	n.d.	n.d.	n.d.	n.d.	84	8
TTK	86	4	81	9	100	5
ULK1	n.d.	n.d.	n.d.	n.d.	108	17
ULK2	n.d.	n.d.	n.d.	n.d.	81	11
VEG-FR	83	4	59	1	80	7
WNK1	n.d.	n.d.	n.d.	n.d.	111	0
YES1	109	2	61	5	95	0
ZAP70	92	0	129	31	95	18

Appendix C- Mass spectrometry data from SILAC experiment from figure 4.4

Table 1 of 2

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
100 kDa coactivator	Q78PY7	426	_VNVTVDYIRPAS(ph)PATETVPAFSER_	2.0497
26S proteasome non-ATPase regulatory subunit 11	Q8BG32	14	_AQS(ph)LLSTDREASIDILHSIVKR_	9.1192
26S proteasome non-ATPase regulatory subunit 2	Q8VDM4	361	_FGGS(ph)GSQVDSAR_	2.4393
26S proteasome non-ATPase regulatory subunit 4	O35226	256	_RAAAASAAEAGIATPGT(ph)EDSDDALLK_	3.0459
40S ribosomal protein S20	P60867	9	_DTGKT(ph)PVEPEVAIHR_	13.9310
6-phosphofructokinase, liver type	P12382	775	_TLS(ph)IDKGF_	9.7890
60S acidic ribosomal protein P1	P47955	101	_KEES(ph)EES(ph)EDDM(ox)GFGLFD_	6.7194
60S acidic ribosomal protein P1	P47955	104	_KEES(ph)EES(ph)EDDM(ox)GFGLFD_	4.8244
60S ribosomal protein L24	Q3UW40	83	_AIT(ph)GASLADIMAK_	8.4185
60S ribosomal protein L7-like 1	Q9D8M4	54	_RLES(ph)FVHDSWR_	4.5002
A-kinase anchor protein 9	Q70FJ1	3676	_DGFGLS(ph)PGIEK_	4.5009
Abelson tyrosine-protein kinase 2	Q4JIM5	936	_VPVLIS(ph)PTLK_	8.5376
Acetylcholine receptor subunit beta	P09690	478	_LILQGEAES(ph)R_	4.1537
Acidic leucine-rich nuclear phosphoprotein 32 family member A	O35381	17	_TPS(ph)DVKELVLDNCK_	28.4010
Acidic nucleoplasmic DNA-binding protein 1	P59328	1077	_VVS(ph)EICETENQEETVKENLDLSKK_	55.6860
Actin-binding protein 1	Q62418	291	_LRS(ph)PFLQK_	4.4441
Actin-binding protein 1	Q62418	277	_AM(ox)S(ph)TTSVTSSQPGK_	2.8880
Actin-binding protein anillin	Q8K298	292	_AS(ph)SPVTAATFITENR_	6.2051
Actin-binding protein anillin	Q8K298	790	_TSVIS(ph)QSEFAPSK_	4.3406
Activated Cdc42-associated guanine nucleotide exchange factor	A2AF47	1352	_SQT(ph)M(ox)PALR_	4.6970
Activated RNA polymerase II transcriptional coactivator p15	P11031	55	_QS(ph)SSSRDDNMFQIGK_	7.9230
Activating transcription factor 2	P16951	72	_NCEEVGLFNELAS(ph)PFENEFKK_	42.1110
Activating transcription factor 3	Q60765	162	_AQNGRT(ph)PEDERNLFIQQIK_	17.6040
Activating transcription factor 4	Q61328	279	_APPDNLPS(ph)PGGSR_	3.9011
Activator protein 1	P05627	63	_NSDLLTS(ph)PDVGLLK_	8.3994
Adaptor-associated kinase 1	Q3UHJ0	621	_VGSLT(ph)PPS(ph)SPK_	3.9064
Adducin-like protein 70	Q9QYB5	423	_HKSDVEIPATVTAFSFEDDSAPLS(ph)PLK_	10.4070
Adducin-like protein 70	Q9QYB5	12	_(ac)SSDTSPAVVTT(ph)PPPPSMPHK_	6.3421
Adenomatous polyposis coli protein	Q61315	2713	_QSVGSGS(ph)PVQTVGLETR_	5.6187
Adenosine monophosphate deaminase 2 (Isoform L)	A2AE27	70	_S(ph)LPGNAPCLK_	27.2500
Adenosine monophosphate deaminase 2 (Isoform L)	A2AE27	160	_AKQDFLKTDS(ph)DSDLQLYK_	15.0210
Adenosine monophosphate deaminase 2 (Isoform L)	A2AE27	138	_QIS(ph)QDVKLEPDILLR_	2.8002
Adhesion and degranulation promoting adaptor protein	O35601	203	_HTFGQKPSLS(ph)TEDSQEENTSK_	26.1100
ADP-ribosylation factor GTPase-activating protein 2	Q99K28	143	_HGTDLWIDSM(ox)NSAPS(ph)HSPEKK_	7.1105
Adrenocortical dysplasia protein	Q5EE38	20	_ELILGS(ph)ET(ph)LSSPR_	2.6217
Adrenocortical dysplasia protein	Q5EE38	22	_ELILGS(ph)ET(ph)LSSPR_	2.6217
Afadin	Q9QZQ1	1726	_TQVLS(ph)PDSLFTAK_	10.7200
Afadin	Q9QZQ1	1777	_S(ph)QDADLPGSSGAPENLTFK_	3.3673
Afadin	Q9QZQ1	655	_YVLSSQHRPDIS(ph)PTER_	2.2574
AHNAK nucleoprotein 2	Q3URZ6	35	_KLS(ph)FSM(ox)PR_	3.0450
Ahnak protein	A0JLR7	2985	_GPSLDIKS(ph)PKLDVNAPDIDVHGPEGK_	41.9810
Ahnak protein	A0JLR7	5325	_GPSFNVAS(ph)PESDFGVSLKGPK_	40.7930
Ahnak protein	A0JLR7	4906	_VPDVDISS(ph)PGINVEAPDIHMK_	36.3920
Ahnak protein	A0JLR7	4686	_MPS(ph)LEVSVPK_	35.7980
Ahnak protein	A0JLR7	4975	_IKAPSFSVSAPQVS(ph)IPDVNVNLKGPK_	35.0310
Ahnak protein	A0JLR7	4740	_VDLETPSLDVHMES(ph)PDINIEGPDVK_	25.0790
Ahnak protein	A0JLR7	4298	_TPQIS(ph)MSDIDLNVK_	18.7780
Ahnak protein	A0JLR7	177	_EGVKDIDIT(ph)SPEFMIK_	15.3540
Ahnak protein	A0JLR7	4879	_MYFPDVEFDIKS(ph)PK_	13.3400
Ahnak protein	A0JLR7	4342	_LDIDT(ph)PDIDIHGPEGK_	9.9429
Ahnak protein	A0JLR7	2381	_ISMPDIDLHLKS(ph)PK_	9.4088
Ahnak protein	A0JLR7	5041	_MEGGSTEIGAQMPS(ph)LEGGLSTSDMK_	9.3720
Ahnak protein	A0JLR7	178	_EGVKDIDIT(ph)SPEFMIK_	8.9735
Ahnak protein	A0JLR7	496	_VKT(ph)PEMIIQKPK_	8.5903

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Ahnak protein	A0JLR7	4950	_VQANLDT(ph)PDINIEGPEAK_	8.3631
Ahnak protein	A0JLR7	5292	_LSSGQIS(ph)GPEIK_	5.9896
Ahnak protein	A0JLR7	5563	_SNS(ph)FSDEREFS(ph)APSTPTGTLEFAGGDAK_	4.6212
Ahnak protein	A0JLR7	5535	_ASLGSLEGEVEAEAS(ph)SPKGK_	4.0238
Ahnak protein	A0JLR7	4890	_FKAEAPLPS(ph)PKLEGEIK_	3.9415
Ahnak protein	A0JLR7	136	_LRS(ph)EDGVEGDLGETQSR_	3.6377
Ahnak protein	A0JLR7	4766	_FGFGAKS(ph)PK_	3.6359
Ahnak protein	A0JLR7	5605	_SKGHYEVT(ph)GSDDEAGKLQGSGVSLASK_	2.2529
Ahnak protein	A0JLR7	159	_RVT(ph)AYTVDVTGR_	2.1025
Ahnak protein	A0JLR7	94	_S(ph)PEPGQTWTHEVFSSR_	2.6105
Ahnak protein	A0JLR7	893	_FGMPGFKAES(ph)PEMEVNLPK_	9.4471
AI607873 protein	B2RWU7	106	_NGQEAGPAT(ph)PTSTTSHMLTSER_	2.3523
Akt substrate of 160 kDa	Q8BYJ6	324	_CSS(ph)VTGVMQK_	5.1961
Akt substrate of 160 kDa	Q8BYJ6	673	_AHGLRS(ph)PLLR_	2.2629
Alkylated DNA repair protein alkB homolog 5	Q3TSG4	362	_RGS(ph)FSSENYWR_	2.1991
Alpha-adducin	Q9QYC0	11	_AAVVT(ph)SPPPTTAPHKER_	4.0882
Alpha-PAK	O88643	212	_SVIEPLPVT(ph)PTR_	14.5440
Amino acid transport system xc-	Q9WTR6	26	_LPS(ph)MGDQEPPGQEK_	3.0970
AMMECR1-like protein	Q3V0N3	161	_MNTASGALS(ph)PLPRPNGTANSTK_	2.6189
Anaphase promoting complex subunit 1	A2ATQ4	688	_SFDFEGSLS(ph)PVIAPK_	2.6709
Ancient ubiquitous protein 1	P70295	319	_LRPQSVQSSFPSPPS(ph)PSSDVQLTTLAHR_	2.5991
Androgen-induced proliferation inhibitor	Q4VA53	1358	_AES(ph)PETSAVESTQSTPQK_	2.7543
Ankrd11 protein	B2RY01	1091	_KAS(ph)FDQLR_	3.9197
Ankyrin repeat domain-containing protein 55	Q8BLD6	474	_SRS(ph)EQDLLNNR_	11.4170
Annexin A1	P10107	37	_GGPGSAVS(ph)PYPSFNVSSDVAALHK_	7.9615
Antigen containing epitope to monoclonal antibody MMS-85/12	O35243	896	_TKS(ph)LLEDKVVSK_	31.7580
Antigen containing epitope to monoclonal antibody MMS-85/12	O35243	1364	_RHLS(ph)EDSQATLLYSK_	17.2800
Antigen containing epitope to monoclonal antibody MMS-85/12	O35243	656	_RTS(ph)TPVILEGAQEETDTR_	6.0708
Antigen containing epitope to monoclonal antibody MMS-85/12	O35243	795	_RLS(ph)VLGR_	4.9062
Aortic preferentially expressed protein 1	Q62407	2413	_RLS(ph)LSLSQK_	2.2089
AP1 subunit gamma-binding protein 1	Q5SV85	576	_TADSVS(ph)PLEPPTKDTFPSAFASGAAQQTQTQVK_	13.2090
AP1 subunit gamma-binding protein 1	Q5SV85	974	_DMMPQTTEQKEFES(ph)GDFQDFTR_	5.4966
APG16-like 1	Q8C0J2	287	_S(ph)VSSIPVPQDIMDTHPASGK_	5.1235
Apoptosis-stimulating of p53 protein 1	Q62415	335	_VNGTSSPQS(ph)PLSTSGR_	5.6452
Apoptosis-stimulating of p53 protein 2	Q8CG79	561	_MLLS(ph)PGAPSGGQDQVLSPASK_	11.2280
Apoptotic chromatin condensation inducer 1	B8JJ87	710	_HLS(ph)HPEPEQQHVIQR_	7.7242
ARF GTPase-activating protein GIT1	Q68FF6	612	_HGSGADSDY(ph)ENTQS(ph)GDPLLGLEGKR_	2.9365
Arfgef1 protein	Q8BKL2	1566	_S(ph)VDIHDSIQPR_	3.2505
Arfip1 protein	A2RSX9	361	_LKT(ph)PGVDAPSWLEEQ_	24.7510
Arginine glutamic acid dipeptide (RE) repeats	A2A7T4	1258	_TLSEYARPHVMS(ph)PTNR_	2.0051
Arhgap21 protein	B2RWX1	928	_KGS(ph)SSEVFSDAAR_	2.0221
ARID domain-containing protein 4B	A2CG63	830	_YCS(ph)ADECLQTGSPGK_	15.2720
Astrocytic phosphoprotein PEA-15	Q62048	116	_YKDIIRQPS(ph)EEEIIK_	2.4348
AT-hook DNA-binding motif-containing protein 1	Q6PAL7	1060	_ASTVS(ph)PGGYMVPK_	4.6963
AT-rich interactive domain-containing protein 1A	A2BH40	1603	_TS(ph)PS(ph)KSPFLHSGMK_	3.4040
AT-rich interactive domain-containing protein 1A	A2BH40	1183	_S(ph)NSVGIQDAFPDGSDPTFQKR_	5.5198
AT-rich interactive domain-containing protein 1A	A2BH40	1185	_S(ph)NSVGIQDAFPDGSDPTFQKR_	3.5919
Ataxia telangiectasia and Rad3-related protein	Q9JKK8	438	_KLS(ph)SSLSSYK_	22.5770
Ataxin-1	Q8C866	62	_HGS(ph)AGTSGEHGLQGMGLHK_	16.4070
Ataxin-2-like protein	Q7TQH0	499	_LSLT(ph)PTDVKELPTKEPSR_	6.6047
Ataxin-2-like protein	Q7TQH0	109	_GPPQS(ph)PVFEGVYNNSR_	2.1097
ATM and Rad3-related-interacting protein	Q8BMG1	14	_KQS(ph)GGLEPFPGLSR_	4.9993
ATP citrate lyase	Q3TED3	455	_TAS(ph)FSESRADEVAPAKK_	3.2462
ATP-dependent DNA helicase 2 subunit 1	P23475	518	_RLGS(ph)LADEFK_	10.0930
ATP-dependent helicase CHD8	Q09XV5	2040	_SRLTS(ph)QDYEVR_	2.8221
ATP-dependent helicase SMARCAD1	Q04692	79	_KAS(ph)LSCFQNQR_	2.6680
ATP-dependent helicase SMARCAD1	Q04692	81	_KAS(ph)LSCFQNQR_	2.3415
ATP-dependent RNA helicase BRIP1	Q5SXJ3	970	_S(ph)DPGLRGESLQTMK_	4.2282
ATP-dependent RNA helicase Dhx29	Q6PGC1	69	_IYSFNS(ph)ANDSGGSANLDK_	38.8500
Autophagy-related protein 2 homolog B	Q80XK6	1571	_DFATAPPT(ph)SPAK_	5.9794

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Autophagy-related protein 2 homolog B	Q80XK6	255	_LS(ph)PSWNPK_	7.3830
Autophagy-related protein 2 homolog B	Q80XK6	379	_KDS(ph)LSMGVSSEK_	2.1052
AVO3 homolog	Q6QI06	1461	_MFS(ph)HDGAGLSSGAGGLVK_	23.5970
Axam2	Q91ZX6	332	_LGS(ph)GSNGLLR_	4.7955
B aggressive lymphoma protein homolog	Q8CAS9	42	_RGHS(ph)EGDYPPLR_	3.3726
B-cell adapter for phosphoinositide 3-kinase	Q9EQ32	629	_DRPPS(ph)SIYDPFAGMK_	2.9633
Barren homolog protein 1	Q8C156	25	_GQQDVLSS(ph)PLER_	9.9552
Basic SAP coiled-coil transcription activator	Q8K4J6	349	_S(ph)LSTSSSPSSGTPGPSGLAR_	3.1417
Basic SAP coiled-coil transcription activator	Q8K4J6	548	_AASCCLS(ph)PGAR_	11.0370
Basic SAP coiled-coil transcription activator	Q8K4J6	423	_AYQDQVS(ph)PAPGAPK_	6.9894
Basophilic leukemia-expressed protein Bles03	Q8VD62	36	_ARS(ph)WVGAER_	4.2455
BCKD-kinase	O55028	31	_S(ph)TSATDTHHVELAR_	5.1256
Bcl-2-associated transcription factor 1	Q8K019	688	_LRCDS(ph)ADLR_	2.1719
Bcl2 antagonist of cell death	Q61337	155	_RMS(ph)DEFEGSFK_	2.3092
Beige-like protein	Q9ESE1	982	_KDSPIS(ph)PHFTR_	2.2366
Beta enolase repressor factor 1	Q61624	311	_GGLLTSEEDSGFSTSPKDNS(ph)LPK_	4.3202
Beta-adrenergic receptor kinase 1	Q99MK8	670	_NKPRS(ph)PVVELSK_	4.5028
Beta-Pix	Q9ES28	598	_KPS(ph)DEEFAVRK_	2.7620
Binder of Rho GTPases 2	Q9CQC5	75	_AHS(ph)GQFPGHNDFFR_	4.4837
Bobby sox homolog	Q8VBW5	242	_QKS(ph)PLFQFAEISSR_	2.6683
BolA-like protein 1	Q9D8S9	45	_AKLEQALS(ph)PEVLELR_	7.9793
BPG-dependent PGAM 1	Q9DBJ1	31	_FSGWYDADLS(ph)PAGHEEAK_	2.9618
Brain-specific gene 4 protein	Q3U1Y4	961	_SLREPS(ph)SPMGR_	3.0299
Breast carcinoma-amplified sequence 3 homolog	Q8CCN5	886	_EGS(ph)IETLSNSSGSTSGSIPR_	2.3181
Breast carcinoma-amplified sequence 3 homolog	Q8CCN5	572	_VKSPPQISPS(ph)K_	4.1473
Bromodomain adjacent to zinc finger domain protein 1A	O88379	1545	_KRQS(ph)TESSPVPLNR_	10.5320
Bruton tyrosine kinase-associated protein 135	Q3UHU8	744	_RPS(ph)TFGIPR_	6.2015
BXMAS1-like protein 1	Q8R4Y0	271	_SPPQTVLQGSTYPKS(ph)PDSR_	15.2230
c-Jun N-terminal kinase 2	Q9WTU6	185	_TACTNFMMTPY(ph)VVTR_	17.7420
C-Jun-amino-terminal kinase-interacting protein 4	Q58A65	705	_DGGS(ph)VVGASVFYK_	10.5380
C2PA	Q9DC04	766	_NGGSMHHLS(ph)LFFTGHR_	9.7578
C2PA	Q9DC04	712	_THS(ph)EGSLLQESR_	4.0243
Cadherin-associated Src substrate	P30999	320	_LRS(ph)YEDMIGEEVPPDQYYWAPLAQHER_	2.8125
Cadherin-associated Src substrate	P30999	349	_GSLAS(ph)LDSLRK_	2.2236
Calcium homeostasis endoplasmic reticulum protein	Q8CGZ0	915	_SYS(ph)FIAR_	10.5600
Calcium-dependent tyrosine kinase	Q9QVP9	579	_YIEDEDY(ph)YKASVTR_	2.6942
Calcium-dependent tyrosine kinase	Q9QVP9	580	_YIEDEDY(ph)YKASVTR_	2.1348
CALNUC	Q02819	456	_KVPEQPPELPQLDS(ph)QHL_	7.4883
CALNUC	Q02819	368	_AQRLS(ph)QETEALGR_	3.3632
CALNUC	Q02819	85	_SGKLS(ph)QELDFVSHNVR_	2.5595
Calpain inhibitor	P51125	219	_EQKPFTPAS(ph)PVQSTPSKPSDK_	4.2860
Cancer susceptibility candidate gene 5 protein homolog	Q66JQ7	794	_IIS(ph)PSDITPGDLDK_	10.1030
CAP-Gly domain-containing linker protein 2	Q9Z0H8	552	_LLS(ph)ASKEHQR_	27.6600
Casitas B-lineage lymphoma proto-oncogene	P22682	450	_QGAEGAPS(ph)PNYDDDDDERADDSLFMMK_	2.8708
Caspase-like protein ESPL1	P60330	1320	_IAS(ph)VPPPLHNSSQK_	5.7834
CCAAT displacement protein	P53564	1471	_RPSSLQS(ph)LFGLPEAAGAR_	8.6033
CCAAT displacement protein	P53564	1506	_AAS(ph)REEPIEWEF_	2.2942
CCAAT-binding transcription factor I subunit A	P62960	210	_RPQYS(ph)NPPVQGEVMEGADNQGAGEQGRPVR_	24.0580
CCAAT-binding transcription factor I subunit A	P62960	126	_YLRS(ph)VGDGETVEFDVVEGEK_	19.2040
CCT-alpha	P49586	342	_WPFSGKT(ph)SPS(ph)SSPASLSR_	5.8661
CD2-associated protein	Q9JLQ0	404	_ASNLLRS(ph)PGAVYPK_	11.3240
CD2-associated protein	Q9JLQ0	458	_S(ph)VDLDAFVAR_	2.5360
Cdc2-related kinase, arginine/serine-rich	Q14AX6	610	_TQVS(ph)ITAAIPHLK_	17.0930
CDC2-related protein kinase 5	Q69ZA1	440	_HSSIS(ph)PSTLTLK_	9.5677
Cell cycle checkpoint protein RAD17	Q6NXW6	70	_GRLS(ph)LEQTHGLETSR_	8.9839
Cell cycle regulatory protein p95	Q9R207	398	_KLS(ph)QETFNIK_	6.6874
Cell death protein RIP	Q60855	415	_RVS(ph)HDPFAQQR_	3.2814
Cell death protein RIP	Q60855	313	_EYPDQS(ph)PVLQR_	2.5335
Cell division protein kinase 14	O35495	78	_TQS(ph)TFDPFEKPANQVK_	7.7761
Cellubrevin	P63024	48	_DQKLS(ph)ELDDRADALQAGASQFETSAAK_	4.1180

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Centaurin-beta-2	Q6ZQK5	521	_YSALLS(ph)PSEQEKR_	5.9467
Centaurin-delta-2	Q4LDD4	431	_LSS(ph)ASVLGVR_	13.5260
Centaurin-gamma-2	Q8BXK8	422	_ATSACAPISS(ph)PK_	4.2775
Centriolin	A2AL36	832	_IHS(ph)PSDVLGK_	13.0710
Centromere protein E	Q6RT24	2426	_SKS(ph)LPAPHPIR_	4.2736
Centromere/kinetochore protein zw10 homolog	O54692	438	_EALPDLPS(ph)PDADHK_	12.4070
Centrosomal protein of 170 kDa	Q6A065	1150	_LGS(ph)LS(ph)ARSDSEATISR_	2.7601
Centrosomal protein of 170 kDa	Q6A065	443	_QKS(ph)EEPSVSLPFLQTALLR_	2.4849
Centrosomal protein of 55 kDa	Q8BT07	426	_S(ph)PSAALNDSLVECPK_	2.8521
Centrosomal protein of 55 kDa	Q8BT07	422	_AT(ph)SPKS(ph)PSAALNDSLVECPK_	2.5577
Centrosomal protein of 55 kDa	Q8BT07	423	_AT(ph)SPKS(ph)PSAALNDSLVECPK_	2.0366
Centrosomin	P23116	584	_LES(ph)LNIQR_	4.0796
Ceramide transfer protein	Q9EQG9	377	_S(ph)SSM(ox)SSIDLVSASDDVHR_	5.2011
Chloride channel CLIC-like 1	A2AEM2	438	_FHS(ph)GNKS(ph)PEVLR_	3.9380
Chromatin assembly factor 1 subunit A	Q9QWF0	776	_LIS(ph)ENSAYEK_	7.6784
Chromatin assembly factor 1 subunit B	Q9D0N7	492	_RVT(ph)LNTLQTWGK_	11.2920
Chromobox protein homolog 5	Q61686	93	_KSS(ph)FSNSADDIK_	4.9591
Chromokinesin	P33174	802	_TFS(ph)YDEIHGQDSGAEDSIAK_	3.7476
Clathrin light polypeptide (Lca)	B1AWD9	236	_MRS(ph)VLISLK_	2.2273
Cleavage and polyadenylation specificity factor subunit 6	Q6NVF9	404	_EMDT(ph)ARTPLSEAEFEEIMNR_	12.4460
CLIP-associating protein 1	Q80TV8	598	_S(ph)RSDIDVNAAASAK_	2.8265
Coactivator independent of AF-2	Q91W39	377	_S(ph)SADSLPGPISR_	4.2875
Coactivator of activating protein 1 and estrogen receptors	Q8VH51	117	_IGLPHS(ph)IK_	10.0810
Cofactor required for Sp1 transcriptional activation subunit 6	Q8VCD5	10	_AVRIS(ph)IESACEK_	7.1002
Cofilin, non-muscle isoform	P18760	41	_AVLFCLS(ph)EDKK_	6.2318
Cohen syndrome protein 1 homolog	Q80TY5	3045	_LVHNLTS(ph)PK_	4.2082
Cohesin subunit SA-2	O35638	1197	_RGT(ph)SLMEDDEEPIVEDVMMSSEGR_	2.0258
Cohesin subunit SA-2	O35638	1198	_RGT(ph)SLMEDDEEPIVEDVMMSSEGR_	2.0020
Coiled-coil and C2 domain-containing protein 1B	Q8BRN9	502	_KPAQT(ph)LVSPSHLLTEPK_	17.2850
Coiled-coil and C2 domain-containing protein 1B	Q8BRN9	499	_KPAQT(ph)LVSPSHLLTEPK_	10.7480
Coiled-coil and C2 domain-containing protein 1B	Q8BRN9	459	_KGSEQDS(ph)VAATLATAQK_	4.5288
Collagen alpha-1(XX) chain	Q923P0	774	_AIS(ph)QVESAEPR_	6.3978
Connecdenn	Q8K382	592	_S(ph)LEDLRAPK_	2.6917
Constitutive coactivator of PPAR-gamma-like protein 2	Q8C3F2	436	_NQMGPIS(ph)PGKPMFSR_	4.1297
Copper pump 1	Q64430	357	_VSIASEVESTASS(ph)PSSSSLQK_	12.2050
Craniofacial development protein 1	O88271	202	_EKPQALVT(ph)SPATPLPAGSGIK_	5.2869
CREB-regulated transcription coactivator 2	Q3U182	434	_VPLS(ph)PLSLPAGPADAR_	4.3559
CREB-regulated transcription coactivator 3	Q91X84	126	_LT(ph)QY(ph)HGGS(ph)LPNVS(ph)QLR_	2.2979
CREB-regulated transcription coactivator 3	Q91X84	393	_S(ph)NPSIQATLSK_	3.7605
CREB-regulated transcription coactivator 3	Q91X84	434	_LFSLS(ph)NPSLSTTNLSGPSR_	2.1424
CRK1	P47811	180	_HTDDEMT(ph)GYVATR_	4.0098
CTTNBP2 N-terminal-like protein	Q99LJ0	488	_DLS(ph)PTLLDNSAAK_	3.7187
CTTNBP2 N-terminal-like protein	Q99LJ0	522	_FTNQGPIKPVS(ph)PNSSPFGTDYR_	2.3207
Cyclic AMP specific phosphodiesterase PDE4D5A	O70286	69	_LSPVIS(ph)PR_	9.9249
Cyclin fold protein 1	Q8BGU5	73	_ASTIFLSKS(ph)QTDVR_	2.4956
Cyclin fold protein 1	Q8BGU5	25	_LES(ph)YRPDTDLSR_	2.2715
Cysteine string protein	P60904	8	_S(ph)LSTSGESLYHVLGLDKNATSDDIKK_	6.5595
Cytoplasmic dynein 1 light intermediate chain 1	Q8R1Q8	414	_SVS(ph)SNVASVSPIPAGSKK_	22.8600
Cytoplasmic dynein 1 light intermediate chain 1	Q8R1Q8	516	_KPASVSPTT(ph)PTS(ph)PTEGEAS_	12.8980
Cytosolic Fe-S cluster assembly factor NUBP1	Q9R060	319	_IRDFCNSHQSHAETLIS(ph)P_	5.4117
Cytosolic prostaglandin E2 synthase	Q9R0Q7	85	_KGESGQS(ph)WPR_	3.4111
Cytospin-B	Q5SXY1	76	_T(ph)STSGAISELTESR_	4.1663
Cytospin-B	Q5SXY1	933	_LLS(ph)ASTGGLKPSK_	16.7090
Cytospin-B	Q5SXY1	847	_TPRS(ph)PLSGIPVR_	3.9045
Cytospin-B	Q5SXY1	810	_GVYVNRTS(ph)PAPSDSATTVK_	2.6403
Dedicator of cytokinesis protein 7	Q8R1A4	1382	_M(ox)NS(ph)LTFKK_	3.9412
Dedicator of cytokinesis protein 7	Q8R1A4	452	_TTS(ph)GDDACNLTSFRPATLTVANFFK_	3.4390
Dedicator of cytokinesis protein 7	Q8R1A4	963	_MSS(ph)HTETSSFLQTLTGR_	2.2405
Dedicator of cytokinesis protein 8	Q8C147	197	_GPLTS(ph)CDFDLR_	25.0010
Dedicator of cytokinesis protein 8	Q8C147	452	_TLS(ph)LEENGVGSNFK_	15.4830

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
DENN/MADD domain containing 4C	A2AJX5	1289	_NLADEIESYMNLKS(ph)PLGSK_	10.0740
DENN/MADD domain containing 4C	A2AJX5	1270	_TSDSEDKLFS(ph)PVISR_	9.8842
DENN/MADD domain containing 4C	A2AJX5	1145	_THS(ph)FENVNCHLADSR_	5.7719
DENN/MADD domain containing 4C	A2AJX5	1370	_STS(ph)LSALVR_	3.0582
DENN/MADD domain containing 4C	A2AJX5	1015	_HLQPT(ph)PEPQS(ph)PTEPPAWGSSIVK_	11.6300
Densin-180-like protein	Q80TH2	829	_VNGLCEDTAPS(ph)PGRVEPQK_	2.3422
Deoxynucleotidyltransferase terminal-interacting protein 2	Q8R2M2	612	_AVIT(ph)PDFEKK_	3.1425
Deoxynucleotidyltransferase terminal-interacting protein 2	Q8R2M2	248	_NMPNVS(ph)DSETYNSDFDDSSPR_	2.0832
DEP domain-containing mTOR-interacting protein	Q570Y9	260	_KSTS(ph)FMSVSPSK_	9.9776
DET1- and DDB1-associated protein 1	Q9D9Z5	33	_RPS(ph)VYLPTR_	5.1187
Deubiquitinating enzyme 10	P52479	364	_CSPPVPS(ph)PLASEK_	8.5877
Deubiquitinating enzyme 20	Q8C6M1	334	_KFS(ph)WGQQR_	2.4217
Deubiquitinating enzyme 24	B1AY13	2558	_TIS(ph)AQDTLAYATALLNEK_	2.0627
Deubiquitinating enzyme 45	Q8K387	452	_KWPS(ph)EEEKTVVTHPK_	2.2487
Diacylglycerol kinase zeta	A2AHK0	900	_LQREPDGAGAKS(ph)PMCHQLSSK_	5.9165
Diphosphoinositol pentakisphosphate kinase 1	A2ARP1	959	_ALQTS(ph)PQPVEGTGLPR_	7.8221
Disabled homolog 2	P98078	727	_S(ph)ADNSLENPFSK_	2.0662
Disabled homolog 2	P98078	32	_KGS(ph)EKTDEYLLAR_	25.7920
Disco-interacting protein 2 homolog B	Q3UH60	99	_YRS(ph)DIHTEAVQAALAK_	2.7006
Disintegrin and metalloproteinase domain-containing protein 17	Q9Z0F8	752	_LQALQPAAMMPPVS(ph)AAPK_	14.5360
Disintegrin and metalloproteinase domain-containing protein 17	Q9Z0F8	735	_IIKPFPAPQT(ph)PGR_	2.0103
DmX-like protein 1	Q6PNC0	922	_ILS(ph)PFSQK_	37.0010
DNA (cytosine-5)-methyltransferase 1	P13864	22	_SKS(ph)DSDTLSVETSPSSVATRR_	4.0818
DNA polymerase alpha 70 kDa subunit	P33611	125	_VS(ph)STPETPLTKR_	3.5501
DNA polymerase delta subunit 3	Q9EQ28	454	_QVS(ph)ITGFFQK_	26.5790
DNA replication licensing factor MCM2	P97310	21	_RRIS(ph)DPLTSSPGR_	8.0594
DNA replication licensing factor MCM2	P97310	25	_RRIS(ph)DPLTSSPGR_	2.1014
DNA replication licensing factor MCM6	P97311	699	_FNGS(ph)S(ph)EDASQETVSKPSLR_	8.0407
DNA replication licensing factor MCM6	P97311	700	_FNGS(ph)S(ph)EDASQETVSKPSLR_	2.1967
DNA topoisomerase 2-beta	Q64511	1439	_SQDFGNLFSFPS(ph)YSQK_	16.8610
DNA topoisomerase 2-binding protein 1	Q6ZQF0	863	_KLSS(ph)PLSEVIVR_	2.0282
DNA-directed RNA polymerase I subunit E	Q8K202	200	_DKLDS(ph)CIEAFGSTK_	20.4360
Docking protein 1	P97465	269	_VGQAQDILRTDS(ph)HDGETEGK_	3.8113
Docking protein 3	Q9QZK7	264	_LPELAM(ox)S(ph)PPCPLPR_	2.1537
Double-stranded RNA-binding protein Staufen homolog 2	Q8CJ67	440	_VTSGTTLSYLS(ph)PK_	10.3690
Down-regulated by CTNNB1 protein A	Q6P9N1	465	_S(ph)PSPAIGCVAGADANR_	8.0352
Down-regulator of transcription 1	Q91WV0	105	_KAS(ph)SRLENLGIPEEELLR_	4.5970
Dual specificity mitogen-activated protein kinase kinase 2	Q63932	295	_ELEASFGRPVVDGADGEPHSVS(ph)PR_	2.1947
Dual specificity mitogen-activated protein kinase kinase 2	Q63932	226	_LCDFGVSGQLIDSMANS(ph)FVGTR_	7.0336
Dual specificity mitogen-activated protein kinase kinase 2	Q63932	23	_RKPVLPALTINPTIAEGPS(ph)PTSEGASEANLVDLQK_	15.1880
Dual specificity mitogen-activated protein kinase kinase 3	O09110	218	_MCDFGISGYLVDS(ph)VAK_	7.4416
Dynactin subunit 4	Q8CBY8	203	_AGAS(ph)ISTLAGLSLR_	4.3400
Dysbindin	Q91WZ8	11	_LLS(ph)VQQDFTSGLK_	14.2170
Dystrophin-like protein	Q8R516	251	_RVS(ph)ADGQPFQR_	3.1106
E3 SUMO-protein ligase RanBP2	Q9ERU9	1438	_S(ph)ASSFVQTSFK_	14.4770
E3 SUMO-protein ligase RanBP2	Q9ERU9	781	_HSTPS(ph)PTKYSLS(ph)PSK_	6.6217
E3 SUMO-protein ligase RanBP2	Q9ERU9	790	_YSLSPS(ph)K_	4.1412
E3 SUMO-protein ligase RanBP2	Q9ERU9	21	_YIASVQGSAPS(ph)PR_	2.2879
E3 ubiquitin-protein ligase HUWE1	Q7TMY8	3759	_LGSSGLGS(ph)ASSIQAAVR_	2.2153
E3 ubiquitin-protein ligase RLIM	Q9WTV7	163	_RLS(ph)VENMESSSQR_	3.7527
E3 ubiquitin-protein ligase TRIM33	Q99PP7	1134	_LKS(ph)DERPVHIK_	5.4858
E3 ubiquitin-protein ligase UBR5	Q80TP3	1549	_RIS(ph)QSQPVR_	5.2401
E4 promoter-binding protein 4	O08750	301	_GPIHS(ph)PVELQR_	2.3066
Echinoderm microtubule-associated protein-like 3	Q8VC03	882	_VLGAGSSGPAPAT(ph)PSR_	2.9360
EGR-1-binding protein 2	Q61127	367	_LHS(ph)EELGGPPLKK_	43.9200
Elongin 110 kDa subunit	Q8CB77	515	_AFSS(ph)PQEEEEAGFTGR_	21.0930
Embryonic large molecule derived from yolk sac	Q8CJF7	528	_CLVAGLLS(ph)PR_	11.6200
Embryonic large molecule derived from yolk sac	Q8CJF7	2198	_LVS(ph)PLASPVDEIK_	2.3432
Ena/vasodilator-stimulated phosphoprotein-like	P70429	335	_SNS(ph)VEKPVSSLLSR_	2.4871
Ena/vasodilator-stimulated phosphoprotein-like	P70429	373	_VKPAGS(ph)VNDVGLDALDLDR_	2.3897

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Endofin	Q80U44	906	_THS(ph)PTVEKPNNGLGDIIR_	2.6648
Endonuclease/exonuclease/phosphatase family domain-containing protein 1	Q3TGW2	31	_KFS(ph)AACNFS(ph)NILVNQER_	4.1417
Engulfment and cell motility protein 1	Q8BPU7	31	_KPLS(ph)AIIK_	21.4870
Enhanced at puberty protein 1	Q8K3X4	127	_S(ph)PGPPPPVGVK_	3.5772
Enhancer of mRNA-decapping protein 4	Q3UJB9	6	_(ac)ASCAS(ph)IDIEDATQHLR_	11.7220
Enhancer of mRNA-decapping protein 4	Q3UJB9	426	_HTEVLPAEEENDS(ph)LGTESSHGAGALESAAGVLIK_	5.0241
Enhancer of mRNA-decapping protein 4	Q3UJB9	620	_LQLDGSLTLNSSSSSLQAS(ph)PR_	2.5689
Epidermal growth factor receptor substrate 15	P42567	324	_NITGSS(ph)PVADFSAIK_	2.9250
Epidermal growth factor receptor substrate 15	P42567	779	_VGT(ph)PTRPCPPPPGK_	2.4477
ERT1	P63085	183	_VADPDHDHTGFLT(ph)EY(ph)VATR_	7.7634
ERT1	P63085	185	_VADPDHDHTGFLT(ph)EY(ph)VATR_	2.6088
ERT2	Q63844	203	_IADPEHDHTGFLT(ph)EY(ph)VATR_	17.6840
ERT2	Q63844	205	_IADPEHDHTGFLT(ph)EY(ph)VATR_	4.4145
Erythrocyte protein band 4.1	A2A841	689	_HHAS(ph)ISELKK_	3.2740
ETS domain transcriptional repressor PE1	Q8R4Z4	175	_FSASSLS(ph)ASGPESGVTTDR_	2.7918
ETS domain-containing transcription factor ERF	P70459	534	_RVS(ph)SDLQHATAQLSLEHRDS_	2.8483
Eukaryotic translation initiation factor 2-alpha kinase 4	Q9QZ05	230	_AAAILHGGS(ph)PDFVGNGK_	6.1477
Eukaryotic translation initiation factor 4E-binding protein 1	Q60876	64	_NS(ph)PVAKTPPKDLPAIPGVTSPTSDEPPMQASQSQLPSSPEDK_	2.9012
Eukaryotic translation initiation factor 5	P59325	10	_SVS(ph)DQFYR_	22.4860
Exportin-4	Q9ESJ0	521	_HQQQFLAS(ph)PGSSTIDNK_	2.2228
Eyes absent homolog 3	P97480	210	_LPS(ph)DSSASPPLSQTTPNK_	25.7010
F-box only protein 4	Q8CHQ0	11	_GAGS(ph)PPPASDWGR_	5.3295
Factor for adipocyte differentiation 49	A2AAY5	291	_LGPS(ph)SPAHSGALDLDGVSR_	8.2831
Far upstream element-binding protein 2	Q3U0V1	182	_VQIS(ph)PDSGGLPER_	5.0426
FCH domain only protein 2	Q3UQN2	391	_VSIGNITLS(ph)PAVS(ph)RHSPVQMNR_	2.3650
FERM domain-containing protein 4A	Q8BIE6	723	_LLGSENDTGS(ph)PDFYTPR_	3.5243
FGFR1 oncogene partner	Q66JX5	158	_EKGPASVEGALDLSDGHPPS(ph)KSPEGK_	4.8118
Filamin-B	Q80X90	983	_LDVTILS(ph)PSRK_	8.6443
Filamin-C	Q8VHX6	2625	_GAS(ph)YSSIPK_	32.4660
Filamin-C	Q8VHX6	2234	_LGS(ph)FGSITR_	12.6110
Filamin, alpha	B7FAV0	2152	_RRAPS(ph)VANIGSHCDLSLK_	3.0094
Filamin, alpha	B7FAV0	968	_SPFSVGVSPS(ph)LDLSK_	2.9010
Filamin, alpha	B7FAV0	2180	_IPEISIQDMTAQVTS(ph)PSGK_	2.8168
FK506-binding protein 15	Q6P9Q6	344	_SNS(ph)LSEQLTVNSNPDTVK_	6.5178
FLI-LRR-associated protein 1	Q3UZ39	302	_AENQRPAEDSALS(ph)PGPLAGAK_	2.4710
Fork head-related protein-like A	Q9DBY0	558	_M(ox)TGS(ph)PTLVK_	2.6559
Forkhead box O3a	Q9WVH4	293	_WPGS(ph)PTSR_	4.2179
Forkhead box protein P1	P58462	319	_RES(ph)LSHEEHPHSHPLYGHGVCK_	11.2780
Formin-1	Q05860	396	_ART(ph)PETALEAFK_	12.8670
Formin-1	Q05860	616	_KLTISLTQLS(ph)PSKDSK_	6.6044
Formin-binding protein 1-like	Q8K012	295	_TIS(ph)DGTISAAKQESGK_	4.7175
Formin-binding protein 11	Q9R1C7	34	_RLS(ph)GSNLCSSSWVSADGFLR_	3.4202
Formin-like protein 1	Q9JL26	509	_ILRGPGDVVSIEILPGAAAT(ph)PSGDDAQAPR_	23.5270
Fragile site-associated protein homolog	A2AAE1	2603	_YTAGSAS(ph)PTPTFK_	4.9389
Friend leukemia integration 1 transcription factor	P26323	241	_GAWNNNMNSGLNKS(ph)PLLGGSQTMGK_	2.3651
Fructose-bisphosphate aldolase	A6ZI44	91	_GILAADES(ph)TGSIAKR_	3.0977
FTS and Hook-interacting protein	Q3U2I3	510	_QQS(ph)LGGSESPGPVPR_	10.3070
FYVE, RhoGEF and PH domain-containing protein 6	Q69ZL1	708	_HTS(ph)CTGDFGPEYENVR_	3.0921
Gag protein	Q6YIY0	530	_GPRPQTS(ph)LLTLDD_	2.5379
Gamma-aminobutyric acid (GABA-C) receptor, subunit rho 2	A3KG53	3	_GWS(ph)VLSSRSRR_	85.5850
Gamma-PAK	Q8CIN4	58	_IIS(ph)IFS(ph)GTEK_	6.3941
GAP-related-interacting partner to E12	Q6GYP7	720	_GWS(ph)RDQPGQAPM(ox)R_	9.0278
GAP-related-interacting partner to E12	Q6GYP7	753	_QKT(ph)VDIDDAQILPR_	3.2070
GAP1(IP4BP)	Q60790	809	_YGS(ph)QEHPIGDK_	8.9464
GAPex-5	Q6PAR5	908	_SRS(ph)SDIVSSVR_	2.9128
Gardner-Rasheed feline sarcoma viral (Fgr) oncogene homolog	Q6GTF2	50	_TSSVFPQPTS(ph)PAFLNTGNMR_	2.0713
GATA zinc finger domain-containing protein 2A	Q8CHY6	185	_EATAQKPTASSGSTVTT(ph)PPPLVR_	2.3728
GDI-1	Q99PT1	62	_VAVS(ph)ADPNVPNVIVTR_	16.6590
Gem-associated protein 7	Q9CWY4	3	_(ac)M(ox)QS(ph)PLTIPVPVPVLR_	10.3900
General transcription factor IIE subunit 2	Q9D902	18	_ALS(ph)TPVVEKR_	4.5777

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
General transcription factor IIF, polypeptide 1	Q8R5B7	384	_GT(ph)SRPGTPS(ph)AEAASTSSTLR_	5.9095
General transcription factor IIF, polypeptide 1	Q8R5B7	385	_GT(ph)SRPGTPS(ph)AEAASTSSTLR_	2.9828
Glucocorticoid receptor	Q06VW2	284	_IQDTGDTILSS(ph)PSSVALPQVK_	12.8080
Glucocorticoid receptor DNA-binding factor 1	Q91YM2	589	_NQKNS(ph)LSDLNIDR_	5.4460
Glucocorticoid receptor DNA-binding factor 1	Q91YM2	1150	_KVS(ph)AVSKPVLYR_	2.8754
Glycogen synthase kinase-3 beta	Q9WV60	216	_GEPNVSY(ph)ICSR_	4.4777
Glycylpeptide N-tetradecanoyltransferase 1	O70310	83	_MTS(ph)LPAER_	18.3830
Golgb1 protein	B2RWW2	651	_ASEAGPLNDAGMELS(ph)SPKLDGVDK_	17.3050
Golgb1 protein	B2RWW2	652	_ASEAGPLNDAGMELS(ph)SPKLDGVDK_	3.9792
Golgin subfamily A member 3	P55937	1479	_LHNQNS(ph)VPRDGLGQ_	25.2850
GPN-loop GTPase 1	Q8VCE2	314	_GNAS(ph)PVLDPSDLILTR_	5.0412
GRB2-associated binder 2	Q9Z1S8	143	_S(ph)SPAEFSSS(ph)QHLLR_	11.9620
GRB2-associated binder 2	Q9Z1S8	532	_NNTVIDELPFKS(ph)PVTK_	4.6448
Growth factor-inducible nuclear protein NUP475	P22893	316	_RLPIFNRIS(ph)VSE_	6.4714
Growth factor-inducible nuclear protein NUP475	P22893	178	_QSIS(ph)FSGLPSGR_	2.5378
GRP1 binding protein GRSP1	Q920B1	693	_S(ph)GSLESQSHLLSEMDSDKPFFTLSK_	4.1843
GTPase Ran	P62827	135	_AKS(ph)IVFHR_	4.6278
GTPase-activating protein RAB7	Q9CXF4	32	_ANDQDS(ph)LISGILR_	3.1370
Guanine nucleotide exchange factor H1	Q60875	646	_LES(ph)FESLRGER_	7.5929
Guanine nucleotide exchange factor H1	Q60875	151	_SVS(ph)TTNIAGHFNDESPLGLR_	6.4508
Guanine nucleotide exchange factor H1	Q60875	122	_ERPTS(ph)AIYPSDSFR_	2.2643
Heat shock protein-binding protein 1	Q99P31	349	_LLQTCFSS(ph)PTDDSMDR_	2.6067
Hematological and neurological expressed 1 protein	P97825	87	_SNS(ph)SEASSGDFLDLK_	4.6084
Hepatoma-derived growth factor-related protein 2	Q3UMU9	459	_KRS(ph)EGLSLER_	13.4470
Heterogeneous nuclear ribonucleoprotein F	Q9Z2X1	104	_HSGPNS(ph)ADSANDGFVR_	9.8420
Heterogeneous nuclear ribonucleoprotein H1	Q8C2Q7	104	_HTGPNS(ph)PDTANDGFVR_	8.6986
Heterogeneous nuclear ribonucleoproteins C1/C2	Q3U6P5	138	_MYS(ph)YPAR_	4.4049
High mobility group box transcription factor 1	Q8R316	392	_RASLS(ph)CGGGPGTGQEFSGSEFSK_	8.6150
High mobility group protein 2	P30681	100	_RPPS(ph)AFFLFCSENRPK_	3.6369
Histone acetyltransferase KAT5	Q8CHK4	191	_KVEVVS(ph)PATPVPSETAPASVFPQNGSAR_	2.0130
Histone deacetylase 1	O09106	409	_ISICS(ph)SDKR_	8.7385
Histone deacetylase 1	O09106	410	_ISICS(ph)SDKR_	8.7385
Histone deacetylase 6	Q9Z2V5	21	_HNPQS(ph)PLQESSATLKR_	2.4886
Histone deacetylase complex subunit SAP130	Q8BIH0	416	_VVPQQITHTS(ph)PR_	4.9384
Histone deacetylase complex subunit Sin3a	Q60520	431	_RHS(ph)GTGATPPVK_	12.8260
Histone deacetylase complex subunit Sin3a	Q60520	10	_RLDDQES(ph)PVYAAQQR_	4.7182
Histone demethylase JARID1C	P41230	317	_NHS(ph)NAQFIESYVCR_	2.0268
Histone H3 methyltransferase DOT1 variant b	Q679P5	1247	_STFS(ph)PISDLGLAK_	2.3296
HIV Tat-specific factor 1 homolog	Q8BGC0	404	_HFS(ph)EHPS(ph)MSNMK_	3.6044
HLA-B-associated transcript 2	Q7TSC1	808	_LAWVGDVFTTTPTDPRPLT(ph)SPLR_	2.3972
HLA-B-associated transcript 2	Q7TSC1	807	_LAWVGDVFTTTPTDPRPLT(ph)SPLR_	2.3972
HLA-B-associated transcript 2	Q7TSC1	1217	_LISGPLS(ph)PMSR_	2.2999
Host cell factor C1	B1AUX1	666	_TITLVKS(ph)PISVPGGSALISNLGK_	6.2506
Host cell factor C1	B1AUX1	598	_VASS(ph)PVM(ox)VSNPATR_	3.9100
Host cell factor C1	B1AUX1	2029	_RPMS(ph)SPEMK_	8.4812
HpaII tiny fragments locus 9a protein	P34022	60	_FAS(ph)ENDLPEWK_	3.5266
I-mfa domain-containing protein	Q8BX65	139	_IQSS(ph)LSVNNDISKK_	2.6066
IK cytokine	Q3TJY5	225	_SKS(ph)YERNELFLPGR_	2.7159
Immediate early protein GLY96	P46694	18	_APS(ph)PAPSTGPELR_	3.5283
Inhibitor of kappa B-related protein	Q6NZL6	873	_LTS(ph)LDGWCAR_	5.5815
Inner centromere protein	Q9WU62	862	_TSS(ph)AVWNSPPLK_	7.4017
Inositol 1,4,5-trisphosphate 3-kinase B	B2RXC2	125	_JLS(ph)PPGPEEAQR_	12.7730
Inositol polyphosphate-5-phosphatase of 145 kDa	Q9ES52	173	_LQS(ph)MDTSGLPEEHLK_	21.2010
Inositol polyphosphate-5-phosphatase of 145 kDa	Q9ES52	889	_NLT(ph)SHDPMRQWEPSGR_	8.8406
Inositol polyphosphate-5-phosphatase of 145 kDa	Q9ES52	935	_S(ph)TLSPDQQLTAWSYDQLPK_	8.1042
Integrator complex subunit 1;	Q6P4S8	40	_RPS(ph)AAAKPSGHPPPGDFIALGSK_	7.5409
Integrin beta-7	P26011	798	_SPS(ph)LSLTR_	5.7534
Integrin-linked protein kinase	O55222	181	_NGT(ph)LNKHSGIDFK_	2.8340
Interacting protein of Rab1	Q80U22	525	_M(ox)LS(ph)CPVR_	2.3797
Interleukin-1 receptor-associated kinase-like 2	Q8CFA1	136	_S(ph)LLDTGPIMAGAQR_	8.1867

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Interleukin-1 receptor-associated kinase-like 2	Q8CFA1	506	_GQLS(ph)LPWSR_	4.2326
IQ motif and SEC7 domain-containing protein 1	Q8R0S2	924	_RSS(ph)AGSLESNVEGSIISSPHMR_	4.9152
IQ motif and SEC7 domain-containing protein 1	Q8R0S2	496	_NS(ph)WDSPAFSNDVIR_	2.9294
IQ motif and SEC7 domain-containing protein 1	Q8R0S2	165	_MQFS(ph)FEGPEK_	2.6188
IQ motif and SEC7 domain-containing protein 1	Q8R0S2	74	_LQHSTS(ph)VLRK_	2.4690
IRS-1 PH domain-binding protein	Q8VDD9	1315	_AQS(ph)YDIQAWKK_	34.9850
IRS-1 PH domain-binding protein	Q8VDD9	674	_VNRGS(ph)VSSTSEVHSPPNIGLR_	5.8098
JmjC domain-containing histone demethylation protein 3C	Q8VCD7	477	_ASAVISPS(ph)QLK_	8.2252
JRAB	Q3TN34	936	_LMDKPEGLKS(ph)PQDR_	5.9916
Jun dimerization protein 2	P97875	148	_TDSVRT(ph)PESEGNPLLEQLDKK_	2.2863
Juvenile spermatogonial depletion-like X-linked protein	Q640M1	182	_ART(ph)PLEQEVFNLLHK_	40.1150
Juvenile spermatogonial depletion-like X-linked protein	Q640M1	205	_NKQPVTDPLLT(ph)PVEK_	24.6310
KH and NYN domain-containing protein	Q80U38	214	_EVGSSGLLS(ph)PQFQGVR_	14.6730
Kinesin-associated protein 3	P70188	60	_LKS(ph)LNANTDITSLAR_	16.3070
Kinesin-like protein KIF21B	Q9QXL1	1150	_AVS(ph)AECLGPPLDSSTK_	6.1907
KRAB-A-interacting protein	Q62318	473	_SRS(ph)GEGEVSGLLR_	13.3040
KRAB-A-interacting protein	Q62318	489	_VS(ph)LERLDLDLTSDSQPPVFK_	10.9680
La ribonucleoprotein domain family member 1	Q6ZQ58	801	_HSS(ph)NPPLESHVGWVMDSR_	2.0833
La ribonucleoprotein domain family member 4B	Q6A0A2	570	_NLS(ph)TDASTNTVPVVGPR_	5.8056
Lamin-A/C	P48678	637	_SVGGS(ph)GGGS(ph)FGDNLVTR_	2.7417
Lamin-B1	P14733	21	_ASAPAT(ph)PLSPTR_	4.5088
Lamin-B2	P21619	494	_QVLEGEDIAYKFT(ph)PK_	2.0880
Lamina-associated polypeptide 1B	Q1EQW1	252	_S(ph)SNSLESRDEATPAAGNHPDSLR_	8.6762
Lamina-associated polypeptide 1B	Q1EQW1	201	_TPEASVMNEDPISNLCRPPLRS(ph)PR_	2.9900
Lamina-associated polypeptide 2, isoforms beta/gamma	P42167	278	_IDGAVISES(ph)TPIAETIK_	2.6884
Lariat debranching enzyme	Q923B1	505	_CGETVES(ph)GDEKDLAKFPLK_	5.5790
Lbc's second cousin	Q61210	964	_LRPLLSQLGGTLS(ph)PNLAAPER_	7.0172
Leucine zipper- and sterile alpha motif kinase ZAK	Q9ESL4	638	_SSS(ph)PTQYGLSR_	3.7345
Leucine zipper- and sterile alpha motif kinase ZAK	Q9ESL4	2	_(ac)S(ph)SLGASFVQIK_	18.5530
Leucine-rich repeat serine/threonine-protein kinase 2	Q5S006	935	_HSNS(ph)LGPVFDHEDLLR_	15.1520
Leucine-rich repeat-containing protein 41	Q8K1C9	276	_APS(ph)RDEGSLLLGSR_	25.3140
Leukemia-associated gene protein	P54227	25	_RAS(ph)GQAFELILS(ph)PR_	13.0330
Leukemia-associated gene protein	P54227	38	_SKESVPDFPLS(ph)PPKK_	2.3114
Leukemia-associated RhoGEF	Q8R4H2	190	_IT(ph)SPVLVGEENNVVHNQK_	41.9510
Luzp1 protein	B7ZW89	992	_NMEATNAYTQRS(ph)PTDFLELEQPR_	3.6571
Luzp1 protein	B7ZW89	556	_TFS(ph)DSTHVSVPNDIVGK_	16.9080
Lymphocyte cytosolic protein 2	Q60787	210	_NHSPLS(ph)PPHPNHEEPSR_	2.4209
Lymphocyte-specific helicase	Q60848	498	_S(ph)INYSELDQFPSELEK_	23.1590
M-phase phosphoprotein 8	A6H600	379	_GISNLELNKLPS(ph)PVFAQTLK_	2.4695
Macropain iota chain	Q9QUM9	17	_HITIFS(ph)PEGR_	12.5020
Macropain subunit C8	O70435	250	_ESLKEEDES(ph)DDDNM_	2.2891
Macropain zeta chain	Q9Z2U1	56	_RITS(ph)PLM(ox)EPSSIEK_	6.0051
Mammalian STE20-like protein kinase 1	Q9JI11	174	_LADFGVAGQLTDT(ph)MAK_	7.3359
Mammary gland factor	P42230	128	_EANNCSS(ph)PAGVLVDAMSQK_	12.7460
MAP kinase signal-integrating kinase 1	O08605	344	_GLPT(ph)PQVLQR_	6.8470
MAPK/ERK kinase kinase 1	P53349	502	_SHDFYSHELSS(ph)PVESPASLR_	2.5762
MAPK/ERK kinase kinase 3	Q61084	166	_HLS(ph)VSSQNPGR_	4.1165
Maternal-embryonic 3	Q9EQH3	7	_PTTQQS(ph)PQDEQEKLLDEAIQAVK_	8.3760
Matrin-3	Q8K310	188	_RDS(ph)FDDRGPSLNPVLDYDHGSR_	8.8611
Matrin-3	Q8K310	533	_MKS(ph)QAFIEMETR_	5.0572
MCG15924, isoform CRA_a	O70349	890	_DKGPAT(ph)PDVPHPDDLIGFK_	13.9080
Mediator of DNA damage checkpoint protein 1	Q5PSV9	1299	_TPEASVPTT(ph)PELQPFTSK_	4.4493
Metastasis inhibition factor NM23	P15532	122	_NIIHGSDS(ph)VK_	5.6600
Metastasis-associated protein MTA1	Q8K4B0	538	_LPEASQS(ph)PLVLK_	2.2262
Methyltransferase-like protein 1	Q9Z120	21	_AHS(ph)NPMADHTLR_	19.2400
MICAL-like protein 1	Q8BGT6	305	_KAS(ph)ESSALTPPTPR_	4.9360
Microphthalmia-associated transcription factor	Q08874	414	_AHGLSLIPSTGLCS(ph)PDLVNR_	2.1836
Microrchidia 3	A6H605	791	_RQCS(ph)ALQQVK_	21.5620
Microsomal endopeptidase	Q91YP2	32	_EAAS(ph)PLQAMSSYTAAGR_	9.0561
Microtubule-actin crosslinking factor 1	B1ARU4	2823	_KIS(ph)VEMEGQR_	7.0353

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Microtubule-actin crosslinking factor 1	B1ARU4	1376	_M(ox)IS(ph)SSDAITQEFMDLR_	14.7100
Microtubule-actin crosslinking factor 1	B1ARU4	3889	_QGS(ph)FSEDVISHK_	7.7334
Microtubule-actin crosslinking factor 1	B1ARU4	534	_KGHFS(ph)SLELVPPSTLTTTHLK_	27.5100
Microtubule-actin crosslinking factor 1	B1ARU4	533	_KGHFS(ph)SLELVPPSTLTTTHLK_	5.7404
Microtubule-actin crosslinking factor 1	B1ARU4	4611	_RQQHEQLNEAAQGILTGPGDMS(ph)PSASQVHK_	3.4793
Microtubule-associated protein 4	P27546	475	_DMS(ph)PLPESEVTLGKDVVILPETK_	3.0097
Microtubule-associated protein 4	P27546	517	_VAEFNNVTPLSEEEVTSVKDMS(ph)PSAETEAPLAK_	2.0439
Microtubule-associated protein 4	P27546	847	_NTT(ph)PTGAAPPAGM(ox)TSTR_	2.4528
Microtubule-associated serine/threonine-protein kinase 2	Q60592	1037	_LLS(ph)GDSIEKR_	11.9500
Microtubule-associated serine/threonine-protein kinase 2	Q8C0P0	389	_EIS(ph)WEARDPDNENMTIDK_	4.1520
Microtubule-interacting protein associated with TRAF3	Q149C2	316	_KLS(ph)DGSFKDVK_	6.5292
Microtubule-interacting protein associated with TRAF3	Q149C2	319	_KLS(ph)DGSFKDVK_	2.9619
Minor histocompatibility protein HA-1	Q3TBD2	886	_QGGS(ph)ESEAATLAMVGR_	19.2090
Mitochondrial dynamics protein MID51	Q8BGV8	58	_AISAPT(ph)SPTR_	2.0966
Mitochondrial import receptor subunit TOM22 homolog	Q9CPQ3	15	_(ac)AAAVAAAGAGEPLS(ph)PEELLPK_	19.2940
mJumpy	Q8VEL2	527	_MGS(ph)SPLEVPKPR_	3.2080
MKIAA0177 protein	Q6A0B1	1452	_FSQGPNNISFS(ph)PK_	10.8950
MKIAA0380 protein	Q5DU33	702	_SLENPTPPFT(ph)PK_	8.6698
MKIAA0629 protein	Q6ZQ80	1563	_ITYAEKLS(ph)PLINEACR_	2.4476
MKIAA0826 protein	Q8CHD0	2273	_AYGVDVGS(ph)PEISFAK_	4.6838
MKIAA0857 protein	Q6ZQ33	626	_ASLAPLAS(ph)PGK_	5.2543
MKIAA0857 protein	Q6ZQ33	1313	_IM(ox)ETSPTLLQIS(ph)PGPPK_	14.9890
MKIAA0857 protein	Q6ZQ33	1212	_T(ph)SLSTALSSGLER_	8.2751
MKIAA1250 protein	Q80TG7	1702	_T(ph)PSTVTLNNNTAPTNR_	4.6090
MKIAA1574 protein	Q69ZG0	582	_T(ph)PENLITDIR_	14.4110
MKIAA1991 protein	Q69Z47	679	_RKGS(ph)VDQYLLR_	4.3570

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Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
MKL/myocardin-like protein 2	P59759	77	_EQLVDQGIMPPLKS(ph)PAAFHEQIK_	9.0808
Mll2 protein	Q3USG3	2299	_ASQVEPQS(ph)PGLGLR_	2.0722
Motor domain of KIF13B	O35063	1409	_GRWES(ph)QQDVSQTLVSR_	2.2670
mRNA (guanine-N(7)-)-methyltransferase	Q9D0L8	34	_LPENT(ph)PPCQQVDQPK_	5.8982
Mszf35	O88252	223	_MEDVAPVLS(ph)PR_	2.9084
Mszf6	O88217	148	_VLPVS(ph)PGAFQNQK_	7.4075
MutY homolog (E. coli)	A2AGE3	27	_ALS(ph)SSQAKPSSLDGLAK_	14.6350
Myc-binding protein 2	Q7TPH6	3550	_SYS(ph)VVASEYDKQHSILPAR_	2.8259
Myc-induced SUN domain-containing protein	Q1HFZ0	723	_KKEGVILTNENAAS(ph)PEQPGDEDAK_	6.9403
Myelin A1 protein	P04370	112	_DRPS(ph)ESDELQTIQEDPTAASGGLDVMASQK_	3.7140
Myelin expression factor 2	Q8C854	511	_LGS(ph)KGNQIFVR_	18.9740
Myocyte-specific enhancer factor 2D	Q63943	121	_RAS(ph)EELDGLFRR_	8.4850
Myomegalin	Q80YT7	195	_SIS(ph)YAPSSR_	10.9260
Myosin phosphatase Rho-interacting protein	P97434	617	_MDIDRS(ph)PGLLGTPDLK_	3.9940
Myosin phosphatase-targeting subunit 1	Q9DBR7	299	_S(ph)PLIESTANMENNQPQK_	42.7770
Myosin regulatory light chain 2, smooth muscle isoform	Q9CQ19	19	_AT(ph)SNVFAMFDQSQIQEFK_	6.6230
Myosin-Ixb	Q9QY06	2036	_GRPTS(ph)FVTVR_	3.2166
Myotubularin-related protein 3	Q8K296	8	_(ac)MDEEMRHS(ph)LECIQANQIFPR_	3.7995
N-myc downstream-regulated gene 1 protein	Q62433	330	_TAS(ph)GSSVTSLEGTR_	3.8189
NAD kinase	P58058	62	_T(ph)RSLHGPCPVTTFGPK_	3.7584
NAD kinase	P58058	64	_T(ph)RSLHGPCPVTTFGPK_	3.5019
NAD kinase	P58058	48	_SRSLS(ph)ASPALGSTK_	2.1757
Nck-associated protein 5-like	Q6GQX2	494	_NSGSDGSPS(ph)PLLAR_	5.7340
Ndrg3 protein	Q8VCV2	344	_THS(ph)TSSSIGSGESPFSR_	2.1261
NEDD4-binding protein 1	Q6A037	299	_KQFS(ph)LENVPEGELLPDGK_	3.3362
Negative elongation factor E	P19426	131	_S(ph)LYESFVSSSDR_	3.3573
Nek1 protein	B2RXX0	251	_DRPSVNS(ph)ILEK_	102.6600
Nercc1 kinase	Q8K1R7	800	_GTMEADRGMEGLIS(ph)PTEAVGNSCGASSSCPGWLR_	16.0620

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Neurabin-2	Q6R891	100	_ASS(ph)LNENVDHSALLK_	29.8050
Neurite outgrowth inhibitor	Q99P72	344	_VVKEDGVMS(ph)PEKTMDIFNEM(ox)K_	2.6404
Neuron navigator 1	Q8CH77	998	_GQLTNIVS(ph)PTAATTPR_	3.4660
Neuropathy target esterase	Q3TRM4	405	_CIS(ph)MPVDISGLQGGPR_	9.3911
Neutrophil cytosol factor 2	O70145	332	_LQLS(ph)PGHK_	8.4491
Neutrophil cytosol factor 2	O70145	385	_LALS(ph)PEHTK_	5.6173
Neutrophil cytosol factor 4	P97369	161	_IKGVS(ph)PQGAIMDR_	6.2677
NF-E2 inducible protein	Q76LS9	103	_VETAEVCSRPQELPQS(ph)PR_	7.0833
NIK- and IKBKB-binding protein	Q3U0M1	953	_MAIQVDKFNFESVPES(ph)PGEKGHFANLK_	6.0856
NSFL1 cofactor p47	Q9CZ44	178	_RRHS(ph)GQDVHVVLK_	2.1097
Nuclear cap-binding protein subunit 1	Q3UYV9	22	_TS(ph)DANETEDHLESLICK_	4.8832
Nuclear fragile X mental retardation-interacting protein 2	Q5F2E7	649	_NDS(ph)WGSFDLR_	2.1338
Nuclear mitotic apparatus protein 1	Q3TH77	398	_LQES(ph)PAPEKGEVLGDALQLDTLKQEAAK_	5.4628
Nuclear mitotic apparatus protein 1	Q3TH77	1844	_LGS(ph)PDDGNSALLSLPGY(ph)RPT(ph)TR_	5.0114
Nuclear protein 220	Q61464	1571	_KEALKISPS(ph)PELNLK_	2.6955
Nuclear protein 220	Q61464	381	_DYQSEADLPIRS(ph)PFGIVK_	25.9600
Nuclear receptor coactivator 6	Q3UQD5	2024	_RNS(ph)RTEEPTMASESVENGHR_	2.0875
Nuclear receptor coactivator 6-interacting protein	Q923W1	103	_MS(ph)SHENFEMSMNAR_	12.6530
Nuclear SET domain-containing protein 2	Q8BVE8	605	_NRAS(ph)ATASSALGFNK_	10.7660
Nuclear transcription factor, X box-binding protein 1	B1AY10	81	_S(ph)PQGFFQSSNK_	2.0843
Nuclear-interacting partner of ALK	Q80YV2	343	_SQDATVSPGSEQSEKS(ph)PGPIVSR_	3.3738
Nucleolar protein 1	Q922K7	59	_AGS(ph)VDVPKPNKSPGIK_	7.8362
Nucleolar protein 56	Q9D6Z1	554	_RKFS(ph)EEPEVAANFTK_	16.8010
Nucleolar protein EMG1 homolog	O35130	16	_RFS(ph)VQEQDWET(ph)TPPKK_	2.6074
Nucleolin	P09405	307	_VEGSEPTT(ph)PFNLFIGNLNPNK_	24.4820
Nucleolin	P09405	616	_GFGFVDFNS(ph)EEDAKAAK_	8.5657
Nucleoporin 153	Q3TA10	339	_RIPSAVS(ph)SPLNS(ph)PLDR_	12.8410
Nucleoporin 153	Q3TA10	520	_VQMTSLGSTGS(ph)PVFTFSSPIVK_	4.3421
Nucleoporin 98	B2RQL0	79	_PIPQT(ph)PESVGNK_	3.4793
Nucleoporin Nup107	Q8BH74	11	_SGFGGMS(ph)SPVIR_	3.3876
Nucleoporin Nup133	Q8R0G9	71	_IFPHHS(ph)ISESVNYDVR_	11.7200
Nucleoporin Nup133	Q8R0G9	40	_RGLSLGS(ph)AVNSPVLFSPAGR_	2.1082
Nucleoporin Nup214	Q80U93	964	_SAFLS(ph)QR_	11.2080
Nucleoporin Nup214	Q80U93	492	_SSAS(ph)VTGEPPLYPTGSDSSR_	3.7366
Nucleoporin Nup35	Q8R4R6	258	_GVLSS(ph)PSLAFTT(ph)PIR_	3.1062
O-phosphoserine phosphohydrolase	Q99LS3	3	_MVS(ph)HSELRK_	6.8846
Oxysterol-binding protein-related protein 3	Q9DBS9	272	_LHSS(ph)NPNLSTLDFGEEK_	6.9326
Oxysterol-binding protein-related protein 3	Q9DBS9	340	_QLMELDTSPS(ph)PSAQVVGLK_	3.7611
p150 target of rapamycin (TOR)-scaffold protein	Q8K4Q0	863	_ILDTSSLTQS(ph)APAS(ph)PTNK_	9.1376
p80	Q62311	532	_AAAPPQPS(ph)PPPTK_	7.9577
p94-Fer	P70451	434	_HSIAGIIKS(ph)PK_	9.5165
PAI1 RNA-binding protein 1	Q9CY58	234	_GGSGSHNWGTVKDELTES(ph)PKYIQK_	2.1421
PAI1 RNA-binding protein 1	Q9CY58	329	_S(ph)KSEEAHAEDSVM(ox)DHHFR_	15.3280
PAI1 RNA-binding protein 1	Q9CY58	327	_S(ph)KSEEAHAEDSVM(ox)DHHFR_	14.3730
PAP-associated domain-containing protein 5	Q68ED3	592	_VGS(ph)QDVSLEVSQAVGK_	10.2850
Paxillin	Q8VI36	83	_YAHQQPPS(ph)PLPVYSSSAK_	5.9487
Pb1 protein	Q8R134	39	_RRLS(ph)NLPTVDPIAVCHELYNTIR_	5.0894
PCF11	A5HLW0	785	_IDGPPT(ph)PGSLR_	10.6010
PCF11	A5HLW0	777	_MIFEGPNKLS(ph)PR_	2.5405
PDH-containing protein JUNE-1	Q8BSN5	398	_RLGGLPKS(ph)GEP_	4.8712
PDZ and LIM domain protein 2	Q8R1G6	210	_FSS(ph)LDLEEDSEVFK_	21.1050
PDZ and LIM domain protein 2	Q8R1G6	199	_VLLHS(ph)PGRPS(ph)SPR_	2.4060
Pericentriolar material 1 protein	Q9R0L6	93	_YM(ox)TQMS(ph)VPEQAELEK_	7.4516
PH domain leucine-rich repeat-containing protein phosphatase 2	Q8BXA7	1207	_RQNS(ph)VNSGILLPANR_	2.2550
PH domain-containing adaptor PHAD47	Q9ERS5	314	_SIS(ph)LTRPGSSTLTSAPNSILSR_	4.6209
Phf3 protein	A0PJJ1	1909	_FYS(ph)DSHHLKR_	50.2070
Phf3 protein	A0PJJ1	1529	_NSGEETSSVGSSSIS(ph)PGPLASLSLR_	16.2510
Phf3 protein	A0PJJ1	1882	_RHS(ph)DPWGR_	5.8488
Phosphatase 2A inhibitor I2PP2A	Q9EQU5	28	_S(ph)AS(ph)PGLPKGEK_	4.0505
Phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase 2	Q6P549	241	_VFDQQSS(ph)PM(ox)VTR_	2.8036

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Phosphatidylinositol 4-kinase type 2-alpha	Q2TBE6	462	_SAS(ph)ESYTQSFQSR_	4.7681
Phosphoacetylglucosamine mutase	Q9CYR6	64	_STIGVM(ox)VTAS(ph)HNPEEDNGVK_	6.5139
Phosphoglucomutase 2	A2CEK3	133	_AIGGIILT(ph)ASHNPGGPNGDFGIK_	6.0035
Phosphoinositide 3-kinase-C2-alpha	Q61194	1553	_SAGAVPFS(ph)PTLGQIGGAVK_	25.1520
Phosphoinositide 3-kinase-C2-alpha	Q61194	261	_ASVCNLQIS(ph)PK_	5.2042
Phospholipase C, beta 4	A2AT93	263	_DGPQTSNSS(ph)MKLQSAN_	2.4159
Phostensin	Q8BQ30	194	_WRLS(ph)PGETPEESLR_	2.5487
Pinin	Q3TUQ5	68	_RGFSDS(ph)GGGPPAK_	19.8540
Plastin-2	Q61233	84	_VFHGLKST(ph)EVAK_	23.1750
Plastin-2	Q61233	291	_ITNFST(ph)DIKDSK_	9.4221
Plastin-2	Q61233	5	_MARGS(ph)VSDEEMMELR_	6.9467
Pleckstrin homology domain-containing family G member 5	Q66T02	938	_SKS(ph)EASLLQLLSGTPAAR_	9.5467
Pleckstrin homology domain-containing family M member 1	Q7TSI1	466	_SAAGLCTS(ph)PVQDTPESR_	15.9130
Pleckstrin homology domain-containing family M member 3	Q8BM47	150	_S(ph)RSDVTHVDWR_	5.5934
Pleckstrin homology domain-containing family M member 3	Q8BM47	350	_SSGLLAS(ph)PVLDSPK_	4.7470
Plectin-1-10	Q9QXS1	4261	_T(ph)QLASWSDPTEETGPVAGILDTETLEK_	5.6297
Plectin-1-10	Q9QXS1	3889	_QIT(ph)VEELVR_	2.0613
Plenty-of-prolines 101	Q52KI8	387	_RLS(ph)PSASPPR_	12.9070
Plenty-of-prolines 101	Q52KI8	389	_RLS(ph)PSASPPR_	4.3213
Poly [ADP-ribose] polymerase 12	Q8BZ20	273	_DSSGPVS(ph)PGTPSQEESEQICLYHIR_	2.1442
Polycomb protein EED	Q921E6	61	_NMS(ph)EREVSTAPAGTDMPAAK_	4.8952
Polypyrimidine tract binding protein 1	Q3U5I2	437	_HQS(ph)VQLPR_	7.5275
PP2A B subunit isoform B56-alpha	Q3ULN8	53	_SQGS(ph)QAELHPLPQLK_	15.0220
PP2A B subunit isoform B56-epsilon	Q61151	7	_(ac)SSAPTT(ph)PPSVDKVDGFSR_	2.2873
PR domain containing 2, with ZNF domain	A2A7B5	1395	_RLS(ph)FNVELSK_	8.3566
Programmed cell death protein 4	Q61823	457	_RFVS(ph)EGDGGR_	22.3480
Programmed cell death protein 4	Q61823	94	_SGVAVPTS(ph)PKGR_	7.4252
Proline-rich AKT1 substrate 1	Q9D1F4	318	_LNT(ph)SDFQK_	8.2387
Proteasome inhibitor PI31 subunit	Q8BHL8	189	_QPAWRDPLS(ph)PFAVGGDDLDPFGCQR_	4.8460
Protein A6	Q91YR1	143	_YLLSQSS(ph)PAPLTAAEEELR_	4.8856
Protein capicua homolog	Q924A2	1644	_SAAAATS(ph)PAPHLVAGPLLGTVGK_	19.6180
Protein capicua homolog	Q924A2	1625	_VPGGS(ph)PMGVSLVYSDKK_	14.0570
Protein capicua homolog	Q924A2	1403	_KVFS(ph)PVIR_	2.0093
Protein DDI1 homolog 2	A2ADY9	194	_LFS(ph)ADPFDLEAQAK_	32.6830
Protein FAM114A2	Q8VE88	119	_AETSLGIPS(ph)PTEISAEVK_	25.3730
Protein FAM122A	Q9DB52	144	_RIDFIPVS(ph)PAPS(ph)PTR_	6.3232
Protein FAM126B	Q8C729	564	_ELLS(ph)PGAPLTK_	13.5080
Protein FAM193A	Q8CGI1	648	_RPPS(ph)IGDVFHGLNK_	2.6787
Protein FAM86A	Q3UZW7	212	_GNVLLNGFSLEPHT(ph)PIDAGSSK_	19.7820
Protein ftsJ homolog 3	Q9DBE9	633	_GRGS(ph)KADEDGFEVVPIQDPVK_	5.0952
Protein HIRA	Q61666	659	_LLPMSLSVQS(ph)PAALSTEK_	9.4993
Protein incorporated later into tight junctions	Q9DCD5	407	_AFVDRT(ph)PPPAAVVQR_	2.0857
Protein kinase C-like 1	Q3UEA6	545	_LIPSAVATGTFS(ph)PNAS(ph)PGAEIR_	10.5040
Protein kinase C-like 2	Q8BWW9	619	_S(ph)KSEYELSIPDSGR_	19.3420
Protein kinase C-like 2	Q8BWW9	305	_SS(ph)VVIEELSLVAS(ph)PTLS(ph)PR_	3.1874
Protein LAP4	Q80U72	821	_MVEPENAVTITPLRPEDDYS(ph)PR_	3.0921
Protein lin-54 homolog	Q571G4	264	_AVTGQTTQAS(ph)PPVVTGR_	10.8030
Protein lin-54 homolog	Q571G4	314	_IAIS(ph)PLKS(ph)PNK_	2.4211
Protein lin-9 homolog	Q8C735	65	_LFS(ph)DEDDRQINTK_	4.0034
Protein lunapark	Q7TQ95	411	_ADS(ph)VPNLEPSEESLVTK_	8.9938
Protein LYRIC	Q80WJ7	491	_TM(ox)S(ph)TSDPAEVLIK_	4.0607
Protein LYRIC	Q80WJ7	96	_KREEAAPPT(ph)PAPDDLAQLK_	2.5546
Protein NEDD1	P33215	411	_NSLGDMFS(ph)PIRDDAVVSK_	17.2010
Protein NEDD1	P33215	379	_SKS(ph)TDIFSK_	7.1658
Protein numb homolog	Q9QZS3	636	_TNPS(ph)PTNPFSSDLQK_	16.4200
Protein RIC1 homolog	Q69ZJ7	1171	_SQS(ph)WLSNIGPTHR_	13.7530
Protein RW1	O70472	2032	_IWS(ph)PTVGR_	5.6217
Protein Smaug homolog 2	Q80XS6	585	_M(ox)GLLS(ph)PSGIGGVSPR_	5.0295
Protein SON	Q9QX47	94	_CVS(ph)VQTDPTDEVPTKK_	6.5585
Protein tyrosine phosphatase, non-receptor type 12	Q80UM4	448	_KVPLQEGPKS(ph)FDGNTLLNR_	5.1905

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
Protein tyrosine phosphatase, non-receptor type 12	Q80UM4	434	_NLS(ph)FEIKK_	2.0152
Protein tyrosine phosphatase, non-receptor type 12	Q80UM4	748	_S(ph)PAEVTDIGFGNR_	10.0730
Protein tyrosine phosphatase, non-receptor type 12	Q80UM4	746	_DQIT(ph)KSPAEVTDIGFGNR_	3.8555
Protein tyrosine phosphatase, non-receptor type 22 (Lymphoid)	B0V3P7	450	_T(ph)KSTPFELIQQR_	9.2823
Protein tyrosine phosphatase, non-receptor type 22 (Lymphoid)	B0V3P7	634	_TPESFIVVEEAGEPS(ph)PR_	3.7353
Protein Wiz	O88286	1015	_GLTHPSS(ph)SPLLKK_	5.6918
PtdIns-4,5-P2 4-Ptase I	Q3TWL2	169	_IINLGPVHPGPLS(ph)PEPQPMGVR_	4.9231
Putative RNA-binding protein 15B	Q6PHZ5	559	_RNS(ph)LEGYSR_	3.9249
Putative uncharacterized protein	Q3U048	557	_SSSQSTFHIPLS(ph)PVEVKPGNVR_	3.3073
Putative uncharacterized protein	Q3U224	564	_S(ph)TRVPGTDAAAQAEDLNVK_	13.2600
Putative uncharacterized protein	Q3U224	34	_EQPPPLQS(ph)PPQSLR_	6.1908
Putative uncharacterized protein	Q3UUE0	540	_VEADVAPPQVQGDLKT(ph)PDLSVQLPSADLELK_	74.7370
Putative uncharacterized protein	Q8BJC6	71	_S(ph)PTGAQPAAAKPPPLSAK_	4.5726
Pyk2/RAFTK-associated protein	Q3UND0	5	_(ac)PNPS(ph)CTS(ph)SPGPLPEEIR_	7.9606
Pyk2/RAFTK-associated protein	Q3UND0	8	_(ac)PNPS(ph)CTS(ph)SPGPLPEEIR_	7.3893
Pyridoxal-dependent decarboxylase domain-containing protein 1	Q99K01	691	_VQGTGVT(ph)PPPT(ph)PLGTR_	2.5262
Pyruvate kinase isozymes M1/M2	P52480	6	_PKPHS(ph)EAGTAFIQTQQLHAAMADTFLEHMCR_	3.4987
R3H domain-containing protein 2	Q80TM6	381	_ASS(ph)FSGISILTR_	3.5321
R3hdm1 protein	B9EHE8	299	_DRIFS(ph)QDSLCSQENYIIDKR_	5.8272
Rab GTPase-binding effector protein 1	O35551	407	_RAQS(ph)TDSLGTSSSLQSK_	3.0421
Rab-3A-interacting protein	Q68EF0	218	_TLVLSSSPTS(ph)PTQEPLAAAK_	3.7894
RAB3 GTPase-activating protein 130 kDa subunit	Q3TPB6	536	_KTS(ph)LSDSTTSAYPGDAGK_	7.8367
Rab3 GTPase-activating protein 150 kDa subunit	Q8BMG7	449	_GGFS(ph)PFGNTQGPSR_	27.7580
RAB34, member of RAS oncogene family	B1AQD4	247	_INS(ph)DDKNLYLTASK_	3.1560
Rab4-interacting protein	Q8BIJ7	52	_SQLPS(ph)PGELR_	9.9709
RACK1	P68040	276	_IIVDELKQEVIS(ph)TSSK_	3.1613
RACK1	P68040	316	_VWQVTIGT(ph)R_	2.5862
Rap1-interacting factor 1 homolog	Q6PR54	1051	_LLEEEKS(ph)TDFVFIPPEGK_	4.0256
Rap1-interacting factor 1 homolog	Q6PR54	1050	_LLEEEKS(ph)TDFVFIPPEGK_	3.6671
Rap1-interacting factor 1 homolog	Q6PR54	1535	_RAS(ph)QGLISAVENSESDSSEAKEEVSR_	2.8987
Rapgef6 protein	B2RUJ6	1076	_S(ph)LSQGSTNSNMLDVQGGAHK_	2.1923
Rapgef6 protein	B2RUJ6	1078	_S(ph)LSQGSTNSNMLDVQGGAHK_	2.1139
Ras and Rab interactor 3	B2RQF8	501	_QATGASWEGLS(ph)PVR_	3.2964
RAS protein activator like 2	Q0VAV5	810	_LKS(ph)PSQDNTDSYFR_	6.4198
RAS protein activator like 2	Q0VAV5	1101	_QQTQQVQSPVDSATMS(ph)PVER_	2.1460
RAS protein activator like-3	Q8C2K5	73	_VLS(ph)APPKESR_	16.9760
RAS protein activator like-3	Q8C2K5	52	_ALS(ph)HQEPMVNSQPAPR_	3.4836
Ras-related protein Rab-1B	Q9D1G1	179	_MGPGAAS(ph)GGERPNLK_	2.7575
Regulation of nuclear pre-mRNA domain-containing protein 2	Q6NXI6	1117	_RMS(ph)GEPIKTVESIR_	5.1425
Regulator of G-protein signaling 10	Q9CQE5	16	_KRPPS(ph)DIHDGDGSSSSGHQSLK_	24.6330
Regulator of ubiquitous kinase	Q8R550	274	_S(ph)IEVENDFLPVEK_	4.5028
Retinoblastoma-like protein 1	Q64701	350	_LTSQAS(ph)VECNLQQHFEK_	13.0210
Rho GTPase activating protein 1	A2AGT9	91	_S(ph)SSPEPVTHLKWDDPYYDIAR_	13.2550
Rho GTPase activating protein 14	Q812A2	837	_NDLQS(ph)PTEHISDYGFGGVMGR_	24.3800
Rho GTPase activating protein 14	Q812A2	858	_LRS(ph)DGAAIPR_	2.9898
Rho GTPase activating protein 25	Q8BYW1	363	_SKDAPIS(ph)PPAQK_	5.6758
Rho GTPase activating protein 30	Q640N3	876	_GVGDHLEEGALSEGPGVELLRVDS(ph)TEEINEQTSEMK_	6.2132
Rho GTPase activating protein 30	Q640N3	966	_IHVAPAS(ph)PCPRPGR_	3.5546
Rho GTPase activating protein 30	Q640N3	996	_NGGSLS(ph)FDAAVALAR_	2.2490
Rho GTPase activating protein 5	B9EKC3	591	_LYHDS(ph)TNIDKVNLFILGK_	23.5020
Rho GTPase activating protein 5	B9EKC3	590	_LYHDS(ph)TNIDKVNLFILGK_	16.3200
Rho GTPase activating protein 5	B9EKC3	968	_ESTHQSEDVFLPS(ph)PR_	3.3943
Rho GTPase-activating protein 27	A2AB59	195	_PLAPS(ph)DSENVYEAIPDLR_	16.2680
Rhomboid family member 2	Q80WQ6	83	_RQAS(ph)LSQSIR_	2.7136
Ribosomal protein S6 kinase alpha-3	P18654	386	_GFS(ph)FVAIT(ph)SDDESQAMQTVGVHSIVQQLHR_	5.4217
Ribosomal protein S6 kinase polypeptide 1	Q3TIM6	28	_SRIS(ph)QTLPGPGPGPQQDSDEAILK_	2.2021
Ribosomal protein S6 kinase polypeptide 1	Q3TIM6	380	_GFS(ph)FVATGLMEDDGKPR_	3.4219
Ribosomal protein S6 kinase polypeptide 1	Q3TIM6	577	_AENGLLM(ox)T(ph)PCYTANFVAPEVLKR_	23.5260
Ribosomal RNA processing protein 1 homolog B	Q91YK2	672	_SILVS(ph)PTGLSR_	2.4447
RIO kinase 3	Q9DBU3	512	_KAASFLKDDGS(ph)PPVLSAD_	5.1255

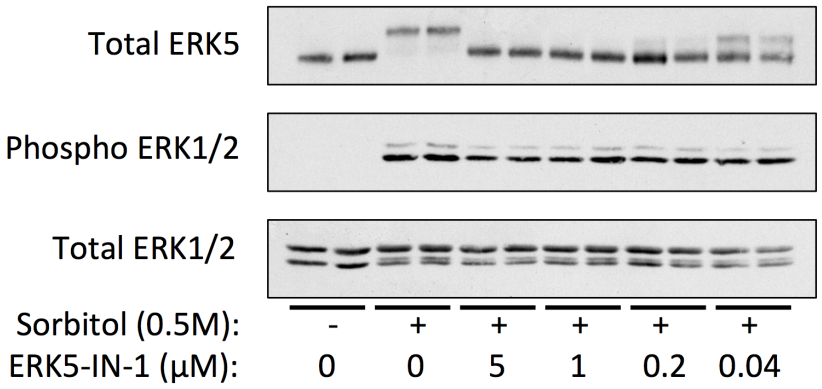
Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
RNA polymerase II subunit A C-terminal domain phosphatase	Q7TSG2	830	_RQPS(ph)MSEAMPLYTLCK_	12.0530
RNA polymerase II-associated protein 3	Q9D706	88	_IKS(ph)YDYDAWAK_	19.1660
RNA-binding motif protein 14	Q8C2Q3	618	_RLS(ph)ESQLSFR_	2.2791
RNA-binding motif protein 14	Q8C2Q3	256	_AQPS(ph)ASLGVGYR_	3.7682
RNA-binding motif protein 14	Q8C2Q3	280	_AQPSVS(ph)LGAPYR_	2.1477
RNA-binding motif protein 7	Q9CQT2	125	_TVGNVS(ph)PTAQMVQR_	5.4646
RNA-binding motif protein 7	Q9CQT2	136	_SFS(ph)SPEDYQR_	3.5978
S100P-binding protein	Q9D5K4	294	_MFS(ph)QSELEK_	17.6010
Salt-inducible kinase 3	Q6P4S6	1218	_NKVPS(ph)RESVLGNCLER_	2.1122
SAPS domain family member 1	Q7TSI3	739	_EADMSSIQIPS(ph)SPPAHGS(ph)PQLR_	2.2484
Sarcolemmal membrane-associated protein	Q3URD3	148	_LRSDVIHAPLPS(ph)PVDK_	10.4710
SEC16 homolog A (S. cerevisiae)	A2AIX1	2101	_APS(ph)LTSDSEGKKPAQAVK_	2.8870
Sec23ip protein	A0AUN0	600	_S(ph)PGSVAVSNGVIK_	4.1039
SECIS binding protein 2	Q3TGQ4	229	_LDFPELQS(ph)PK_	13.0950
Serine/arginine repetitive matrix protein 2	Q8BTI8	2224	_TPAAAAAMNLAS(ph)PR_	4.2931
Serine/threonine kinase 10	A1A553	416	_SRPLS(ph)MDAR_	5.2346
Serine/threonine-protein kinase 24	Q99KH8	4	_(ac)AHS(ph)PVQSGLPGMQNLKADPEELFTKLEK_	15.1790
SH3 domain-binding protein 1	P55194	182	_SLTSLDTALAELRDNHNQADHS(ph)PLTTAAPFSR_	2.8813
SH3 domain-binding protein 1	P55194	553	_RLPAS(ph)PVISNMPAQVDQGVATEDR_	3.7840
Shootin-1	Q8K2Q9	494	_LTAEADSS(ph)SPTGILATSESK_	5.5390
Shootin-1	Q8K2Q9	3	_(ac)MNS(ph)SDEEKQLQLITSLK_	2.6554
Signal transducer and activator of transcription 1	P42225	733	_LQTTDNLLPMS(ph)PEEFDEMSR_	3.7019
Smad-interacting protein 1	Q9R0G7	647	_GLTS(ph)PINPYKDHM(ox)SVLK_	5.8764
Smith-Magenis syndrome chromosomal region candidate gene 8 protein	Q3UMB5	416	_VLISVGS(ph)YK_	3.0089
SNARE-associated protein Snapin	Q9Z266	14	_(ac)AAAGSAAVSGAGT(ph)PVAGPTGR_	2.8061
Solute carrier family 25 member 46	Q9CQS4	45	_SFGSGTELGHWVTT(ph)PPDIPGSR_	5.3396
Solute carrier family 7 member 6	Q8BGK6	8	_(ac)MEAQELGS(ph)PTPTYHLLPK_	8.8644
Son of sevenless homolog 1	Q62245	1120	_SAS(ph)VSSISLSK_	8.8987
Sorting nexin-2	Q9CWK8	104	_ELILSSEPSPAVTPVT(ph)PTTLIAPR_	13.5560
Sorting nexin-9	Q91VH2	175	_SSS(ph)PYFKDSEPAEAGGIQR_	2.6030
Spectrin alpha 2	A3KGU5	1197	_WRS(ph)LQQLAEER_	3.8006
SPEN homolog, transcriptional regulator (Drosophila)	A2ADB0	1075	_LNS(ph)ALSPK_	10.0980
Spleen tyrosine kinase	P48025	270	_IGAQMGHPGS(ph)PNAHPVT(ph)WSPGGIISR_	10.6490
Splicing factor that interacts with PQBP-1 and PP1	Q923D5	600	_GATAVPQRRS(ph)EDDSAVPVAK_	37.8100
Striatin	O55106	259	_AKS(ph)VIDTSTIVR_	2.2206
Sucrose nonfermenting protein 2 homolog	Q91ZW3	136	_QNLLS(ph)VGDYR_	3.6756
Sugen kinase 223	Q571I4	131	_GLQKPAS(ph)PLACTDGNSR_	2.8420
Sugen kinase 223	Q571I4	472	_TIYLSS(ph)PDSAVGVQWPR_	2.5135
Sugen kinase 223	Q571I4	802	_AAS(ph)SPDGFFWTQGS(ph)PKPR_	2.0800
Symplekin	Q80X82	510	_RLS(ph)VQGQAISVVGSQSTMS(ph)PLEEEVPQAK_	57.7190
Syntaxin-4	P70452	36	_LGS(ph)PDDEFFQK_	2.6491
SZT2	A2A9C3	1115	_SSIS(ph)AQPPQWHCYAR_	15.1750
SZT2	A2A9C3	2433	_ANTFPCTPVSGEPVT(ph)PPSK_	7.8526
Target of EGR1 protein 1	Q9D2E2	349	_S(ph)LQSQPGTQTLAEAEDGPPTK_	10.9010
Target of Myb protein 1	Q3UDC3	160	_KGLEFPMTDLDMLS(ph)PIHTPQR_	38.5490
Targeting protein for Xklp2	A2APB8	482	_VIPAT(ph)VPKSPVFALK_	4.4393
TBC1 domain family member 5	Q3U325	546	_SES(ph)MPVQLNK_	4.7294
TBC1 domain family, member 17	Q3TCA5	20	_GGVYLHT(ph)SARK_	17.8180
TBP-associated factor 3	Q5HZG4	669	_EM(ox)ALPLFS(ph)PSAVR_	12.7080
Telomeric repeat-binding factor 2-interacting protein 1	Q91VL8	43	_RLS(ph)TLILHGGGTVCR_	21.2580
Tensin-3;Tensin-like SH2 domain-containing protein 1	Q5SSZ5	648	_GIS(ph)NGPNPPDTQQLCPGK_	14.9560
Terminal uridylyltransferase 4	B2RX14	176	_T(ph)PRSPLEPENVPSLLLK_	2.2269
Testicular zinc finger protein	Q99PP2	1878	_VVIELS(ph)PSLPSKR_	3.8501
Tether containing UBX domain for GLUT4	Q8VBT9	279	_LGGPSASLRPLT(ph)SPSANSSK_	6.9735
THO complex 5	Q5SVF8	340	_RRPT(ph)LGVQLDDK_	10.2260
THUMP domain-containing protein 1	Q99J36	119	_FQS(ph)VESGANNVVFIR_	14.2010
THUMP domain-containing protein 1	Q99J36	88	_FIDKDQQPS(ph)GS(ph)EGEDDDAEAALKK_	12.4680
THUMP domain-containing protein 1	Q99J36	86	_FIDKDQQPS(ph)GS(ph)EGEDDDAEAALKK_	2.3066
Thyroid hormone receptor-associated protein 3	Q569Z6	253	_SPALKS(ph)PLQSVVVR_	4.0951
Thyroid hormone receptor-associated protein 3	Q569Z6	572	_MDS(ph)FDEDLARPSGLLAQER_	7.1723

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
TNF receptor-associated factor 2	P39429	11	_(ac)AAASVTSPGS(ph)LELLQPGFSK_	4.4737
TNFAIP3-interacting protein 1	Q9WUU8	279	_EGLCGQPSS(ph)PKPEGAGK_	2.0393
TopBP1-interacting checkpoint and replication regulator	Q8BQ33	1763	_FLS(ph)AKEESEYK_	4.7921
Tpr protein	Q05CS8	2204	_TVPS(ph)TPTLVVPHR_	10.1200
Tpr protein	Q05CS8	2223	_TDGFAEAIHS(ph)PQVAGVPR_	2.2129
Tpr protein	Q05CS8	453	_KGAILSEEELAAMS(ph)PTAAAVAK_	7.0957
Tpr protein	Q05CS8	1730	_GIAS(ph)TSDPPTANIKPTPVVSTPSK_	70.6920
Tra1 homolog	Q80YV3	2049	_GLS(ph)VDSAQEVKR_	16.9470
Tra1 homolog	Q80YV3	2073	_S(ph)QSLPGADSLLAKPIDKQHTDTVVNFLIR_	4.3810
TRAF family member-associated NF-kappa-B activator	P70347	258	_GLGRDEEDTS(ph)FESLSK_	17.2570
Trafficking protein particle complex subunit 10	Q3TLI0	809	_RQES(ph)GSSLEPPSGLALEDGAHVLR_	2.2456
Transcription factor E2F8	Q58FA4	20	_GLMKS(ph)PLHPSSK_	2.1076
Transcription factor EB, isoform CRA_b	Q3U327	524	_RS(ph)SFSMEEGDVL_	2.4345
Transcription factor HIVEP3	A2A884	555	_GS(ph)YS(ph)FDDHVADPEVPS(ph)R_	5.4459
Transforming acidic coiled-coil-containing protein 1	Q6Y685	284	_GSGAQRS(ph)PLNLK_	4.1761
Transforming acidic coiled-coil-containing protein 2	Q9JJG0	763	_QALYLMFDTPQES(ph)PVKSPPVR_	3.6910
Trinucleotide repeat-containing gene 6B protein	Q8BKI2	422	_NGNTNSLNLS(ph)SPNPMENK_	15.2230
Triosephosphate isomerase	P17751	130	_VTNGAFTGEIS(ph)PGMIK_	5.8263
tRNA-intron endonuclease Sen54	Q8C2A2	178	_FQLSSVVS(ph)PYER_	2.9312
Tuberin	Q61037	1367	_AIS(ph)SEGARPAVDLSFQPSQPLSK_	25.2700
Tuberin	Q61037	1130	_VRS(ph)MSGGHGLR_	19.4160
Tuberin	Q61037	1366	_AIS(ph)SEGARPAVDLSFQPSQPLSK_	13.8190
Tuberin	Q61037	1254	_SLS(ph)VPAAGTAKPPTLPR_	6.0723
Tudor domain-containing protein 3	Q91W18	438	_DTPTSKS(ph)TVENSVLSR_	17.2800
Tumor protein D52	Q62393	158	_ASAAFSSVGS(ph)VITK_	14.3170
Tumor protein D52-like 2	A2AUD5	12	_(ac)MDSASQDINLNS(ph)PNKGVLSDFMTDVPVDPGVVHR_	2.0183
Twinfilin-2	Q9Z0P5	349	_LIRGPGENGEDS(ph)_	2.4880
Type III PIP kinase	Q9Z1T6	307	_SAS(ph)ITNLSLDR_	2.2847
Tyrosine-protein phosphatase non-receptor type 6	P29351	536	_GQES(ph)EYGNITYPPAVR_	3.0442
Tyrosine-protein phosphatase non-receptor type 7	Q8BUM3	147	_AQSQEDS(ph)DYINANYIR_	17.3690
U2 small nuclear ribonucleoprotein A'	P57784	178	_S(ph)KTFNPGAGLPTDKK_	2.5192
Ubiquitin-activating enzyme E1	Q02053	13	_RVS(ph)GPDPKPGSNCSPAQSALSEVSSVPTNGMAK_	2.5835
Ubiquitin-associated protein 1	Q8BH48	146	_VLS(ph)PPHTK_	10.9450
Ubiquitin-associated protein 2-like	Q80X50	43	_LAQMIS(ph)DHNDADFEEK_	18.1550
Ubiquitin-associated protein 2-like	Q80X50	630	_YPSS(ph)ISSS(ph)PQKDLTQAK_	2.9576
Ubiquitin-conjugating enzyme E2O	A2A7X3	836	_NMTVEQLLTGSPTS(ph)PTVEPEKPTR_	3.5821
UBX domain-containing protein 7	Q3UGV7	280	_SES(ph)LIDASEDSQLEAAIR_	2.6837
UDP-glucose 6-dehydrogenase	O70475	474	_RIPYT(ph)PGEIPK_	23.6320
Uncharacterized protein C10orf78 homolog	Q8BP27	141	_S(ph)SSHSFCSVVK_	2.1859
Uncharacterized protein C12orf35 homolog	Q5DTW7	1482	_SLS(ph)ADEFEILQNPVK_	7.9504
Uncharacterized protein C3orf63 homolog	Q69ZR9	1078	_VVS(ph)ISSSDFSAK_	34.6290
Uncharacterized protein FLJ45252 homolog	Q6PIU9	115	_AS(ph)DDLGEPDVFATAPFR_	5.2484
UPF0414 transmembrane protein C20orf30 homolog	Q8CIB6	24	_LAS(ph)TDDGYIDLQFKK_	3.2235
UPF0485 protein C1orf144 homolog	Q6NXN1	107	_ILGSAS(ph)PEEEQEKPILDRPTR_	5.0958
UPF0557 protein C10orf119 homolog	Q8R3C0	154	_VS(ph)PSTSYTPSR_	3.3294
UPF0684 protein C5orf30 homolog	Q8VEB3	256	_S(ph)LDYLNLDK_	2.1975
UPF0688 protein C1orf174 homolog	Q80WR5	20	_S(ph)YSSASLASAR_	12.7080
USP6 N-terminal-like protein	Q80XC3	671	_HVPTAHSGFVSTQIS(ph)PRPQINPSR_	6.0824
Vacuolar protein sorting-associated protein 13C	Q8BX70	1397	_EVS(ph)TPQDVHTTQGVPAAR_	19.7370
Vacuolar protein sorting-associated protein 13C	Q8BX70	1396	_EVS(ph)TPQDVHTTQGVPAAR_	4.2443
Vacuolar protein sorting-associated protein 26B	Q8C0E2	304	_SMS(ph)HQAAIASQR_	5.5528
Vimentin	P20152	426	_ET(ph)NLESLPLVDTHSKR_	17.0780
WASH complex subunit FAM21	Q6PGL7	866	_IRS(ph)SVPSGGSLFGDDEDDDLFSSAK_	10.3130
WASH complex subunit FAM21	Q6PGL7	833	_TDSRPKS(ph)TGVFQDEELLFSHK_	27.4260
WASH complex subunit FAM21	Q6PGL7	284	_SKRPTS(ph)FADELAAR_	20.2260
WASH complex subunit FAM21	Q6PGL7	832	_TDSRPKS(ph)TGVFQDEELLFSHK_	6.3458
WASH complex subunit FAM21	Q6PGL7	723	_VPLLFS(ph)DEEDSEVPSGVKPEDLK_	2.4678
WASH complex subunit FAM21	Q6PGL7	533	_GLFS(ph)DEEDSEDLFSSQSSSKPK_	2.1323
WD repeat and FYVE domain-containing protein 3	Q6VNB8	970	_VHKPSS(ph)LSFEPEMR_	4.2021
WD repeat and FYVE domain-containing protein 3	Q6VNB8	818	_HAFHCVST(ph)PPVYPAK_	2.6557

Protein Name	Uniprot	Position	Peptide Sequence	Ratio M/L
WD repeat domain 47	A2AGC7	304	_RPQS(ph)ADAYMTR_	13.5340
WD repeat domain 47	A2AGC7	296	_LSPYPS(ph)SPM(ox)R_	2.0465
WD repeat domain 47	A2AGC7	297	_LSPYPS(ph)SPM(ox)R_	2.0465
WD repeat-containing protein 13	Q91V09	79	_AYS(ph)NSIVR_	7.2705
WD repeat-containing protein 70	Q3TWF6	641	_TMFAQVES(ph)DDEESKNEPEWK_	2.1590
Wdr20a protein	Q3ULP2	492	_S(ph)SDKLNLVTK_	8.6536
YTH domain family 1	A2AWN8	209	_APGMNS(ph)LEQGMVGLK_	7.2693
YTH domain family 2	Q3UNH5	39	_DGLNDDDFEPYLS(ph)PQAR_	2.9350
Zinc finger CCCH domain-containing protein 11A	Q6NZF1	741	_RLS(ph)S(ph)ASTGKPPLSVEDDFEK_	73.5500
Zinc finger CCCH domain-containing protein 11A	Q6NZF1	289	_KLS(ph)VGGDSDPPLKR_	3.8626
Zinc finger CCCH domain-containing protein 14	Q8BJ05	343	_RPS(ph)LPPSK_	3.6450
Zinc finger CCCH domain-containing protein 4	Q6ZPZ3	1276	_TGTGSPFAGNS(ph)PAREGEQDAGSLKDVFK_	2.2737
Zinc finger CCCH type containing 13	B9EHN9	1427	_LIS(ph)DPMER_	12.4620
Zinc finger CCHC domain-containing protein 2	Q69ZB8	455	_SIS(ph)SESQHNFNNLQSSLK_	9.1330
Zinc finger MYM-type protein 2	Q9CU65	305	_QQGVDSLS(ph)PVASLPK_	15.5150
Zinc finger protein 451	Q8C0P7	429	_ILS(ph)VKESSAEDCIVPTKK_	15.5520
Zinc finger protein 462	A2SW42	351	_S(ph)PHNSGLVNLTER_	6.2775
Zinc finger protein 609	Q8BZ47	467	_TNS(ph)MGSATGPLPGTK_	2.3661
Zinc finger protein 687	Q9D2D7	1120	_SSGS(ph)AEQSLVGLR_	27.1150
Zinc finger transcription factor Trps1	Q925H1	830	_TLRDS(ph)PNVEAAHLAR_	2.1444
Zinc finger ZZ-type and EF-hand domain-containing protein 1	Q5SSH7	1535	_LLS(ph)FRS(ph)M(ox)EETRPVPTVK_	3.9386
Zinc finger, C3H1-type containing	B2RT41	356	_RLS(ph)ASDIVSEK_	3.3305
Zinc finger, C3H1-type containing	B2RT41	1049	_S(ph)FLESNSFTKPNLK_	3.1372
Zyxin	Q3TCR9	336	_S(ph)PGGPGPLTLK_	12.2680

Appendix D- ERK5 in macrophages

One of the original aims of this thesis was to examine the role of ERK5 in cytokine production in macrophages. Studies with a LRRK2 inhibitor demonstrated an off-target effect of this inhibitor was to inhibit cytokine production in LPS-stimulated macrophages (data not shown). Screening of this inhibitor identifed ERK5 as being strongly inhibited. A more selective ERK5 inhibitor was obtained (Deng et al., 2013). Sorbitol treatment of HeLa cells led to band shift of ERK5 which is indicative of activation by MEK5 which results in further autophosphorylation events (Mody et al., 2003). Treatment of cells with 5 μM ERK5-IN-1 completely blocked ERK5 bandshift (See Appendix D- Figure 1). Activation of ERK1/2 was unaffected by ERK5-IN-1.

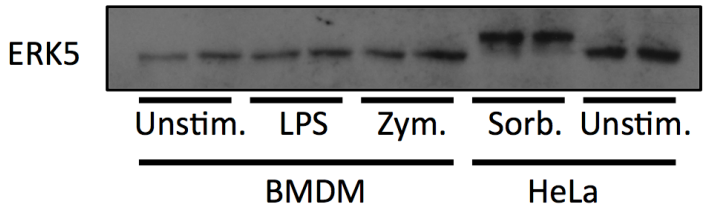


Appendix D- Figure 1: ERK5-IN-1 blocks ERK5 autophosphorylation

Cells were serum-starved overnight and then incubated with indicated concentrations of ERK5-IN-1 for 1 hour. Cells were then stimulated with 0.5M Sorbitol for 30 mins. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to ERK5, phospho ERK1/2 and ERK1/2. Results are representative of three independent experiments.

The LRRK2 inhibitor blocked cytokine production in macrophages independently of LRRK2 (unpublished observations- Nic Dzamko/Dario Alessi). ERK5 was suggested as a potential target of this inhibitor and therefore the role of ERK5 in macrophages was investigated. Macrophages were stimulated with LPS and Zymosan and ERK5 bandshift was examined. Sorbitol treatment of HeLa cells caused an upward shift of the ERK5 band compared to unstimulated cells. Neither LPS or Zymosan treatment of BMDMs caused

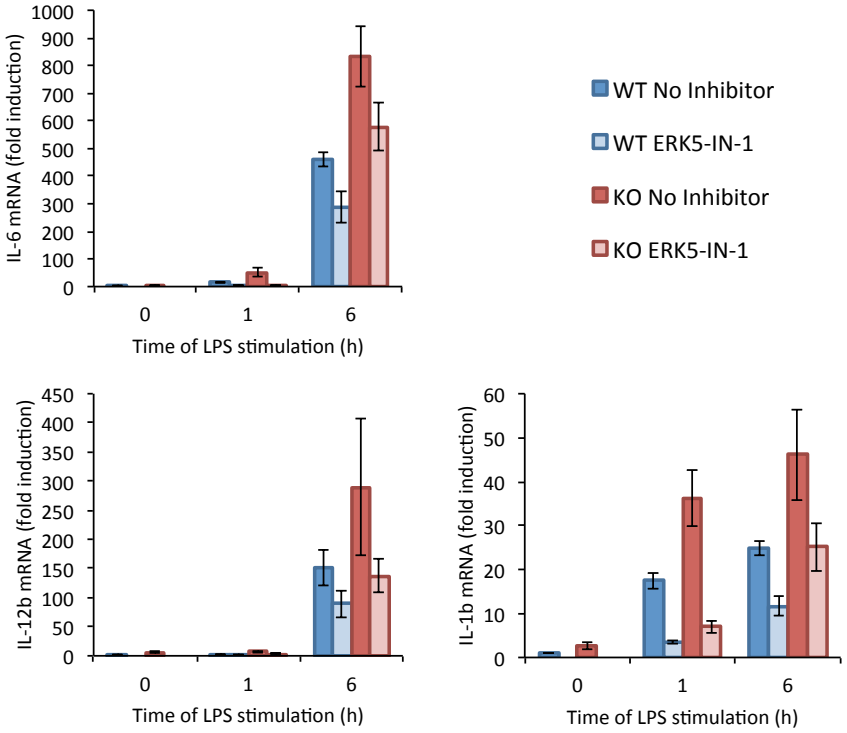
an upward mobility shift of the ERK5 band, which suggests ERK5 is not activated in response to these agonists (see Appendix D- Figure 2).



Appendix D- Figure 2: ERK5 does not bandshift in LPS-stimulated cells

BMDMs were stimulated with 100 ng/ml LPS or 200 μg/ml Zymosan for 30 mins. HeLa cells were stimulated with 0.5M Sorbitol for 30 mins. Cells were lysed and proteins separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies to ERK5. Results are representative of three independent experiments.

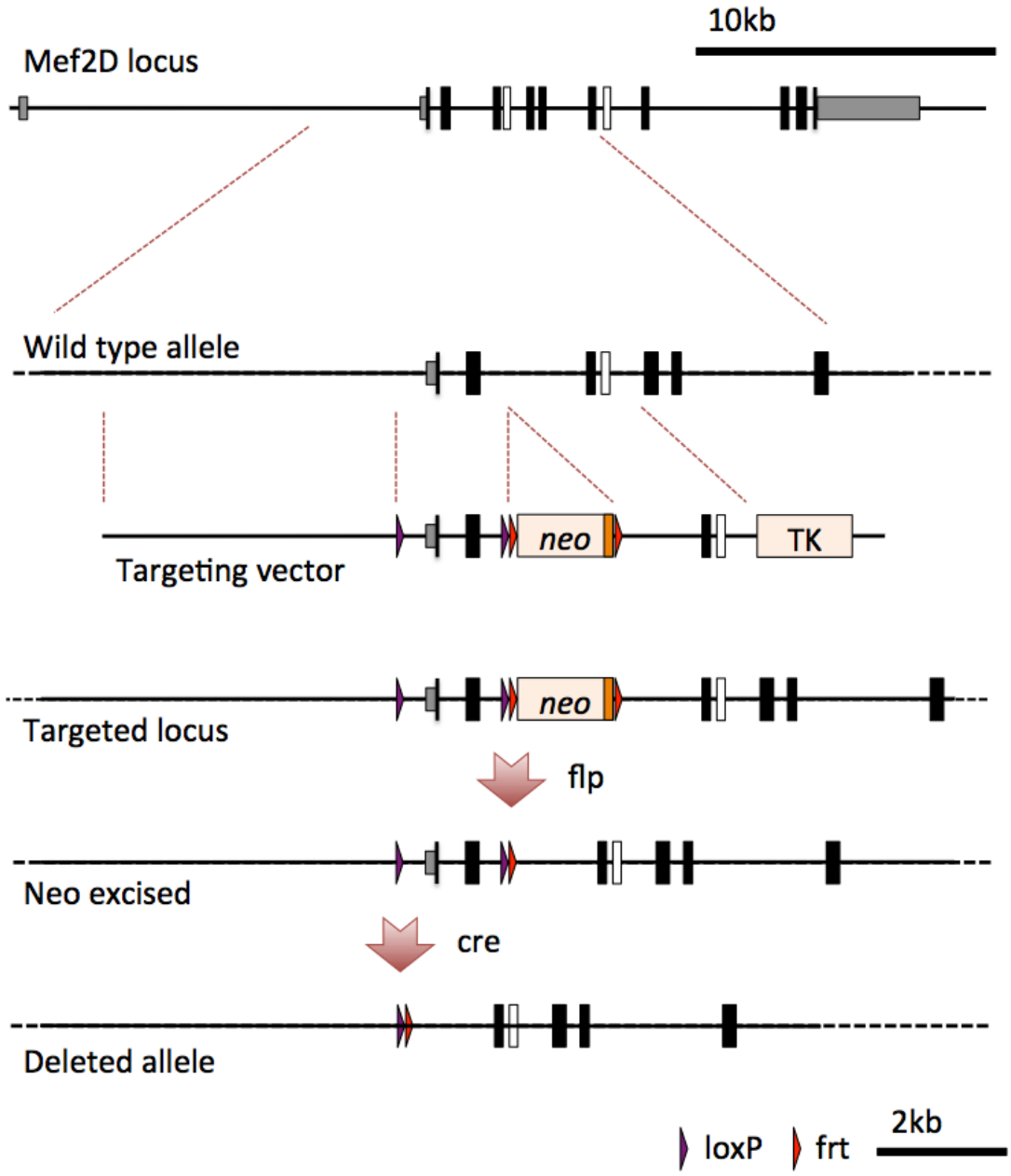
In order to exclude a role for ERK5 in promoting cytokine production in LPS-stimulated macrophages, BMDMs were cultured from ERK5 conditional knockout mice and stimulated with LPS in the presence or absence of ERK5-IN-1. LPS stimulation induced transcription of IL-6, IL-12b and IL-1β in wildtype macrophages during the timecourse. Addition of ERK5-IN-1 reduced cytokine transcription in wildtype cells (see Appendix D- Figure 3). Macrophages lacking ERK5 were stimulated with LPS and transcription of IL-6, IL-12b and IL-1b was induced to similar or higher levels as seen in wildtype macrophages. Treatment of ERK5-deficient macrophages with ERK5-IN-1 reduced transcription of cytokines in response to LPS compared to LPS-stimulated ERK5 cKO macrophages. These data suggest that ERK5-IN-1 suppresses cytokine transcription in response to LPS independently of ERK5.



Appendix D- Figure 3: ERK5-IN-1 represses cytokine production in the absence of ERK5

Wildtype or ERK5 conditional KO BMDMs were stimulated with 100 ng/ml LPS for the times indicated in the presence or absence of 5 μM ERK5-IN-1. Cells were lysed with RLT buffer (Qiagen) and total RNA purified using the Qiagen microRNeasy kit. Total RNA was reverse transcribed using iScript (Bio-Rad) and qPCR was carried out using SYBR-green based detection methods. Levels of 18S were used as a normalization control, and fold induction was calculated for IL-6, IL-12b and IL-1β mRNA. n=4, error bars represent the standard deviation from independent cultures from four mice per genotype.

Appendix E: MEF2D targeting strategy



Appendix E- Figure 1: MEF2D targeting strategy

A targeting vector was constructed to delete exons 2-3 of the murine MEF2D gene through replacement with a neomycin selection cassette (NEO). The thymidine kinase cassette (TK) acts as a negative selection marker. The following primers were used to generate targeting vectors: ARM1 SENSE TGCGGCCGC-CGCCTAATGAGAGCAGTTCCCTAGCTG,ARM1 ANTISENSE TCATATGATAACTTCGTATAGCAT-ACATTATACGAAGTTATAAAGCTTATGCACTGTATTGTTGTACGCTGCCGAC, DELETION SENSE TGCGGCCGCTTATTCATATGAATACAGCCCAACAAGCTCTATAGAGAAC, DELETION ANTI-SENSE AAAGCTTGGCCGGCCATAACTTCGTATAGCATACATTATACGAAGTTATAGCCCAGTC-CCTTAACATTTCACAGATGAC, ARM2 SENSE TGGCGCGCCTCTGGCCTTTCCTTGCCATGT-GACTTTAG, ARM2 ANTISENSE CTCGAGTCGACGCACTTTGCAATACAGCTCTGACCCAAG, SD SENSE AACCGGTGTGCCATGGACAAGGTGCTGCTCAAGTACAC, SD ANTISENSE AACCG-GTACCTCCTCAGACTGGCAGATCACAG.Vectors were injected into embryonic stem cells and crossed to flp mice to remove the neomycin selection casette. Mice were then crossed to Bal1 cre mice to delete exons 2 and 3 from the MEF2D gene.