

REGULATION OF IRF5 ACTIVITY IN INFLAMMATORY DISEASES: NOVEL KINASES



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ABSTRACT

Interferon regulatory factor 5 (IRF5), is a multifunctional regulator of immune responses, with a critical role in controlling pro-inflammatory responses of macrophages. IRF5 contributes to the pathogenesis of several inflammatory and autoimmune diseases. Given the importance of IRF5 function in physiology and disease, IRF5 represents a therapeutic target. However, the mechanisms regulating IRF5 activation and the kinases responsible for its phosphorylation *in vivo* have been unclear. The purpose of this study was to identify novel IRF5 kinases as potential targets for specific control of IRF5-driven inflammatory conditions. Based on a kinase inhibitor library screen and rounds of validation PYK2 and MEKK3 have been prioritised as candidate IRF5 kinases.

Using the CRISPR-Cas9 system I have generated PYK2 and MEKK3 deficient macrophages. Deficiency in PYK2 had an effect on IRF5 activation, IRF5 recruitment to the genomic loci and transcription of IRF5 target inflammatory genes. Moreover, I have identified the PYK2 dependent tyrosine site within IRF5 and demonstrated its importance in IRF5 activation. PYK2 inhibition led to a comparable impact on macrophage transcriptomic signature as IRF5 deficiency, and suppressed cytokine production in biopsies taken from the inflamed gut mucosal tissue of patients with ulcerative colitis. The molecular dissection of the PYK2/IRF5 axis carried out in this thesis has a direct translational potential for inflammatory conditions such as ulcerative colitis, where both the PYK2 and IRF5 gene loci have been associated with increased genetic risk.

Deficiency in MEKK3 had an effect on IRF5 activity and inflammatory gene expression. In the absence of MEKK3 inhibitors, clinically validated kinase inhibitors with off-target MEKK3 potency, were used as initial leads to design MEKK3-specific inhibitors. Chemical modifications of the lead compound Neratinib resulted in the identification and synthesis of analogs with comparable biochemical potency on MEKK3, improved selectivity on a small panel of pathway relevant kinases, with reduced IRF5-driven TNF activation in an IRF5- and MEKK3- specific manner.

DECLARATION

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All experimental data described in this thesis are original and have been performed by myself, unless stated below or indicated in the text. Some of the text and figures in the general introduction have been published with me as a first author in a literature review article (1).

Experiment and analyses described below were performed by or with help from others. Grigory Ryzhakov carried out the kinase inhibitor library screens and initial round of validation with overexpression systems (Sections 3.2.2 and 3.2.3.1 - 3.2.3.4). Mass spectrometry sample preparation and run was carried out by Sarah Bonham at Target Discovery Institute (TDI), Nuffield Department of Medicine (NDM). Mass spectrometry data analyses were carried out by Raphael Heilig and Roman Fischer at TDI, NDM (Sections 4.2.4, 5.2.7). mRNA-sequencing was conducted by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, Oxford. Tariq Khoiratty performed computational analysis of genomic datasets (Section 4.2.6.5). *In vivo* work and human biopsy work was carried out by Alastair Corbin (Section 4.2.6.9). Mouse colon explants in Figure 4.2.5 were prepared by Claire Pearson (Powrie Group, Kennedy Institute). MEKK3 probes in Chapter 5.2 were developed by LAB282 (Evotec).

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ABBREVIATIONS

Ab	antibody
AID	autoinhibitory domain
APC	antigen presenting cell
ATM	ataxia telangiectasia mutated
BM	bone marrow
BMDM	Bone marrow-derived macrophages
bp	base pairs
Cas9	CRISPR associated protein 9
ChIP	Chromatin immunoprecipitation
CIA	collagen- induced arthritis
CLR	C-type lectin receptor
CRISPR	clustered regularly interspaced short palindromic repeats
CSN	COP9 signalosome
CTLD	C-type lectin domains
DAMPs	damage associated molecular patterns
DBD	DNA-binding domain
DCs	dendritic cells
DD	dimerisation domain
DOK1	docking protein 1
EC	endothelial cell
ELISA	enzyme-linked immunosorbent assay
EtBr	ethidium bromide
FBS	Foetal Bovine Serum
FcyR	Fc gamma receptor

FOXO3a	forkhead transcription factor
GM-CSF	granulocyte macrophage colony-stimulating factor
GWAS	genome-wide association study
Hh	Helicobacter hepaticus
HIPK4	homeodomain interacting protein kinase 4
hMDM	human primary monocyte-derived macrophages
HSV-1	herpes simplex virus type 1
IAD	IRF association domain
IBD	inflammatory bowel disease
IFN	interferon
IKK α	Inhibitor of nuclear factor kappa-B kinase subunit alpha
IKK β	inhibitor of nuclear factor kappa-B kinase subunit beta
IP	immunoprecipitation
iPSdM	induced pluripotent stem cell-derived macrophages
IRF	interferon regulatory factor
IRF5	interferon regulatory factor 5
ISRE	interferon stimulated response element
ISR1	insulin receptor substrate 1
ITAM	immunoreceptor tyrosine-based activation motif
JNK2	Jun N-terminal kinases 2
KO	knockout
LNP	lipidoid nanoparticles
LPS	lipopolysachharide
M-CSF	macrophage colony-stimulating factor
MEKK3	mitogen-activated protein kinase kinase kinase 3
MDP	muramyl dipeptide
MI	myocardial infarction

MyD88	myeloid differentiation primary response 88
NDV	newcastle disease virus
NES	nuclear export signal
NETs	neutrophil extracellular traps
NF- κ B	nuclear factor kappa-light-chain enhancer of activated B cell
NHEJ	Non homologous end joining
NLR	NOD-like receptor
NLS	nuclear localisation signal
NK	natural killer
OGN	oligonucleotide
OSM	oncostatin M
PAM	protospacer adjacent motif
PAMP	pathogen-associated molecular pattern
PGN	peptidoglycan
PEST	Proline-, Glutamic acid-, Serine-, Threonine-rich
Pol II	RNA polymerase II
PRD1	positive regulatory domain 1
PRR	pathogen recognition receptor
PTMs	post-translational modifications
PYK2	Protein tyrosine kinase 2
RA	rheumatoid arthritis
RIP2	Receptor-interacting serine/threonine-protein kinase 2
RLR	RIG-I-like receptors
ROS	reactive oxygen species
SCI	spinal cord injury
SFK	Src family kinase
SLE	systemic lupus erythematosus

SS	steady state
TAMPs	tumour-associated molecular patterns
TBK1	TANK-binding kinase 1
TF	transcription factor
TRIF	TIR-domain containing adapter-inducing interferon- β
TIR	Toll/interleukin-1 receptor
TIRAP	TIR-domain containing adaptor protein
TiO ₂	titanium dioxide
TLR	toll-like receptor
TMT	tandem mass tag
TRAM	TRIF-related adaptor molecule
TRIM21	TRIfpartite Motif21
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
UC	ulcerative colitis
VCAM-1	vascular cell adhesion molecule-1
VSV	vesicular stomatis virus
WB	western blot
WGP	Whole glucan particle
WCE	whole cell extract
WT	wildtype

1.0. GENERAL INTRODUCTION

1.1. INNATE IMMUNITY

The host's ability to detect invading pathogens rapidly and efficiently is essential in mounting an effective immune response against them. Physical and physiological barriers provide the hosts first line of defence against foreign pathogens. These barriers include skin or mucosal surfaces of the respiratory and gastrointestinal tract, mucociliary clearance mechanisms, acidic pH and bacteriolytic lysozyme in tears, saliva and other secretions. When these cellular barriers are overcome, an effective innate immune response is initiated to eliminate pathogens. Incoming pathogens are recognised by immune cells such as dendritic cells, macrophages and neutrophils via distinct pathogenic components termed pathogen associated molecular patterns (PAMPs). In addition, cells can recognise danger signals released by stressed cells or cells undergoing necrosis known as damage associated molecular patterns (DAMPs).

The ability to sense PAMPs/DAMPs through germline-encoded pathogen recognition receptors (PRRs) is a critical first step in the hosts defence against pathogenic infection. Well-known examples of PRRs include the families of toll-like receptor (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (**Figure 1.1**). More specifically TLRs recognise extracellular or endosomal nucleic acids, flagellin, lipopolysaccharide (LPS) and lipopeptides, CLRs sense glycoproteins and glycolipids from fungi, NLRs responds to structural components of peptidoglycan and RLRs recognise intracellular nucleic acids. Effective sensing of PAMPs by PRRs, rapidly induces complex intracellular signalling pathways leading to the activation of transcription factors (TFs) such as nuclear factor kappa-light-chain enhancer of activated B cell (NF- κ B), mitogen-activated protein kinases (MAPKs) and interferon regulatory factors (IRFs) to elicit inflammatory responses. Transcription factors may act independently or cooperatively to produce

inflammatory cytokines, chemokines and type I interferons to eliminate the pathogen. Whilst the innate immune system is the primary, immediate defence to incoming pathogens, it initiates an adaptive response via B and T lymphocytes, which is long-lasting and confers immunological memory against the pathogen.

1.1.1. Cells of the innate immune system

Innate immune cells include a range of myeloid and lymphoid cell types which originate from the haematopoietic system. They function within the immune system to defend and protect the human body.

1.1.1.1. Macrophages

Macrophages are phagocytic cells which patrol the body and have the ability to sense signals released during infection and damage, and efficiently respond to microenvironmental cues. They exist as a heterogeneous cell population, with many macrophage subpopulations now known. Macrophages also demonstrate a great degree of plasticity in which they alter their phenotype and physiology in response to cytokines and microbial signals. For example, colony stimulating factors (CSFs) including macrophage CSF (M-CSF) and granulocyte-macrophage CSF (GM-CSF) play a key role in inducing macrophage phenotypes. M-CSF is constitutively secreted by host tissue cells, such as fibroblasts, stromal cells, osteoblasts, even in the absence of inflammation and has a homeostatic and resolving role, whereas GM-CSF is produced by the same cells during inflammation and has a more pro-inflammatory effect on macrophages (2). The macrophage populations that result from culture with GM-CSF or M-CSF have been likened to M1-(classically activated) or M2 (alternatively activated) macrophages, respectively. M1 macrophages produce high levels of the pro-inflammatory cytokines interleukin 12 (IL-12), IL-23, TNF and low levels of IL-10. This sets up an inflammatory environment for expansion of other immune cell types to mediate tissue destruction and resistance to pathogens. On

the other hand, M2 macrophages produce high levels of IL-10 and low levels of IL-12 and IL-23 to promote tissue repair and remodelling as well as tumour progression (3).

1.1.1.2. Dendritic cells

Dendritic cells (DCs) are phagocytic cells, derived from monocytes and can be found in lymphoid and non-lymphoid organs. As professional antigen presentation cells (APCs), DCs uptake antigens and present them to T-cells in tissues and in the lymph node.

1.1.1.3. Natural killer cells

Natural killer (NK) cells, originate from the lymphoid lineage and do not attack pathogens directly. Instead, these cells recognise abnormal cells such as tumour cells or infected host cells. Upon cell recognition, NK cells release contents of their cytoplasmic granules including perforin and proteases in an action known as degranulation, which induces apoptosis or lysis of the incoming pathogen.

1.1.1.4. Granulocytes

Granulocytes are innate polymorphonuclear cells with an abundance of granules in the cytoplasm. They can be divided into neutrophils, eosinophils, and basophils. They possess distinct functions such as phagocytosis of invading pathogens and killing of parasites too large to endocytose. Eosinophils are rare in healthy individuals, but become elevated in Th2 driven inflammation such as parasitic infection and allergic inflammation. Upon cell activation they release a large array of cytokines, growth factors, and chemokines. Basophils are the rarest granulocytes and contribute to protective immunity to pathogens and to the development of acute and chronic allergy and autoimmunity. Despite sharing certain features with tissue-resident mast cells, basophils have distinct *in vivo* roles and differ in their location and lifespan.

1.1.1.4. Neutrophils

Neutrophils are rapidly recruited to the site of infection where they phagocytose and kill pathogens via cytotoxic mechanisms including the production of reactive oxygen species (ROS), and the release of antimicrobial peptides. Moreover, neutrophils are capable of releasing neutrophil extracellular traps (NETs) composed of decondensed chromatin, histones and granule proteins to bind and trap pathogens. Traditionally, neutrophils were considered short-lived and terminally differentiation phagocytes. However, recent findings have expanded our knowledge on the role of neutrophils as they exhibit a certain degree of heterogeneity, plasticity and dynamics.

1.1.2. Pathogen recognition receptors

1.1.2.1. Toll-like receptors (TLRs)

TLRs are the most widely and best studied PRRs, with 10 and 12 family members identified in humans and mice, respectively, with TLR1-9 conserved in both humans and mice. TLR10 is expressed in humans but not in mice because of a retroviral insertion in the murine TLR10 gene, and TLR11-12 are expressed in mice but not humans. Some TLRs (TLRs 1, 2, 4, 5, and 6) are expressed on the cell surface from which they detect microbial cell surface marker PAMPs, whilst others (TLRs 3, 7, 8 and 9) are expressed in intracellular compartments such as endosomes from which they detect intracellular microbial nucleic acids (**Figure 1.1**).

All TLRs share common domains; a transmembrane domain, an extracellular or endosomal domain containing leucine-rich repeat (LRR) motifs for PAMP recognition, and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain that initiates downstream signalling cascades through adaptor molecules. Although TLR members share similar extracellular LRRs, each TLR senses different PAMPs. For example, TLR3 recognises viral double

stranded RNA, TLR4 recognises bacterial LPS, whereas TLR5 recognises bacterial flagellin.

Following TLR engagement, TLRs recruit different TIR-domain-containing adaptors such as myeloid differentiation factor 88 (MyD88), TIR-domain adapter-inducing interferon- β (TRIF), TIR domain-containing adapter protein (TIRAP), and TRIF-related adaptor molecule (TRAM) to the TIR domain (4). TLR signalling can be divided into two pathways; MyD88 dependent and MyD88 independent/TRIF dependent. All TLRs excluding TLR3 signal via the adaptor protein MyD88 to initiate MyD88-dependent signalling. In addition to MyD88, TLRs 1, 2, 4 and 6 recruit TIRAP during MyD88-dependent signalling. TLR3 signals exclusively via the TRIF pathway during MyD88-independent signalling. TLR4

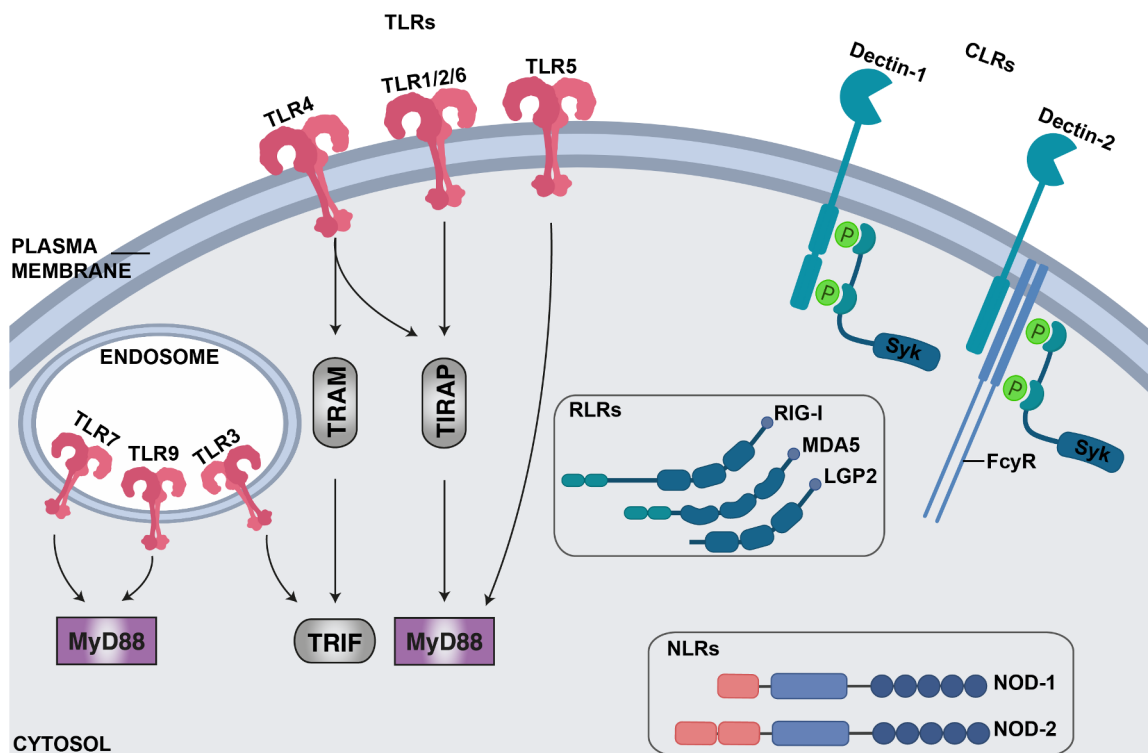


Figure 1.1. Pathogen recognition receptors and associated proteins of the innate immune system Toll like receptor (TLR) 1/2/4/5/6 are plasma membrane-localised, and TLRs 3/7/9 are endosomal localised. All TLRs excluding TLR3 recruit the adaptor molecule MyD88 to activate MyD88-dependent pathways. TLRs 1/2/4/6 recruit TIRAP, prior to MyD88 recruitment. TLR3 recruits TRIF to activate MyD88-independent pathways. TLR4 also activates MyD88 independent signalling via an additional adaptor molecule, TRAM. RIG-I-like receptors (RLRs) including RIG-I, MDA5 and LGP2 recognise intracellular viral PAMPs. NOD-like receptors including NOD1 and NOD2 are cytosolic sensors. Activation of C-type lectin receptors localised on the plasma membrane leads to intracellular signalling via Syk. Syk directly binds to phosphorylated intracellular regions on Dectin-1 or via phosphorylated regions on FcγR- which is associated with Dectin-2.

signals via both pathways and recruits TRIF via an additional adaptor molecule TRAM. Recruitment of these adaptor molecules is responsible for coupling TLR ligation to the activation of various transcription factors that culminate in an innate response against pathogens (**Figure 1.1**).

1.1.2.2. *C-type lectin receptors (CLRs)*

C-type lectin receptors are a large superfamily of signalling transmembrane receptors and play an essential role in antifungal immunity. They are characterised by the presence of one or more C-type lectin domains (CTLDs) in the extracellular region that binds to fungal PAMPs and a immunoreceptor tyrosine-based activation motif (ITAM) in their intracellular cytoplasmic tails (5). Specifically CLRs sense carbohydrate structures found in fungal cell walls including β -glucan and mannan (6).

CLRs can be divided into two groups based on their downstream signalling potential: 1) receptors that utilise an ITAM-like motif within their cytoplasmic tails (such as Dectin-1, Clec-2 and DNGR-1), and 2) receptors that utilise a ITAM-bearing FcR γ adaptor molecule (such as Dectin-2, CLECSF8 and Mincle) (7). Signalling via ITAMs in myeloid cells is mediated by the recruitment of the protein spleen tyrosine kinase (Syk) to phosphorylated tyrosines on the cytoplasmic domain, which results in immune regulation through transcription factors. Other receptors couple to Syk via the Fc receptor γ -chain to induce an immune response (**Figure 1.1**).

For example, dectin-1 is a lectin receptor for β -glucan that is essential for innate macrophage recognition of fungi and induces the respiratory burst and the production of inflammatory mediators. Dectin-1 has been demonstrated to synergise with TLR2 in response to yeast PAMPs to elicit a strong inflammatory response via phosphorylation of the membrane cytoplasmic domain and recruitment of the protein tyrosine kinase Syk (8–

10). In addition to this crosstalk behaviour, Dectin-1/Syk and Dectin-1/TLR2 signalling pathways can operate independently.

1.1.2.3. *NOD-like receptors (NLRs)*

NLRs are a family of soluble receptors composed of more than 20 members, which recognise a wide range of PAMPs and DAMPs. In contrast to TLRs, which are membrane bound, NLRs recognise PAMPs in the host cytosol. Structurally, members of the NLR family share a tripartite structure which includes (1) a variable N-terminal domain, (2) a central nucleotide domain that mediates the formation of self-oligomers, and finally (3) a C-terminal leucine-rich repeat that detects PAMPs and modulates NLR activity (**Figure 1.1**). Based on the N-terminal domain, NLRs can be subdivided into 2 main subtypes; NLRC and NLRP proteins. The NLRC proteins (such as NOD1 and NOD2) have one or more caspase recruitment (CARD)-containing domains. The CARD containing NODs respond to antimicrobial PAMPs in the cytosol and signal to the activation of NF- κ B and IRFs to elicit a response. The NLRP proteins contain pyrin domains (PYD) at the N-terminus and upon activation these receptors interact with caspase-1 and drive IL-1b production.

1.1.2.4. *RIG-I-like receptors (RLRs)*

The RLR family of receptors are cytoplasmic sensors of viral nucleic acid and consists of three members including retinoic acid-inducible gene-I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (**Figure 1.1**). Structurally, RIG-I and MDA-5 contain two N-terminal CARD domains that are essential for downstream signalling, and a DExD/h RNA helicase domain, which is required for ligand binding. In contrast, LGP2 lacks the CARD domain and possesses only the helicase domain. Following recognition of viral RNA, RIG-I/MDA-5 recruits the adaptor protein IPS-1 (also known as MAVS) leading to activation of NF- κ B via the IKK complex, and IRF3/7 via the TBK1/IKKi complex. The activation of the transcription factors drives

expression of inflammatory cytokines and type I interferons. LGP2 positively regulates RIG-I and MDA5-mediated signalling pathways.

1.2. INTERFERON REGULATORY FACTOR FAMILY OF TRANSCRIPTION FACTORS

Interferon Regulatory Factors (IRFs) are a family of transcription factors consisting of nine mammalian family members (IRF1-IRF9). IRFs comprise of multi-domains; a N-terminal DNA-binding domain (DBD) and a C-terminal domain (**Figure 1.2**). All members of the family consist of 115 amino acids in their DBD, which shares structural homology with the MYB family. The DBD contains a conserved repeat of five tryptophan (W) residues each separated by 10-18 amino acids. This forms a helix-turn-helix motif and recognises DNA sequences corresponding to the IFN-stimulated response element (ISRE) (A/GNGAAANNGAAACT), of which 5'-GAAA-3' is the consensus IRF recognition sequence (11, 12). The ISRE can be found in the promoters of type 1 IFN, IFN-inducible genes and other genes involved in immune responses (13).

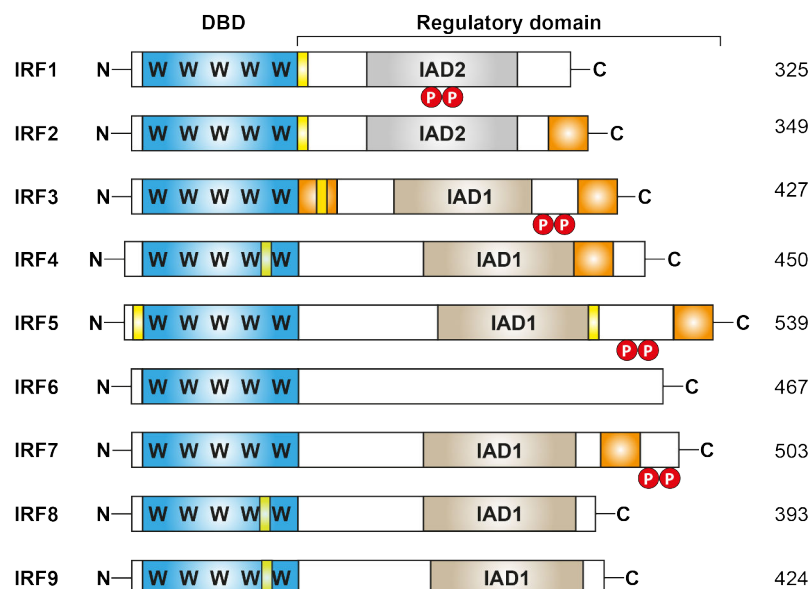


Figure 1.2. Domain structure of interferon regulatory factor (IRF) family of transcription factors IRFs consist of an N-terminal DNA-binding domain (DBD; blue) containing 5 tryptophan (W) repeats and a regulatory domain (white). Most IRFs contain an IRF-association domain (IAD) of either type 1 (IAD1; grey) or type 2 (IAD2; brown). Some IRFs contain a nuclear localisation signal (yellow) and a repression domain (orange). IRFs 1,3,5 and 7 contain phosphorylation sites essential for activation. The number of amino acids for each IRF is indicated. N, amino terminus; C, carboxyl terminus.

The C-terminal region of IRFs are less well conserved, which supports their distinct functions. Two types of IRF association domains (IAD) have been identified within the C-terminal region of IRFs (14): IRF-association domain 1 (IAD1), which is present in most of the IRFs (excluding IRF1 and IRF2) and shares structural similarity with the Mad-homology 2 (MH2) domains of the Smad family of transcription factors (15, 16); and IRF association domain 2 (IAD2) found in IRF1-2 (**Figure 1.2**). The IAD mediates homo- and heteromeric interactions between IRFs as well as other proteins to form transcriptional complexes (11, 12). The transactivation functions of the IAD of IRF3, IRF5 and IRF7 have shown to be suppressed by an autoinhibitory domain (AID). Other domains present in IRFs include a nuclear localisation signal (NLS), nuclear export signal (NES) and regulatory domain.

Each IRF plays distinct roles in many aspects of the immune response including cell development and differentiation and regulating responses to pathogens. The first IRFs, IRF1 and IRF2 were originally identified as factors that regulated the IFN- β promoter through their ability to bind to positive regulatory domain 1 (PRD1). IRF1 and IRF2 were assumed to function as an activator and repressor of the IFN- β promoter, respectively. However, mouse knock-out studies revealed that both IRFs were dispensable for activation of IFN- β genes (17). IRF2 also functions as a transcriptional activator for vascular cell adhesion molecule-1 (VCAM-1) and cell cycle regulated histone H4 genes (18, 19).

IRF3 and IRF7 share similar domain structures and are the key IRFs that mediate production of type I interferons upon PRR activation. IRF7's ubiquitous expression is dependent on IFN α/β signalling, whereas IRF3 is constitutively expressed. IRF3 is activated in infected cells upon sensing of dsRNA to elicit an innate antiviral response. IRF3 resides in the cytoplasm and migrates to the nucleus upon infection. Following PRR engagement, the adaptor proteins MAVS, STING, and TRIF which are activated by viral RNA, cytosolic DNA and bacterial LPS, respectively activate the kinases IKK and TBK1

(20). This leads to IRF3 recruitment and phosphorylation by TBK1. Phosphorylated IRF3 then dimerises and enters the nucleus to induce type I IFNs.

IRF4 plays a critical role in lymphocyte development and function. Furthermore, IRF4 acts as a negative regulator of TLR signalling by competing with IRF5 for MyD88 to antagonise IRF5 activation (21). Studies have shown that knockdowns in IRF4 leads to increased expression of IRF5 and conversely overexpression of IRF4 represses IRF5 expression (22).

Initially, IRF5 was reported to be activated during antiviral responses and induce type I IFN responses (23), but is now known to contribute to the plasticity of macrophage polarisation and play a major role in inflammation (24). IRF5 is involved in TLR-mediated induction of proinflammatory cytokines such as IL-6, TNF- α , and IL12, rather than type I IFNs. Further discussion on the role and functions of IRF5 are included in Section 1.3.

Unlike other IRFs, IRF6 does not appear to function in innate immunity and interferon signalling, but plays a role in normal epidermal development and differentiation. IRF8 is expressed exclusively in myeloid and lymphoid cell lineages and is induced by IFN γ , but not IFN- α/β . IRF8 binds to PU.1 TF and plays a critical role in the regulation of the immune system and oncogenesis. IRF9 is expressed in many tissues and plays an essential role in the IFN antiviral response.

1.3. INTERFERON REGULATORY FACTOR 5

1.3.1. Gene structure of IRF5

The human *Irf5* gene is located on chromosome 7q32. IRF5 exists as multiple distinct isoforms (V1–V11) that are generated by alternatively spliced transcripts. Each isoform utilises one of three promoters, giving rise to transcripts containing non-coding exons 1A, 1B or 1C, and displays differences in the composition of other exons (**Figure 1.3**). For example, IRF5 isoforms V1-V4 and V6-V10 have distinct deletion patterns in exon 6; V1

has a deletion of 30 nucleotides (nts), isoforms V2, V6, V7 and V10 contain a 48 nt deletion, transcripts V3-V4 lack both the 30- and 48-nt regions, isoform V8 contains a deletion in exons 6-7, transcript V9 contains a premature stop codon in exon 6, and transcript V10 contains a 48 nt deletion in exon 6 and an in-frame deletion of 30 nt at the beginning of exon 7. Transcript V11, which includes exon 1C, has a 28-nt deletion of exon 3, predicted to encode a truncated protein translated from an alternate reading frame (25). IRF5 isoforms show cell-type specific expression, subcellular localisation and function (26). For example, isoforms containing exon 1A and exon 1B are constitutively expressed in plasmacytoid dendritic cells and B cells, whereas isoforms containing exon 1C are inducible by type I IFN. More specifically, transcripts V1-V4 are expressed in human primary pDCs and macrophages, V5-V6 were identified in healthy human primary peripheral blood mononuclear cells (PBMCs) and immortalised B and T cell leukaemias. Expression of V7-V9 transcripts have only been detected in haematological malignancies and transcripts V10 and V11 were identified in human PBMCs from SLE patients (25).

Multiple GWAS studies have identified polymorphisms in the *Irf5* locus, leading to expression of alternatively spliced isoforms of *Irf5* that are associated with risk of autoimmune diseases such as systemic lupus erythematosus (SLE) in humans (27, 28). For example, IRF5 isoforms generated from exon 1B (v2, v9, v10) are strongly linked to overexpression of IRF5 and to susceptibility to SLE, whereas elevated expression of IRF5 in the absence of exon 1B does not confer risk (25). Different IRF5 protein isoforms may lead to different stability, affinity for transcriptional cofactors and activation properties leading to increased risk of SLE in individuals with exon 1B transcripts. Several IRF5 isoforms contain splicing variations in and around exon 6, which encodes for a proline-, glutamic acid-, serine- and threonine-rich (PEST) domain are thought to be important for protein stability in the IRF family of proteins (29). Unlike the complicated spliced variations of human IRF5, murine IRF5 located on chromosome 6 is predominantly expressed as a full-length transcript and is homologous to human transcript V5 (30). A single splice variant

is detected in very low levels in the bone marrow of C57BL/6J mice and contains a 288-nucleotide deletion from exons 4-6 and exhibits reduced transcriptional activity (30).

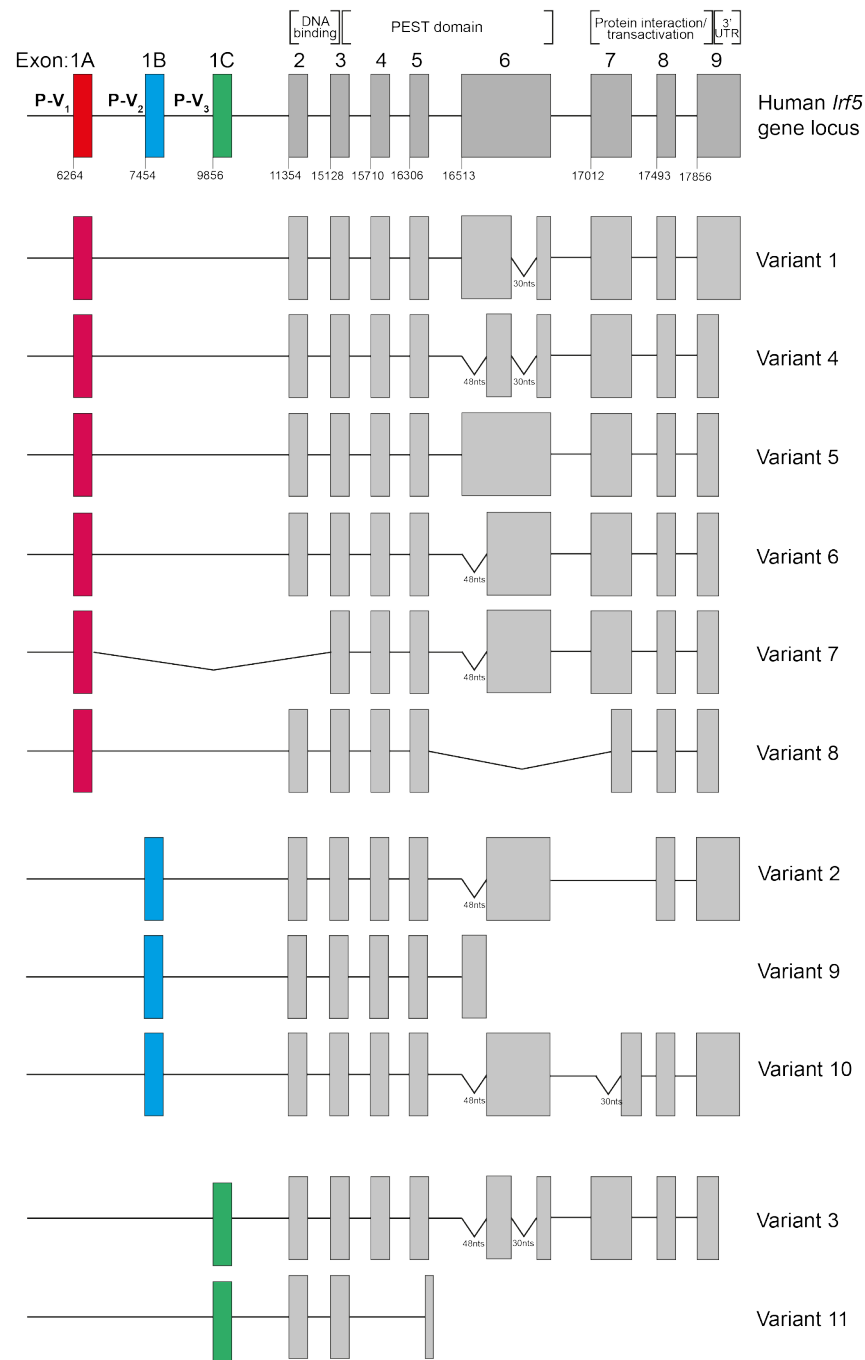


Figure 1.3. A schematic of the splice variants of the human IRF5 gene The first three 5' noncoding exons are depicted in coloured boxes (red, blue and green) with promoter regions labelled P-V₁, P-V₂ and P-V₃ and coding exons depicted in grey boxes. Each isoform variant 1-11 includes exon 1A, 1B or 1C. Some isoforms have 30/48 nucleotide deletions in exon 6. Variant 7 displays a deletion in exon 2 and Variant 8 displays deletions in exon 6-7. Variants 9 and 11 contain a premature stop codon. The location of the exons encoding the DNA-binding, PEST and protein interaction domains, as well as the 3' untranslated region are annotated. Exon/intron structure is not shown to scale.

1.3.2. Protein structure of IRF5

IRF5 is a 60 kDa protein that resides predominantly in the cytoplasm of uninfected cells. It is a multi-domain protein consisting of an N-terminal DBD, an IAD in the middle, a serine-rich region (SRR), an autoinhibitory domain (AID), and a regulatory domain at the C-terminus. IRF5 possesses two nuclear localisation signals (NLS) - one in the N-terminal region and another in the C-terminal region. IRF5 also contains a functional nuclear export signal (NES) that controls dynamic shuttling of IRF5 between the cytoplasm and nucleus. The NES is dominant in unstimulated cells which ensures cytoplasmic retention of IRF5 (31).

1.3.3. Role of IRF5 in immune cells

IRF5 is expressed in several immune cells including B cells, dendritic cells, monocytes and macrophages. The role of IRF5 in different immune cells is discussed briefly below and summarised in **Table 1.1**.

1.3.3.1. IRF5 and B cells

Several studies demonstrate that IRF5 plays a role in B cell activation and proliferation (32, 33). IRF5 acts downstream of TLR signalling to drive proliferation and differentiation to antibody secreting cells (33). IRF5 knockdown in isolated naïve B cells resulted in reduced activation, clonal expansion, plasmablast differentiation, and IgG1/3 secretion (33). Moreover, TLR9-stimulated *Irf5*^{-/-} B cells undergo less proliferation, with reduced *cc13* and *cc14* expression compared to *Irf5*^{+/+} B cells, suggesting that IRF5 may promote the clonal expansion of auto-reactive B cells (32).

1.3.3.2. IRF5 and dendritic cells

IRF5 is responsible for mediating type 1 IFN responses in both conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (34, 35). IRF5 along with NF-kB p50 are required for the

induction of IFN- β and IL-6 in TLR9-stimulated human pDCs (36). Moreover, IRF5 plays an essential role in the production of pro-inflammatory cytokines and chemokines in DCs. For example, induction of IL-6, IL-12 and TNF α by various TLR ligands was severely impaired in spleen-derived cDCs and pDCs from *Irf5*^{-/-} mice (37) and induction of Flt3-ligand induced BM-derived DCs from *Irf5*^{-/-} mice (38). In pristane-induced model, IRF5 is involved in the recruitment of pDCs to inflammatory sites with reduced expression of chemokines CCL19 and CCL21 in *Irf5*^{-/-} mice (39).

1.3.3.3. *IRF5 and monocytes*

IRF5 plays a role in monocytes with enhanced expression and activation found in monocytes of SLE patients (40, 41). In particular, elevated IRF5 protein expression in the monocytes from SLE patients was associated with the *IRF5* SLE homozygous risk haplotype (40). Monocytes from SLE patients have constitutively elevated levels of nuclear IRF5 triggered by autoantigens composed of apoptotic/necrotic material (41). In pristane-induced mice, IRF5 is required for the recruitment of Ly6C^{high} monocyte subsets to sites of inflammation (42). Expression of chemokine receptors CXCR4 and CCR2 was dysregulated in *Irf5*^{-/-} monocytes and less responsive to their respective ligands, CXCL12 and CCL2 (42). Another study, demonstrated that peritoneal exudates from pristane-induced *Irf5*^{-/-} mice have increased levels of Ly6C^{low} monocytes and decreased levels of Ly6C^{high} monocytes (39). TLR8-mediated sensing of *S. aureas* RNA in human primary monocytes resulted in IRF5 activation via TAK1 and IKK β and was necessary for induction of IFN- β (43). Likewise, TLR8 activation resulted in the induction of inflammatory cytokines via a IRAK4-TAK1-IKK β -IRF5 axis in human monocytes (44)

1.3.3.4. *IRF5* and macrophages

IRF5 is a major factor defining the pro-inflammatory macrophage phenotype (24). It directly regulates the secretion of specific inflammatory mediators including IL-12, IL-23, TNF and IL-1a (24). The secretion of pro-inflammatory cytokines subsequently set up the environment for expansion of Th1/Th17 cells, which are important players in rheumatoid arthritis (RA). Moreover, IRF5 can inhibit expression of anti-inflammatory cytokines such as IL-10 (24). IRF5 has been shown to cooperate with p65 RelA to regulate target genes including TNF- α (45, 46). Gain of function mutation genes in IRF5 have been identified as a genetic risk factor for a host of several inflammatory conditions including RA, inflammatory bowel disease (IBD) and systemic lupus erythematosus (SLE).

Cells	Role of IRF5	References
B cells	• B cell activation and proliferation	(32, 33)
Dendritic cells	• Type 1 IFN production • IFNβ production in response to microbial infection • Production of pro-inflammatory cytokines and chemokines	(34, 35) (36) (37–39)
Monocytes	• Increased expression and activation in SLE patients • Recruitment of monocytes to inflamed sites • Production of IFNβ downstream of TLR8 • Pro-inflammatory cytokine production downstream of TLR8	(40, 41) (39, 42) (43) (44)
Macrophages	• Promote inflammatory macrophage phenotype • Induce expression of pro-inflammatory cytokines • Specific marker of inflammatory macrophages	(24) (24, 46) (47)

Table 1.1. Reported role of IRF5 in immune cells

1.3.4. IRF5 in disease pathogenesis

There is overwhelming evidence that IRF5 plays a key role in numerous conditions based on the phenotype of IRF5 knockout mice in disease models. Mice lacking *Irf5* are resistant to lethal endotoxin-induced shock with reduced expression of proinflammatory cytokines (30, 37). *Irf5*-deficient mice are also protected from arthritis and lupus in murine models of

inflammatory arthritis and pristane-induced lupus, respectively (48–50). For example, *Irf5*-deficient mice exhibit reduced knee swelling when challenged with methylated BSA in the acute antigen-induced arthritis (AIA) murine model (50). *Irf5*^{-/-} mice demonstrate impaired expression of IL-12b and enhanced expression of IL-10 in their affected joints (47). Moreover, mice lacking IRF5 accumulate less neutrophils at the site of inflammation due to reduced levels of chemokine secretion important for neutrophil recruitment. During AIA, pro-inflammatory monocyte-derived macrophages with IRF5 expression are specifically detected in the affected knees (47). Somewhat contradictory to these results was a report describing no differences between WT and *Irf5*^{-/-} mice in a model of collagen-induced arthritis (CIA) (32). The mice on C57BL/6 background express the b haplotype of the major histocompatibility complex (MHC) class II and need MHC class II Aq to develop CIA dependent on autoreactive T cells (51, 52). When C57BL/6 *Irf5*^{-/-} mice were crossed with the strain carrying MHC class II Aq, a significant reduction in the number of mice developing the pathology was observed (*H. Eames, unpublished data*), suggesting that the conclusion of no role for IRF5 in the CIA induced pathologies needs to be revisited.

Gain-of-function polymorphisms in the *IRF5* gene leading to increased IRF5 protein expression have been associated with increased susceptibility to IBD (53). The role of IRF5 has further been highlighted by studies using various mouse models of colitis where IRF5 deficiency confers disease protection. IRF5 knockouts displayed reduced intestinal inflammation in the T cell transfer colitis model, with reduced Th1 and Th17, and increased Th2 cytokines (54). Moreover, IRF5 deficiency ameliorates immunopathology during *Helicobacter hepaticus* (*Hh*)-induced colitis assessed by reduction in pathology, inflammatory cellular infiltrate, production of inflammatory cytokines and Th1/Th17 response (55).

Increased IRF5 levels are associated with better prognosis of pulmonary disease (56). In murine asthma models with house dust mite (HDM) exposure, *Irf5*^{-/-} demonstrate impaired

lung function and extracellular matrix deposition, but mice overexpressing IRF5 were protected from allergic inflammation (56). Recent studies have also highlighted important contributions of IRF5 to neuropathic pain (57), vascular diseases (58, 59), obesity (58) and hepatic and skin fibrosis (60, 61). For example, *Irf5*^{-/-} mice on a high fat display beneficial expansion of subcutaneous adipose tissue and retain their insulin sensitivity (62).

Other reported roles for IRF5 include cell cycle arrest and apoptosis (63), microbial infection (64, 65), and glycolysis (66). IRF5 is reported to act as a tumour suppressor as it can induce the expression of p21, Bax and Caspase 8 in response to DNA damage independently of p53 (63, 67). Furthermore, in response to DNA damage mouse embryonic fibroblasts (MEFs) from *Irf5*^{-/-} mice are resistant to apoptosis but not cell cycle arrest (68). Ectopic IRF5 expression reduces the proliferation of chronic myeloid leukaemia cells *in vitro* as a target of BCR-ABL kinase (69). In line with these effects, loss of IRF5 is associated with progression of several cancers including gastric cancer, breast cancer and chronic myeloid leukaemia (69–72). Although, IRF5 is usually considered a tumour suppressor, in thyroid malignancies, IRF5 displays tumour promoting activity by promoting proliferation and survival of thyroid cancer cells by upregulating the proto-oncogene B-raf (73). Several studies have also found that autoantibodies against IRF5 were able to cross react with homologous peptides from *Mycobacterium avium* subspecies paratuberculosis and Epstein–Barr virus (74–76). Antibodies against these peptides were significantly higher in the cerebrospinal fluid and serum of multiple sclerosis (MS) patients due to molecular mimicry (74, 75).

From these studies and given the fact that in humans IRF5 gene polymorphisms related to higher IRF5 expression (25, 77–80) have been associated with susceptibility to inflammatory and autoimmune diseases including RA, IBD, SLE, MS, and Sjorgens syndrome, IRF5 has emerged as an attractive target for therapeutic intervention. Inhibition of IRF5 would specifically affect the expression of proinflammatory cytokines and would

result in fewer effector T cells. A benefit of targeting IRF5, is that it acts in a cell-type and activity-specific manner. Both IRF5 and NF- κ B transcription factors are essential for the induction of proinflammatory genes (46). Due to the broader functional activities of NF- κ B and its ubiquitous nature, there are concerns of the detrimental effects that might result from blockage of NF- κ B activity. In comparison, targeting IRF5 may well be more beneficial and offer less adverse effects to general cell function. Despite the strong rationale for targeting IRF5, inhibitors that interfere with the IRF5-specific pathway remain elusive.

1.3.5. IRF5-signalling pathways

IRF5 is involved in innate immune signalling pathways via various PRRs including TLRs, CLRs, NLRs, and RLRs. Upon recognition of PAMPs, activated PRRs trigger intracellular signalling cascades. IRF5 is proposed to exert its molecular function via a cascade of events involving its ubiquitination, phosphorylation, dimerisation, nuclear translocation and selective binding to its target genes to enables their expression (81). **Figure 1.4** provides a schematic overview of the main IRF5-activating pathways.

1.3.5.1. TLR-MyD88 pathway

Several TLRs converge on IRF5 regulation in the innate immune response including TLR4 found on the cell surface membrane and TLR7, TLR8, and TLR9 found within endosomal membranes. Upon ligation, these TLRs recruit and utilise the adaptor protein MyD88 to initiate signal transduction. Upon binding of LPS, the adaptor protein MyD88 is recruited to the receptor via its own TIR domain. This leads to the assembly of a signalling protein complex that includes IRAK4 and the E3 ligase TRAF6 (TNF receptor associated factor 6). Activated IRAK4 phosphorylates TAK1 to activate IKK β . IRF5 then undergoes post-translational modifications including Lysine-63 (K63) polyubiquitination mediated by TRAF6, and phosphorylation by IKK β and other unknown kinases. Subsequently, active

IRF5 undergoes homodimer formation, translocation to the nucleus, binding to gene promoters and induction of inflammatory gene transcription.

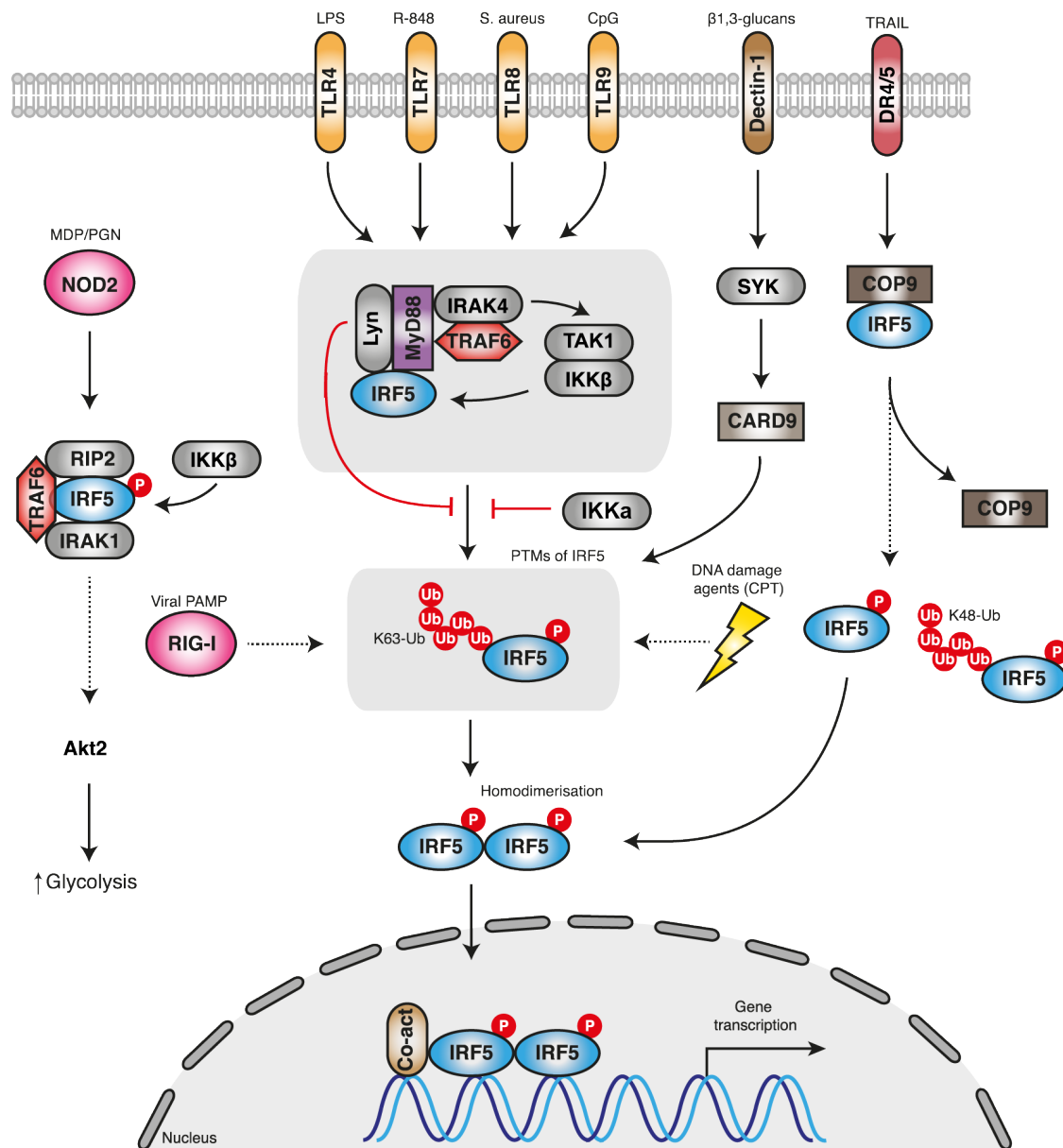


Figure 1.4. A schematic representation of the proposed mechanisms of IRF5 activation The stimulation of toll-like receptors (TLRs) on cell membranes by pathogen-derived products or RIG-I-like receptors (RLRs) in the cytosol by viral PAMPs trigger signalling cascades. IRAK4 regulates the TLR/MyD88 response via TAK1 and IKKβ. Interferon regulatory factor 5 (IRF5) is activated from its latent state by PTMs that include ubiquitination by the ubiquitin ligase TRAF6 and phosphorylation by IKKβ. These modifications trigger IRF5 homodimerisation, translocation to the nucleus and binding to gene promoters along with coactivator proteins (p300). Lyn kinase and IKKα block IRF5 PTMs. Alternatively, DNA damage agents (CPT) can induce IRF5 phosphorylation and transcriptional activation. Another model proposes that the Dectin-1- induced IFN-β production is mediated by a Syk-Card9-IRF5-dependent pathway. In another model, IRF5 interacts with the COP9 signalosome (CSN). Upon stimulation with agonists such as TRAIL, an unknown kinase phosphorylates IRF5 which leads to its dissociation from CSN. Loss of the CSN-IRF5 interaction leads to K48-linked ubiquitination of IRF5 and degradation by the ubiquitin-proteasome pathway. Some activated IRF5 migrates to the nucleus and activates target genes. An alternative model suggests that upon NOD2 stimulation IRF5 associates with RIP2, IRAK1, TRAF6 and IKKβ to increase glycolysis through Akt2 activation.

Lyn kinase which belongs to the Src family kinase (SFK) was found to bind to and inhibit the activity of IRF5 in the TLR-MyD88 pathway (38). Even though Lyn is capable of phosphorylating IRF5, its phosphorylation did not alter IRF5 protein activity. Instead, Lyn is thought to inhibit IRF5 in a kinase independent manner by blocking the PTMs of IRF5. Another more recent study has shown that Fc gamma receptor (FcγRs) synergise with TLRs to amplify pro-inflammatory cytokine production and induce glycolytic reprogramming via IRF5 (82). Two pathways are thought to converge on IRF5 activation where TLR stimulation induces IRF5 phosphorylation and FcγR stimulation induces IRF5 nuclear translocation.

1.3.5.2. *Dectin-1-SYK-IRF5 pathway*

Another study has demonstrated a role for IRF5 in induction of IFN-β following fungal infection by the pathogen *Candida albicans*. During *C. albicans* infection, dectin-1 on dendritic cells is activated and a signalling pathway involving the tyrosine kinase Syk, the adaptor protein Card9 and IRF5 leads to the production of IFNβ (65). Another study has reported the importance of Dectin-1-IRF5 signalling in innate immune responses against cancer where it enhanced NK cell-mediated tumour killing (83). Dectin-1 expressed on myeloid cells including dendritic cells and macrophages recognises N-glycan structures on tumour cells to activate IRF5 and subsequently activate NK cells. *In vivo*, mice lacking Dectin-1 or IRF5 were characterised by massive growth of tumour-associated molecular patterns (TAMP)-expressing tumours.

1.3.5.3. *COP9 signalosome*

An alternative model suggests that the COP9 signalosome (CSN) interacts with and stabilises IRF5 (84, 85). In unstimulated cells, IRF5 resides in the cytoplasm and interacts with CSN subunits at both the carboxyl- and amino-terminal ends of IRF5. This is thought to mask and stabilise the DBD of IRF5 by protecting IRF5 from degradation. Stimulation of the death receptor by TRAIL ligand leads to phosphorylation of the complex by an

unknown kinase and loss of the CSN/IRF5 interaction. IRF5 subsequently undergoes K48-linked ubiquitination and is targeted for degradation by the 26S proteasome. Some of the phosphorylated IRF5 migrates to the nucleus and transactivates target genes.

1.3.5.4. NOD2-IRF5 pathway

Stimulation of the intracellular NOD2 receptor has been shown to enhance glycolysis through IRF5 signalling. Upon NOD2 activation with muramyl dipeptide (MDP), IRF5 associates with RIP2, IRAK1 and TRAF6 in human monocyte-derived macrophages (hMDMs) (66). IRF5 and IKK β are proposed to cross-regulate phosphorylation of each other where NOD2-induced IKK β phosphorylation is dependent on the IRF5/RIP2/IRAK1/TRAF6 complex and IRF5 phosphorylation is dependent on IKK β activation. Each of the molecules in the complex along with IKK β are required for NOD2-induced IRF5 serine phosphorylation, which increases glycolysis through Akt2 activation.

1.3.5.5. Alternative IRF5 activating pathways

Other modes of IRF5 activation include viral infection by Newcastle disease virus (NDV), vesicular stomatitis virus (VSV) and herpes simplex virus type 1 (HSV-1) (30, 68, 86, 87). IRF5 phosphorylation on residues Ser-427 and Ser-430 (numbered as in the human isoform v3/v4) was detected in NDV-infected cells by 32P metabolic labelling (88, 89). VSV and HSV-1 can activate IRF5, leading to its nuclear translocation, and induction of IFN α genes. Other reported modes of IRF5 activation include induction by DNA-damaging agents (90) or ionising radiation (91). For example, ionising radiation-induced pro-inflammatory macrophage phenotype has been shown to be dependent on ataxia telangiectasia mutated (ATM) and IRF5 (91). Enhanced expression of IRF5 was observed in macrophages after moderate doses of γ -irradiation. This was accompanied by increased IRF5 serine phosphorylation and expression of IRF5-dependent target genes. In their model, the study proposed that ionising radiation increased NOX2 expression leading to the generation of

ROS double stranded breaks. This induced ATM activation in an unknown mechanism leads to IRF5 activation to favour the functional switch to a pro-inflammatory macrophage phenotype.

1.3.6. IRF5 post-translational modifications

IRF5 undergoes PTMs including polyubiquitination and phosphorylation. **Figure 1.5** and **Table 1.2.** provides an overview of reported sites, target proteins and site functions.

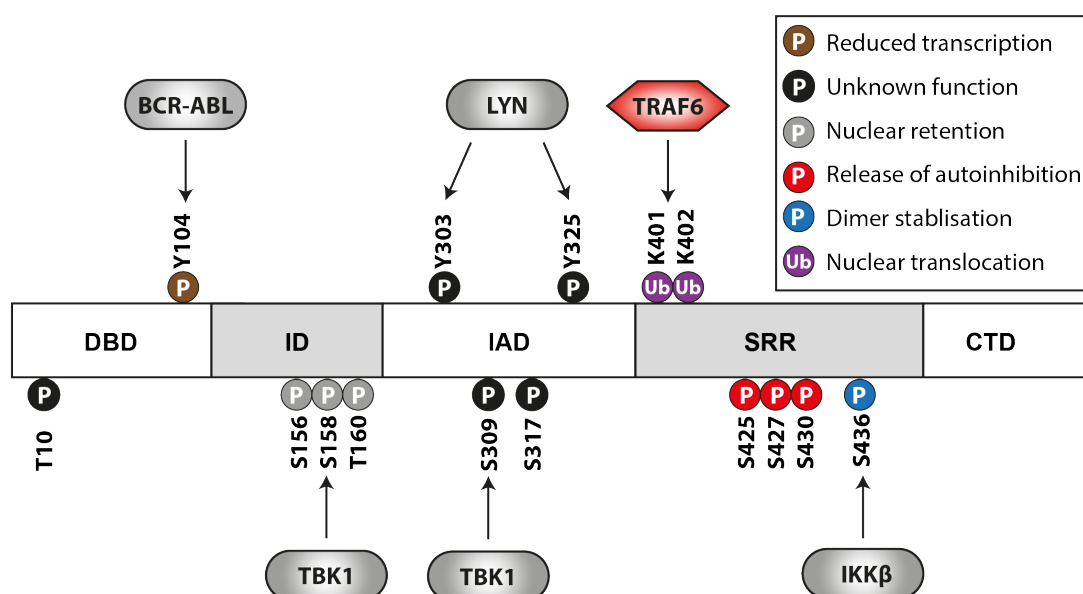


Figure 1.5. Schematics of putative IRF5 phosphorylation and ubiquitination sites Reported IRF5 (human isoform v3/v4) PTM sites including phosphorylation (P) and ubiquitination (Ub) and their positions with respect to functional domains. Suggested targets and functions of mapped sites are also shown.

Site (IRF5 isoform v3/v4)	Suggested target/ Function of site
T10	Unknown function (92)
Y104	Target of BCR-ABL (69) Transcription, inhibited (69)
S156	Nuclear retention (31)
S158	TBK1 kinase site (92) Nuclear retention (31, 92)
T160	Nuclear retention (31)
Y303	LYN kinase site (38)
S309	TBK1 kinase site (92)

S317	Unknown function (92)
Y325	LYN kinase site (38)
K401-ub	Nuclear localisation (93) Transcription, inhibited (38)
K402-ub	Nuclear localisation (93) Transcription inhibited (38)
S425	Transcription, induced (89) Release of IRF5 autoinhibition (94)
S427	Transcription, induced (89) Release of IRF5 autoinhibition (94)
S430	Transcription, induced (89) Release of IRF5 autoinhibition (94)
S436	IKK β kinase site (95, 96) Dimer stabilisation (92) Transcription, induced (89) Apoptosis, induced (92)

Table 1.2. Reported IRF5 phosphorylation and ubiquitination sites Phosphorylation on serine (S), threonine (T), and tyrosine (Y) residues and ubiquitination on lysine (K) residues on IRF5 (isoform v3/v4).

1.3.6.1. Ubiquitination of IRF5

Post-translational modification of proteins by ubiquitination is the process of adding ubiquitin chains to a lysine residue of target proteins. IRF5 has been shown to undergo both lysine-48 (K48)-linked and lysine-63 (K63)-linked polyubiquitination. K48-linked polyubiquitin chains are the most abundant linkage chain in living cells, and are known to target proteins for degradation by the 26S proteasome. The second most common linkage chain are K63-linked polyubiquitin chains and regulates proteasome-independent pathways such as signal transduction.

During IRF5 signalling, IRF5 is subjected to TRAF6-mediated K63-linked ubiquitination at positions 401 and 402 of IRF5 (of human isoform v3/v4) (93). However, to date, there is some controversy in the literature on the functional consequence of this ubiquitination. Mutagenesis studies suggest ubiquitination of IRF5 is essential for nuclear translocation and target gene regulation (93). However, several lines of evidence using double KK/RR mutants, and the A20-K63 ubiquitination enzyme have indicated that ubiquitination is not essential for IRF5 transcriptional activity (92). These ubiquitination sites are located near the C-terminal phosphorylation sites of IRF5, and their mutagenesis may potentially

interfere with dimerisation and other interactions with co-factors. Furthermore, the A20 de-ubiquitinating enzyme possesses K48 ubiquitin ligase activity, which was not addressed in the study (92). Instead, the carboxyl terminal phosphorylation of IRF5 is thought to be the critical PTM that determines IRF5 transcriptional activity.

The ubiquitin E3 ligase Pellino-1 has been reported to induce K63-linked ubiquitination of IRF5 via direct interaction and enhance IRF5 nuclear translocation during M1 macrophage polarisation (97). Furthermore, Pellino-1 mediated ubiquitination of IRF5 in M1 macrophages is reported to regulate glucose intolerance in obesity. The study proposes that TRAF6-mediated K63-linked IRF5 ubiquitination minimally occurs during macrophage polarisation as Pellino-1 competes with TRAF6 for binding to IRF5.

Other reported IRF5 ubiquitin ligases include the E3-ubiquitin ligase TRIM21 (TRIM21) and COP9 signalosome which have both been demonstrated to target IRF5 for degradation (84, 98). Following TLR7 activation, TRIM21 mediates degradation of IRF5 in an isoform specific manner, that is, it targets isoforms v1/v5 but isoforms v2/v3 are resistant to degradation. Moreover, TRIM21 targets IRF5 isoforms with different types of ubiquitin chains as it was still capable of ubiquitinating IRF5 with K48- and K63-linked ubiquitin mutants.

1.3.6.2. Phosphorylation of IRF5

Protein phosphorylation directed by kinases is one of the most common PTMs affecting protein activity. During phosphorylation protein kinases covalently attach phosphate to the side chain of either serine, threonine, or tyrosine sites of target protein substrates. Such phosphorylation of proteins can influence its activity, interaction with other proteins and molecules, and its localisation. In the case of IRF5, protein phosphorylation is essential for its activation.

In unstimulated cells, IRF5 is predominately localised in the cytoplasm in an inactive conformation. The IRF5 C-terminal autoinhibitory domain (AID) is thought to either mask the N-terminal DNA binding domain and/or the C-terminal IRF association domain (AID) required for homo/heterodimerisation. Based on the crystal structure of the transactivation domain of pseudophosphorylated human IRF5, phosphorylation is thought to induce a conformational change in a C-terminal autoinhibitory region, to enable dimerisation (94).

Phosphorylation sites towards the C-terminal serine-rich region of IRF5 include S425, S427, S430 and S436 (human isoform v3/v4). Phosphorylation of S436 contributes to the stabilisation of the activated dimer, whereas phosphorylation of S425, S427 and S430 are essential for release of the C-terminal autoinhibitory conformation (86, 94). Mass spectrometry analysis confirmed phosphorylation of S425 and S436, and extensive mutagenesis studies have revealed their contribution to IRF5 activity (92). Phosphomimetic S425D, and S436D displayed an increase in *IL-12p40* promoter-controlled luciferase activity. In contrast, the loss of function mutation with S436A reduced RIP2- induced activation of the luciferase reporter, and the double mutant S427A, S436A also failed to induce promoter activity in cells stimulated with peptidoglycan (PGN) or muramyl dipeptide (MDP). Together, these studies indicate that phosphorylation of carboxyl serine residues are essential for IRF5 function, and provide a rationale for targeting the kinases involved.

1.4. THERAPEUTICALLY TARGETING IRF5

Given the complexity of IRF5 signalling, finding an effective and feasible way of targeting IRF5 function presents a challenge. Potential strategies for modulation of IRF5 activity and expression include (1) Modulating IRF5 expression, (2) Interfering with the PTMs that modulate IRF5 function including phosphorylation and ubiquitination, and (3) Interfering

with IRF5 association with protein partners, disrupting dimer formation or DNA binding (**Figure 1.6**).

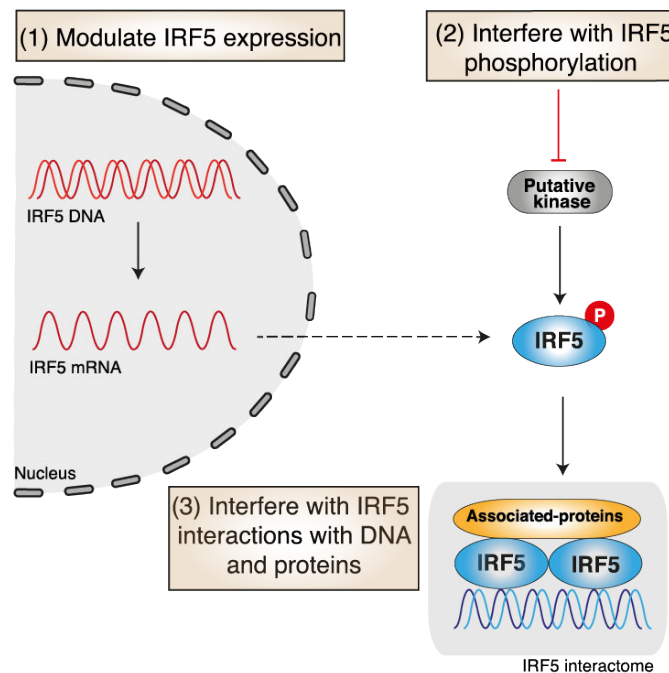


Figure 1.6. Strategies to target IRF5 (1) Modulating IRF5 expression, (2) Interfering with the upstream kinases that phosphorylate IRF5 and (3) Interfering with IRF5 interactome including protein partners, disrupting dimer formation, or DNA binding.

1.4.1. Modulating IRF5 expression

Several genetic manipulation technologies exist with the potential to control IRF5 expression levels and help reduce the severity of IRF5-affected conditions (**Figure. 1.7**). Strategies for reducing IRF5 expression include small interfering RNA (siRNA) for genetic knockdowns, clustered regularly interspaced short palindromic repeats-associated Cas9 nuclease (CRISPR-Cas9) technology for genetic knockouts, and adenoviral delivery for IRF5 overexpression. These strategies are particularly useful for mechanistic and target validation experiments that will provide a platform for the development of specific therapeutic agents targeted towards IRF5, but could also be explored as therapeutic strategies themselves. One approach to target IRF5 is to inhibit the expression of IRF5 using siRNA that are specific for sequences in the *Irf5* mRNA (**Figure 1.7a**). An *in vivo* study in an animal model of myocardial infarction (MI), demonstrated the use of siRNA to

suppress IRF5 expression in cardiac macrophages (99). Intravenous administration of siRNA packaged lipidoid nanoparticles (LNP) in mice, attenuated M1 macrophage polarisation with a decrease in M1 macrophage-associated genes without increasing M2 genes. Furthermore, siRNA treatment ameliorated inflammation following MI, and improved infarct healing.

Nano-particle-delivered IRF5 siRNA has also been used during spinal cord injury (SCI). In mice this promoted M1 to M2 transition in the wound after SCI and attenuated demyelination and neurofilament loss (100). Given that the delivery of siRNA-encapsulated LNP has proven safe and effective in humans, RNAi drugs could be a therapeutic prospect (101–103). Despite this, a number of hurdles must be overcome before RNAi drugs can be therapeutically used including off-target effects, toxicity and delivery. To date, RNAi-based

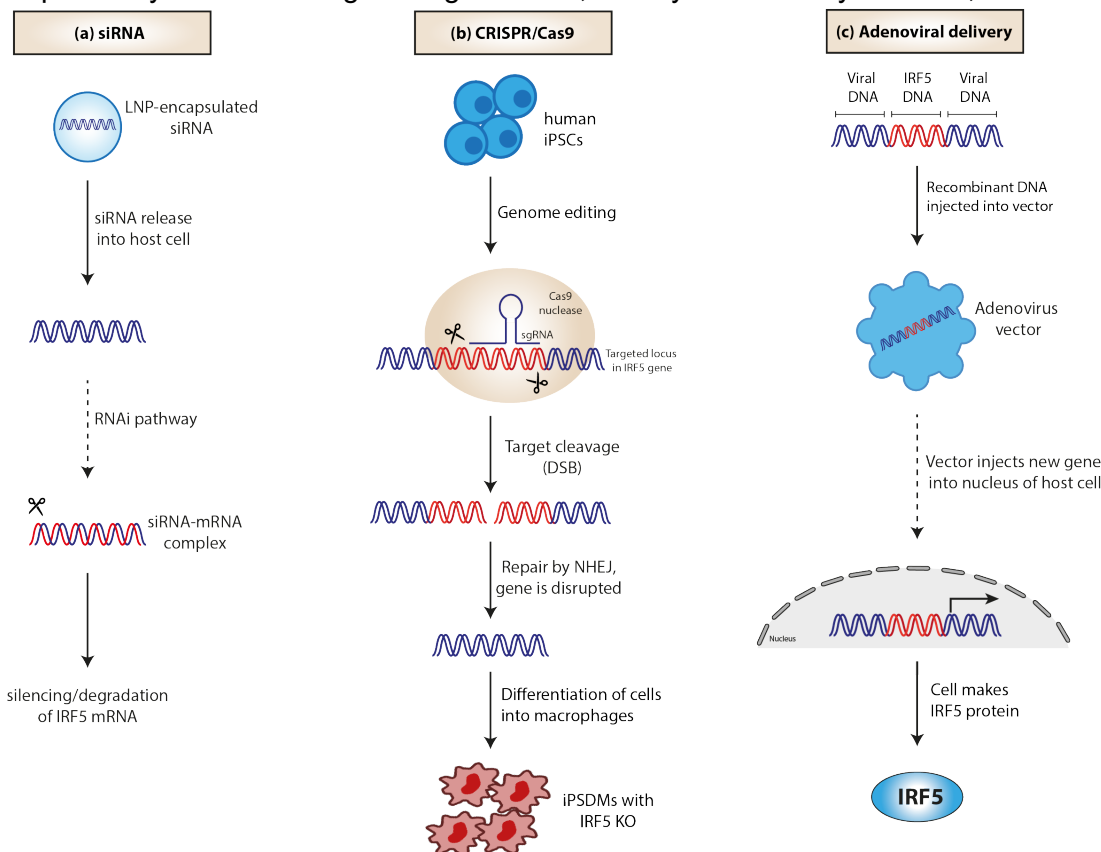


Figure 1.7. Strategies to modulate IRF5 expression (a) Small interfering RNA (siRNA)-mediated therapy using lipidoid nanoparticles (LNP) delivery to modulate the expression of IRF5 through RNA interference (RNAi). (b) IRF5 knockouts in human-induced pluripotent stem cell-derived macrophages using the CRISPR/Cas9 system. (c) IRF5 gene delivery with an adenoviral vector to increase levels of IRF5.

therapeutics to modulate the expression of target proteins are currently at the clinical trial stages (104).

A recent study utilized CRISPR/Cas9 to study the role of IRF5 in Chlamydia infection by generating IRF5 knockout mutations in macrophages derived from human-induced pluripotent stem cells (iPSdMs) (105) (**Figure 1.7b**). IRF5 iPSdM knockouts were more susceptible to Chlamydia infection, highlighting a role for IRF5 in limiting Chlamydia infection. Although the CRISPR-Cas9 approach was used for mechanistic validation, the present study opens the possibility of achieving therapeutic genome editing of IRF5 in human macrophages. While still in its infancy, therapeutics using CRISPR/Cas9 are being developed. For example, genome editing by CRISPR to delete the PD-1 gene in T cells from lung cancer patients is reportedly underway (106).

IRF5 overexpression in the airway lumen was shown to enhance immune responses in the lung following allergen exposure (56). In this study, an adenoviral vector expressing IRF5 (AdIRF5) was used to increase IRF5 expression in the lungs (**Figure 1.7c**). In response to HDM, IRF5 overexpression ameliorated airway hyperresponsiveness, reduced mucus production and reduced goblet cell hyperplasia. Moreover, overexpression of IRF5 resulted in diminished production of type 2 cytokines and decreased eosinophilia. Thus, localised adenoviral delivery of IRF5 (e.g., via intranasal administration) to enhance IRF5 expression in macrophages and stimulate their immune potential could be a promising therapeutic strategy in eosinophilic asthma and would circumvent a concern related to the systemic administration of adenoviruses which results in hepatic tropism independent of the primary receptors (107). Adenovirus-based vectors are a widely used therapeutic platform for gene delivery, especially in the field of cancer gene therapy, where they demonstrate a good safety record and a great promise in preclinical studies (108). IRF5 overexpression could represent a promising approach to enhance cancer therapies through the reprogramming of tumour-infiltrating macrophages, which are primarily M2-polarised.

1.4.2. Inhibiting IRF5 post-translational modifications

Other than strategies to module IRF5 expression, blocking the enzymatic action of regulators of IRF5 signalling including kinases and E3 ubiquitin ligases, could provide attractive targets for therapeutic intervention. Although IRF5 undergoes ubiquitination, phosphorylation of IRF5 is thought to be the critical modification that determines IRF5 transcriptional activity. Targeting kinases in a signalling pathway with small molecule inhibitors is typically the most straightforward approach to block a protein. Kinases have been established as an important drug target. Despite a high degree of similarity in their catalytic core with the ATP-binding pocket, kinases are amenable to blockade by small molecule agents with high selectivity. Moreover, high-throughput kinase inhibitor libraries can identify potential candidate kinases.

Several kinases including TBK1, RIP2, IKK ϵ , IRAK4, TAK1, and IKK β have been proposed to phosphorylate and activate IRF5 (44, 109–111), while IKK α inhibits IRF5 (111) (**Figure 1.8**). Lyn, a Src family kinase has been shown to negatively regulate IRF5 in the TLR-

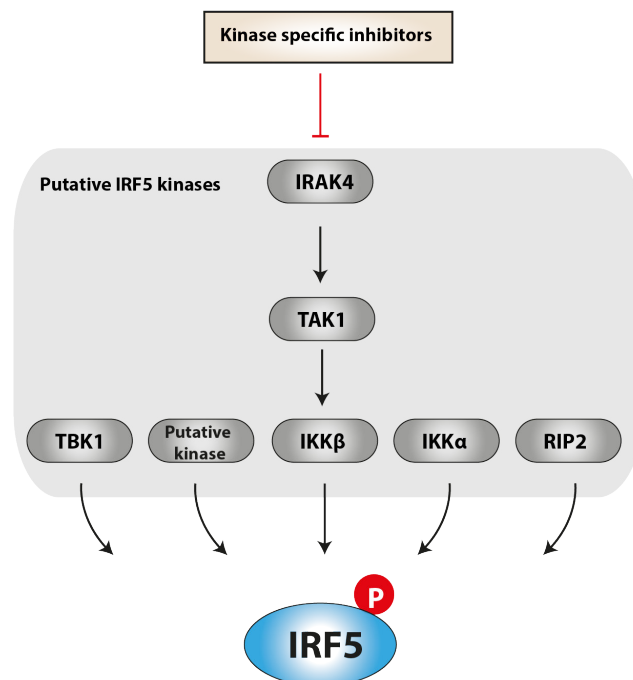


Figure 1.8. Inhibition of IRF5 phosphorylation through the use of kinase inhibitors Kinases reported to phosphorylate IRF5 include IRAK4, TAK1, RIP2, IKK α , IKK β , IKK ϵ and TBK1.

MyD88 pathway in a kinase independent manner via direct binding to IRF5 (38). Phosphorylation of IRF5 by TBK1 or IKK ϵ fail to induce nuclear translocation of IRF5 (31). Furthermore, macrophages from TBK1-deficient mice produce normal levels of proinflammatory cytokines in response to LPS (112). Thus, these kinases can be excluded as suitable IRF5 candidate kinases. Stimulation of the NOD2 intracellular receptor with MDP or PGN, can induce IFN β expression via IRF5 and RIP2 (113). Using overexpression systems, a later study identified S436 on IRF5 as a RIP2 target residue (92). More recently, endogenous IRF5 serine phosphorylation in response to MDP was shown to be dependent on RIP2 and IKK β , where IRF5 associates with RIP2, IRAK1 and TRAF6 (66). IRF5 and IKK β cross-regulate each other's phosphorylation, but the kinase in the IRF5 complex responsible for IKK β activation is unknown in this model (66). Since IKK β phosphorylation of IRF5 is known, it could be an interesting pharmacological target.

Earlier studies based on the use of IKK β loss of function mutations, indicate that IKK β activity is required for the activation of IRF5. Two groups have shown that phosphorylation by IKK β on S436 induces IRF5 nuclear translocation and an IFN response (95, 96). Numerous inhibitors which are specific to IKK β , and do not inhibit other IKK-related kinases are available, but as IKK β is involved in the activation of several targets its inhibition is unlikely to result in the specific inhibition of IRF5 function. Besides, the well documented role of IKK β in phosphorylating I κ B in the NF- κ B pathway, other reported substrates include forkhead transcription factor (FOXO3a), 14-3-3 β , insulin receptor substrate 1 (ISR1) and docking protein 1 (DOK1) (114–117).

IKK α is another kinase from the IKK family reported to phosphorylate IRF5. Unlike IKK β , IKK α -mediated phosphorylation of IRF5 negatively regulates IRF5 in the MyD88-IRF5 pathway by blocking TRAF6-mediated IRF5 ubiquitination (111). IKK α could represent a therapeutic target in conditions with low IRF5 activity. However, no potent commercially

available IKK α -specific inhibitors have been reported to date. Another reported kinase involved in IRF5 activation is TGF-B-activated kinase 1 (TAK1). Upstream of IKK β , TAK1 can induce IFN-B and IL-12 in response to *Staphylococcus aureus* RNA (43). Several inhibitors for TAK1 reported to inhibit IRF5 include NG-25, and 5Z-7-oxozeaenol, which both block nuclear translocation of IRF5 in monocytes stimulated with TLR8 ligands (43). However, as TAK1 plays an essential role in several signalling pathways including the MAPK cascade and IKKs (118), inhibition of TAK1 might not offer the best approach for inhibiting IRF5 function.

In a recent report, IRAK4 was identified as a kinase that activates IRF5 via an IRAK4-TAK1-IKK β axis in human monocytes (44). Chemical inhibition of IRAK4 with a potent and selective IRAK4 inhibitor blocked IRF5 nuclear translocation and IRF5 transcriptional activity at the promoters of inflammatory cytokines, such as IL-1, IL-6, and TNF in human monocytes. Despite blocking IKK β activation, the IRAK4 inhibitor had no effect on NF-kB nuclear translocation and transcriptional activity. In their proposed model, the kinase activity of IRAK4 kinase regulates the IRF5 pathway, whereas the scaffolding function of IRAK4 regulates the NF-kB pathway. The requirement of IRAK4 kinase activity in regulating IRF5 supports the rationale for IRAK4 inhibitor use. Inhibition with IRAK4-specific inhibitors could be sufficient to block IRF5 activity without having an impact on NF-kB activation. This is a promising study and future studies will have to establish the efficiency of the inhibitor in disease mouse models. Mice harbouring the IRAK4 kinase-dead mutant are protected from disease models of RA (119), emphasising IRAK4 as an attractive therapeutic target in the context of IRF5.

1.4.3. Interfering with IRF5 interacting partners

Transcription factors are traditionally considered as 'non-druggable' targets. Blocking their activity with small molecules presents a challenging task, given the large surface areas for

binding proteins or DNA, the lack of hydrophobic pockets and the absence of enzymatic activity (120). Despite these obstacles, therapeutic modulation of transcription factor activity has been well documented by interfering with their ability to bind to DNA, partner proteins or their ability to dimerise. For example, the small molecule agent Nutlin can disrupt the p53–MDM2 interaction, thereby increasing p53 levels and p53 target gene expression (121). Another example is the MAML1-derived stapled peptide which disrupts the NOTCH transcription complex and represses Notch transcriptional function (122).

One such method for blocking protein–protein interactions is the use of peptide inhibitors. These consist of short amino acid sequences that competitively inhibit the interaction between proteins. Several advantages that peptide inhibitors offer are their affordable synthesis, specificity, potency and activity (123). However, several drawbacks to consider are their low proteolytic stability and low conformational stability, which could decrease target binding (124). Despite these issues, ongoing efforts have been undertaken to develop effective peptide inhibitors to disrupt protein–protein interactions.

With regard to IRF5, peptide-based inhibitors to decrease myocardial inflammation and fibrosis have been developed. In a murine model of systemic scleroderma, IRF5 bound to an apoAI mimic 4F, a peptide that inhibits myocardial inflammation (125). Hearts from 4F-treated Tsk2/+ mice are less inflamed with a decrease in IRF5 expression, phosphoserine levels and nuclear localisation. Whether these effects are mediated in part by the ability of 4F to directly bind to IRF5 or to other targets is unclear. It is also noted in the study that the changes observed may be due to differences in immune cell content. Nevertheless, the possible involvement of 4F binding to IRF5 warrants further exploration.

A more recent promising study developed a peptide inhibitor specifically designed from the peptide sequence of IRF5 (59). The synthesised decoy peptide, termed IRF5D is derived from the IRF5 C-terminal dimerisation domain with the original sequence ELSWSAD-

SIRLQISNPD replaced by the 17 amino acid-long sequence ELDWDADDIRLQIDNPD. The aspartate substitution mimics activated IRF5, whereby IRF5D associates with IRF5 and prevents nuclear translocation of IRF5. In the study, Tsk/+ mice were used as a murine model of myocardial inflammation and fibrosis. IRF5D-treated Tsk/+ mice produced reduced levels of ICAM-1 and IRF5 expression in the heart, reduced leucocyte infiltration of the myocardium, improved endothelial vasodilation and IRF5 nuclear translocation was reduced in cultured Tsk/+ myocytes. Moreover, IRF5D treatment had no effect on endothelial cell (EC) proliferation and apoptosis. Thus, this study demonstrates that IRF5 is a druggable target and based on the published 3D structure of IRF5 (94), it is possible to develop IRF5-specific peptide inhibitors.

Alternatively, peptide inhibitors may target other interfaces such as the IRF5/RelA-binding interaction. A previous study has demonstrated that IRF5 is recruited to the TNF α gene via interaction with NF- κ B subunit RelA (45, 46). The requirement for RelA for induction of proinflammatory genes with IRF5 supports the concept of developing inhibitors to block the IRF5–RelA interaction. This association has been mapped to the IAD of IRF5 and the dimerisation domain (DD) of RelA (46). Based on this information, along with the crystal structure of the IRF5 IAD (94), peptide inhibitors have been developed to sterically inhibit the IRF5/RelA interface but showed no significant inhibitory effects on IRF5- dependent gene expression (126). Further work to optimise peptide delivery and modifications to protect the peptide from degradation are worth pursuing to develop effective IRF5 inhibitors.

Other IRF5-interacting proteins thought to be involved in IRF5 activation include CBP/p300 and histone deacetylases (127, 128), as well as signalling molecules KAP1, RIP2, TRAF6, MyD88, IRAK1 and IRAK4 (30, 37, 66, 129) (**Table 1.3**). Thus, binding sites on IRF5 could be a potential peptide target and would require identifying the peptide sequences that are crucial for the IRF5–protein interactions. Another IRF5-binding partner that has been shown to interact with and stabilise IRF5 is the CSN, which is thought to protect IRF5 from

degradation by the ubiquitin–proteasome pathway (84). This interaction was mapped to the carboxyl and amino termini of IRF5. Disruption of the CSN–IRF5 interaction was shown to reduce IRF5 transactivation ability, whereas inhibition of IRF5 degradation by the proteasome enhances its transcriptional ability. Therefore, peptide inhibitors that target the CSN–IRF5 binding interaction could destabilise IRF5 and reduce IRF5 activity.

Negative gene regulation	Positive gene regulation	IRF5 activation
KAP1 TRIM21	RelA CBP/p300 Histone deacetylase	RIP2 TRAF6 MYD88 IRAK1 IRAK4 CSN LYN

Table 1.3. Summary of the IRF5 interactome

The recent identity of Lyn from the Src family of tyrosine kinases as an IRF5-binding partner that negatively regulates IRF5 in the TLR-MyD88-IRF5 pathway could be an interesting target to increase IRF5 activity in conditions such as asthma (38, 56). In a kinase-independent manner, Lyn inhibits IRF5 by directly binding to IRF5 and preventing PTMs. Lyn deficiency in mice suffering from SLE leads to IRF5 hyperactivation, but reducing IRF5 levels ameliorates the disease development. Consistent with the elevated activity of IRF5, Lyn-deficient mice exhibit profound intestinal inflammation and colitis (130). Like IRF5, Lyn is highly expressed in immune cells (DCs, monocytes, macrophages and B cells), making it an attractive therapeutic target in inflammatory conditions. Since Lyn association with IRF5 is not activation or phosphorylation dependent, available Lyn allosteric activators such as MLR-1023 (131) are unlikely to affect IRF5 function. Instead, peptides could potentially bind and regulate IRF5 activation. The use of Lyn peptide inhibitors have been reported to bind to Lyn and block its association with the Bc receptor (132). *In vitro*, the Lyn peptide inhibitor blocked Lyn-dependent functions of IL-5 (132), and MD-2 (Myeloid differentiation) tyrosine phosphorylation (133). Moreover, the peptide inhibitor blocked eosinophil differentiation, survival and airway influx in a murine model of asthma (132). Therefore,

peptide inhibitors that block the Lyn–IRF5 interaction could be used in the context of asthma to enhance IRF5 function. The Lyn–IRF5 binding interaction has been mapped to Lyns unique and kinase domains (LYN UD-IRF5 DBD and Lyn KD-IRF5 IAD) (38). Based on the amino acid sequence of these domains, peptides could be developed and tested for their ability to increase IRF5 activity.

An alternative approach is the use of oligonucleotides (ODNs) containing the consensus binding site of a transcription factor. Use of decoy ODNs to bind to IRF5 offers another way to inhibit its activity, and has recently been investigated (134). IRF5 and an ODN termed MS19 share a consensus AAAG repeat-binding site. In a mouse model of septic peritonitis, MS19 treatment prolonged survival and reduced expression of iNOS, IL-6 and TNF- α , suggesting that it might be useful in treating inflammatory conditions. *In vitro* treatment of LPS-stimulated RAW264.7 macrophages, also reduced expression of iNOS, IL-6, TNF- α and IRF5, with reduced nuclear IRF5 levels. MS19 is thought to bind to IRF5, prevent its nuclear translocation and subsequent induction of target genes. MS19 as an ODN to interfere with IRF5 function could be a potential therapeutic, that warrants further investigation. An efficient means to deliver MS19 to target cells, and its specificity towards IRF5 should be considered in future studies.

1.4.4. Future perspectives on IRF5 therapeutics

Peptide inhibitors are an attractive therapeutic strategy as they offer the advantage of targeting IRF5 directly but there are issues with delivery and stability. A more promising strategy at this stage is the use of specific inhibitors to the components of the IRF5-signalling pathway, for example, kinase inhibitors. Kinase inhibitors offer several benefits over biologic therapies, principally, they are easier to administer (orally), less expensive to produce, and easier to store. Much progress has been made in the use of kinase inhibitors in the treatment of cancer. One such success story is Imatinib (Novartis) which inhibits the

tyrosine kinase BCR-ABL, the causative target of chronic myelogenous leukaemia. A greater understanding of inflammatory signalling cascades has led to kinase inhibitors taking centre stage in the pursuit for new anti-inflammatory agents. Thus, targeting IRF5 kinases could provide a potential avenue for the development of therapeutic agents.

1.5. HYPOTHESIS OF INVESTIGATION

The goal of this study is to identify novel kinase(s) for drug targeting IRF5 activity in inflammatory disorders. This project will authenticate kinases identified in a preliminary study by analysing their role in regulating IRF5 function in macrophages and test the hypothesis that targeting IRF5 kinases will block IRF5 activity to restore macrophage homeostasis, thus reduce inflammation. Chapter 3.0 provides an overview of the kinase inhibitor screen and validation of candidate kinases in overexpression and endogenous systems. Chapter 4.0 describes PYK2 as a lead IRF5 kinase with the use of PYK2 deficient cells and PYK2-specific inhibitors. Chapter 5.0 presents MEKK3 kinase as a lead IRF5 kinase with initial development and testing of MEKK3-specific inhibitors. Finally, chapter 6.0 summarises my findings and provides insight for future studies.

1.5.1. Specific aims for kinase screen and validation (Chapter 3.0)

- 1) Characterise the endogenous expression of IRF5 and candidate kinases in macrophages including RAW264.7 cells and BMDMs
- 2) Perform endogenous binding assays of candidate kinases with IRF5
- 3) Generate knockout cell lines of IRF5 and lead kinases in RAW264.7 cells by CRISPR/Cas9
- 4) Test the effect of kinase deficiency on the IRF5-driven TNF promoter driven firefly luciferase reporter assay
- 5) Establish the kinetics of IRF5 phosphorylation in RAW264.7 cells over a time course of LPS stimulation
- 6) Optimise protocols to study the nuclear translocation of IRF5

On the basis of the initial validation round with overexpression systems and secondary validation round with endogenous systems I narrowed down on PYK2 and MEKK3 as lead candidate kinases.

1.5.2. Specific aims for PYK2 kinase validation (Chapter 4.0)

- 1) Investigate the effects of PYK2 inhibition or deletion on cytokine levels and TNF-reporter activity
- 2) Explore whether PYK2 is involved in IRF5 recruitment to IRF5-dependent genes
- 3) Compare the transcriptome of WT and IRF5 KO macrophages treated with PYK2 inhibitor
- 4) Study the effects of PYK2 inhibition on IRF5 nuclear translocation
- 5) Identify PYK2 dependent phosphorylation sites on IRF5 by mass spectrometry and explore their functional importance
- 6) Examine the impact of PYK2-specific inhibitors in human macrophages and biopsies

1.5.3. Specific aims for MEKK3 kinase validation (Chapter 5.0)

- 1) Investigate the effects of MEKK3 deletion on cytokine levels and TNF-reporter activity
- 2) Test MEKK3 inhibitor compounds developed by LAB282 (Evotec) in cell based assays
- 3) Investigate the global phosphoproteome of WT and kinase deficient cells to map out substrate specificity of candidate kinases

2.0. MATERIALS AND METHODS

2.1. CELL CULTURE

2.1.1. Maintenance of cell lines

Cell lines were incubated in a 5% CO₂ humidified atmosphere at 37°C. RAW264.7 cells, a mouse leukemic monocyte macrophage cell line were purchased from Invivogen USA and cultured in DMEM (Lonza) supplemented with 10 % v/v Foetal Bovine Serum (FBS; Gibco) and 1 % v/v Penicillin/Streptomycin (P/S; Lonza). HEK293-TLR4-CD14/Md2 (HEK293-TLR4) cells were cultured in DMEM (Lonza) supplemented with 10 % v/v Foetal Bovine Serum (FBS; Gibco), 1 % v/v Penicillin/Streptomycin (P/S; Lonza), 50 µg/ml Hygromycin B (Invivogen) and 10 µg/ml Blasticidin S (Invivogen). RAW264.7 and HEK-TLR4 cells were cultured in 10 cm tissue culture dishes (Falcon, UK) up to a confluency of 80 %, and passed for no more than 10 passages. Cells were regularly tested for *Mycoplasma* contamination.

Passage procedure - Culture media was aspirated from the cells, and gently rinsed with Dulbecco's Phosphate Buffer Saline (PBS; Lonza). Trypsin-EDTA (Life Technologies) was then added to the dish and the cells were incubated for 5 minutes at 37°C to detach the adherent cells. RAW264.7 cells were gently scraped with a cell lifter to dislodge the cells. Cells were resuspended with twice the volume of trypsin with complete media to stop the reaction, and centrifuged at 1,500 rpm for 5 minutes. The cell pellet was resuspended in fresh media and seeded onto 10 cm plates at a 1:5 dilution or cells were counted and seeded for experiments as necessary.

2.1.2. Cryopreservation of cell lines

To freeze cells- Cells were resuspended in FBS with 10% v/v dimethylsulfoxide (DMSO; Invitrogen) at a cell count of 2×10^7 million cells/ml. The cell suspension was transferred as

1 ml aliquots into cryovials, and stored in an insulated freezing container at -80°C overnight to ensure slow freezing. The cryovials were then transferred to liquid nitrogen for long-term storage.

To defrost cells- Vials of frozen cells were thawed quickly by swirling in the 37°C water bath until just defrosted. The contents were transferred into pre-warmed media (10 ml total) and centrifuged at 1,500 rpm for 5 minutes to remove the DMSO. The cell pellet was resuspended in 10 ml media and seeded onto a 10 cm plate. Cells were passed for at least two passages before any experiments were carried out to allow recovery from the freeze/thawing stress.

2.2. GENERATION OF BONE MARROW-DERIVED CELLS

2.2.1. Isolation of whole bone marrow

Wild-type mice on a C57BL/6 background were sacrificed by Schedule 1 method by CO₂ inhalation followed by cervical dislocation. Femurs and tibias were dissected and removed by dislocating the bones at the ankle and pelvis. Excess muscle tissue surrounding the bones were removed with scissors, followed by soaking the bones in 70% v/v ethanol for 5 minutes, then removing the remaining muscle using a tissue (Kimtech). Cleaned bones were cut at both ends, and the bone marrow flushed out into a 50 ml tube (Falcon) with 10 ml of PBS in a 10 ml syringe with a 27G needle until the bones appeared clear and white. All the bone marrow flushed from a single mouse was collected in the same tube and then centrifuged at 1,500 rpm for 5 minutes at room temperature. The resulting pellet was resuspended in 1 ml of bone marrow culture media consisting of RPMI-1640 medium with L-glutamine (Lonza) + 10 % v/v FBS (Gibco) + 1 % v/v P/S (Lonza) supplemented with 0.01 % 2-mercaptoethanol (Gibco). A 10 µl aliquot of the cell suspension was counted using the CasyTon and the cells diluted to a concentration of 5×10^6 cells/ml in BM media. BM cells

were seeded in a bacterial petri dish (Sterilin) and incubated in a 5 % CO₂ humidified atmosphere at 37°C.

2.2.2. Bone marrow-derived macrophages

For the generation of *in vitro* differentiated macrophages, bone marrow from wild type mice were cultured in BM media with either recombinant murine GM-CSF (20 ng/ml; Peprotech) or recombinant human M-CSF (100 ng/ml; Peprotech).

For M2 macrophages, BM progenitors were treated with M-CSF on Day 0, with no further addition of M-CSF during the 8-day culture period. For M1 macrophages, BM progenitors were treated with fresh GM-CSF on days 0, 3 and 6. On day 3, 10 ml of BM media supplemented with 20 ng/ml murine GM-CSF was added to the culture dishes (20 ml media total). On day 6, 10 ml of the cell culture suspension was transferred to a 50 ml tube, centrifuged at 1,500 rpm for 5 minutes and the pellet resuspended in fresh BM media supplemented with 20 ng/ml murine GM-CSF. The cell suspension was then returned to each dish.

After 8 days of differentiation, cells were washed with 10 ml PBS and then harvested by Versene-EDTA (Lonza). Cells were resuspended in BM media, seeded in tissue culture plates (BD) as required. Macrophages were stimulated with LPS (100 ng/ml; Enzo). For GM-CSF differentiated macrophages (GM-BMDMs), medium contained 10 ng/ml GM-CSF during LPS stimulation, whereas M-CSF differentiated macrophages (M-BMDMs) did not require additional M-CSF.

2.3. REAGENTS

RAW264.7 cells were stimulated with LPS from *E. coli*, Serotype EH100 (Ra-mutant) (Enzo) at indicated concentrations and bone marrow derived macrophages were stimulated with

LPS (Enzo) at a final concentration of 100 ng/mL. WGP Dispersible (InvivoGen) was made up to a 1 mg/ml stock in endotoxin-free water (Sigma-Aldrich) and used at a final concentration of 100 µg/mL. For CRISPR lentiviral transductions, RAW264.7 cells were selected in 4 µg/ml puromycin (InvivoGen).

Kinase inhibitors (**Table 2.1**) were dissolved in DMSO to make a 10mM stock and stored as aliquots at -20°C. Prior to treatment inhibitors or vehicle solution (DMSO) were diluted in ten-fold dilutions in cell culture media to make 10 µM and 100 µM stocks. Lysis buffers were routinely supplemented with protease inhibitor tablets (1 tablet per 10 ml; cComplete™ Protease Inhibitor Cocktail tablets; Roche), phosphatase inhibitor (1:100; Phosphatase Inhibitor cocktail 2; Sigma-Aldrich), and benzonase nuclease (1 µl per 1 x 10⁷ cells; Sigma-Aldrich) prior to use.

Name	Target	Supplier
PF-431396 hydrate	PYK2/FAK	Sigma
Defactinib/PF-04554878/VS-6063	PYK2/FAK	Cayman chemical
Ruxolitinib / EOAI3445270 / EV'008	JAK1/2	Evotec
Neratinib / EOAI3411488 / EV'010	HER2 EGFR	Evotec
PP121 / EOAI3444793 / EV'015	PI 3-K	Evotec
5n / EOAI3983773 / EV'030	ALK	Evotec
EOAI3999039	EV'010 derivative	Evotec
EOAI3999037	EV'010 derivative	Evotec
EOAI4001041	EV'010 derivative	Evotec
EOAI3983902	EV'010 derivative	Evotec
EOAI3411487	EV'010 derivative	Evotec
EOAI4006691	EV'010 derivative	Evotec
EOAI4002355	EV'010 derivative	Evotec
EOAI4006768	EV'010 derivative	Evotec
EOAI4006693	EV'010 derivative	Evotec
EOAI4003272	EV'010 derivative	Evotec
EOAI4004328	EV'010 derivative	Evotec
EOAI4004326	EV'010 derivative	Evotec
EOAI4005432	EV'010 derivative	Evotec
EOAI4005475	EV'010 derivative	Evotec
EOAI4005433	EV'010 derivative	Evotec
EOAI4005476	EV'010 derivative	Evotec
EOAI4006771	EV'010 derivative	Evotec
EOAI4006694	EV'010 derivative	Evotec
EOAI4006692	EV'010 derivative	Evotec
EOAI4006690	EV'010 derivative	Evotec

Table 2.1. List of kinase inhibitors used in this study

2.4. CELL VIABILITY ASSAY

Cell viability was measured using Cell-Titer-Glo luminescent cell viability assay (Promega) per the manufacturer's protocol. The kit determines the number of viable cells based on amount of ATP present, an indicator of metabolically active cells. Briefly 5×10^4 cells were cultured in poly-lysine (Sigma-Aldrich) coated 96-well plates and incubated overnight. The kinase inhibitor was added at indicated concentrations and timepoints the following day. Plates were washed once with PBS, and then 75 μ l PBS and 75 μ l of CellTiter-Glo reagent was added to lyse the cells. After a 3-minute incubation at room temperature on a shaker, samples were transferred to a luminescent-plate (ThermoScientific), and the luminescence was recorded in a luminometer (BMG FLUOstar). The luminescence signals were normalised to the luminescence signal obtained from untreated cells.

2.5. TRANSFECTIONS AND LENTIVIRAL INFECTION

2.5.1. Nucleofection of RAW264.7 cells

RAW264.7 cells were transfected by electroporation using the Amaxa Cell Line Nucleofector Kit V (Lonza) according to the manufacturers recommendation. Briefly, 2×10^6 cells was resuspended in 100 μ l of Nucleofector solution, followed by the addition of 2 μ g of total DNA plasmid. The cell/DNA suspension was transferred to a cuvette and placed in a Nucleofector II device (Amaxa) programmed to D-032. The sample was removed from the cuvette and plated onto a 6-well plate for overnight incubation.

2.5.2. Transfection of RAW264.7 cells for luciferase assay

All kinase plasmids were prepared and provided by Dr. Grigory Ryzhakov. Prior to transfection, plasmids were run on a 1 % w/v agarose gel by EtBr and UV excitation to check they were supercoiled.

RAW264.7 cells were seeded into poly-lysine coated 96-well plates at a density of 3×10^4 cells/well in 100 μ l media and incubated overnight. For a luciferase reporter assay for 6 wells (**Section 2.9.5**) the reagent quantities and protocol were followed:

1. Prepare 600 ng of total DNA plasmid in 40 μ l of Optimem media (Gibco)
2. Add 19 μ l optimem and 1 μ l plus reagent. Incubate for 15 minutes at room temperature
3. Add 18 μ l optimem and 2 μ l LTX reagent. Incubate for 30 minutes at room temperature
4. Add 12.5 μ l of the transfection mixture per well and incubate cells overnight

2.5.3. Transfection of HEK-TLR4 cells for luciferase assay

HEK293-TLR4 cells were seeded (50,000 cells/well) in poly-L-lysine (Sigma) 96-well tissue culture plates and incubated overnight at 37°C. In order to transfect 6 wells for a luciferase reporter assay (**Section 2.9.5**) the reagent quantities and protocol below were followed:

1. Prepare 1200 ng total DNA plasmid in 176 μ l Optimem media (Invitrogen)
2. Add 7 μ l Lipofectamine 2000 reagent (Invitrogen) in 176 μ l Optimem media, and incubate for 20 minutes at room temperature
3. Add 55 μ l of the transfection mixture per well of a 96 well-plate (6 wells total)

2.5.4. CRISPR/Cas9 lentiviral infections

The CRISPR/Cas9 system consists of a single Cas9 protein and a non-coding guide RNA (gRNA). The Cas9 endonuclease can be programmed with a single gRNA, directing a DNA double-strand break (DSB) at a desired genomic location. The DSB is repaired by DNA repair processes such as non-homologous end joining (NHEJ) and the repair introduces mutations to generate a knockout. RAW264.7 cells were transfected with lentiviral particles containing an all-in-one lenti CRISPR vector containing both gRNA and Cas9 elements

where Cas9 open reading frames are flanked by puromycin and GFP elements. Infected cells were selected by puromycin treatment.

Lentiviral transfections were carried out on RAW264.7 cells of low passage number. 5,000 cells/well were seeded in 96-well plates and infected the following day with IKBKB, PTK2B, TBK1, HIPK4, MAP3K and IRF5 lentiviral particles (pLV-U6g-EPCG) provided by Sigma-Aldrich (**Table 2.2**). The volume of virus needed to reach desired dose was determined by the following formulas:

1. # cells x desired MOI (*Multiplicity of Infection*) = total TU (*Transducing Units*) needed.
2. (Total TU needed) / (TU/ml) = total ml of virus needed to reach desired dose.

Where:

#cells = 5000

MOI = 10

TU/ml= As provided on the data sheet (**Table 2.2**)

Cells were transduced with virus particles in medium containing 8 µg/ml polybrene (Sigma) and spun at 1,500 x g for 1 hour. After an overnight incubation, media was replaced with fresh media. Cells were selected every 2-3 days with puromycin (4 µg/ml) for 2 weeks and checked under the microscope for GFP expression. When cells reached 80 % confluence under puromycin selection, cells were transferred to bigger plate formats (from 24 well plates to 6 well plates, then to a 10-cm plate). Cells were expanded to several 10-cm plates and frozen down (**Section 2.1.2**) for long-term storage.

Target ID	Gene	Sequence Verified	Titer (TU/mL)
MM0000139716	IKBKB	GGAAATGAAAGAACGCCTGGGG	1.4 X 10 ⁷
MM0000145196	PTK2B	CCCTATTTCGCCACTCAGGAGG	8.6 X 10 ⁶
MM0000365661	TBK1	TTGAACATCCACTGGGCGAAGG	1.0 X 10 ⁷
MM0000329202	MAP3K3	GACTCGATCATGAAGGACCTGG	1.2 X 10 ⁷
MM0000299177	IRF5	ACCCTGGCGCCATGCCACGAGG	1.4 X 10 ⁷
MM0000299181	IRF5	CTGAGGTGGCATGTCCCGAGGG	1.4 X 10 ⁷

Table 2.2. List of candidate kinases and IRF5 lentiviral particles

2.6. DNA SEQUENCING

1x10⁶ cells were plated on a 6 well plate and genomic DNA (gDNA) was isolated from WT and KO cells lines using the DNeasy Blood and Tissue Kit (Qiagen) in accordance with the manufacturer's protocols. The eluted DNA was subsequently sequenced via Sanger sequencing (Source Bioscience). Target gene of interest were amplified by designing primers approximately 300 bp from the CRISPR PAM sequence. All primer sequences can be found in **Table 2.3**. SnapGene View software was used to analyse and align the sequencing results.

Target of interest	Forward primer sequence (5'→3')	Reverse primer sequence (5'→3')
IRF5_177	GTTTCCTTCTCCCCTAACAGAACTC	GGATAGCCTTGATCCCTAGCACAAA
IRF5_181	GCAGCAGTGAGTAGAATTCATTGAG	CCCAAAGAAGAGATGTGGAGGACAA
PYK2	ATCTGGTCTGGAAGTGTAACTTAGA	ATCTTCTAGAAAAGTGGAGTGGCTT
TBK1	ACACCTCTCTTTCTAGTGACACTTC	ACTGGTGATCTCTATGCTGTCAAAGT
MEKK3	ATTCAGATTGCTTTAGCTTGACCTT	AGAGGATCACTGTACTCCAAGTATG

Table 2.3. List of primers for DNA sequencing

2.7. PROTEIN DETECTION

2.7.1. Protein isolation from cultured cells

Cells were harvested from tissue culture plates by gently scraping with a cell lifter and pelleting in 1 ml of PBS in a microcentrifuge by centrifugation at 1,500 rpm. Cell pellets were lysed in cold 1 % Tx-100 lysis buffer (1 % v/v TX-100, 10 % v/v glycerol, 1mM EDTA, 150 mM NaCl, 50 mM Tris pH 7.8) supplemented with protease and phosphatase inhibitors. Lysates were incubated on ice for 30 minutes and cleared by centrifugation at 13,000 rpm for 10 minutes at 4°C to remove cellular debris. Supernatants were transferred into new tubes and stored at -80°C for long term storage.

Subcellular fractionation by salt extraction were prepared as described by Schreiber *et al* (135). Briefly 1x10⁷ cells were pelleted by centrifugation, and resuspended in a hypotonic

cold buffer A (**Section 2.13**). The cells were allowed to swell on ice for 15 minutes, followed by the addition of 60 μ l of a 10 % solution of NP-40 (Sigma), and vigorously vortexed for 10 seconds. The homogenate was centrifuged at 13,000 rpm for 30 seconds in a microfuge at 4°C, with the supernatant containing the cytoplasm. The nuclear pellet was resuspended in 100 μ l of ice-cold buffer B (**Section 2.13**), and the tube was vortexed intermittently for 15 minutes. The nuclear extract was centrifuged at 13,000 rpm for 5 minutes at 4°C and the supernatant collected as the nuclear fraction.

Subcellular fractionation by sucrose method were prepared by plating 1×10^7 cells. Cell pellets were collected and washed three times with PBS. The pellet was resuspended in 200 μ l cytoplasmic lysis buffer (**Section 2.13**). Cells were incubated on ice for 10 minutes, and layered on top of 500 μ l cold sucrose buffer (**Section 2.13**). The nuclear pellet was collected by centrifugation at 12,000 rpm for 10 minutes at 4°C. The supernatant was collected as the cytosolic fraction and the nuclear pellet was resuspended in 200 μ l of RIPA buffer (**Section 2.13**). The nuclear lysate was sonicated for 10 cycles (30 seconds on/30 seconds off) on the Biorupter Pico, followed by centrifugation at 13,000 rpm for 5 minutes at 4°C. The supernatant was collected as the nuclear fraction.

Subcellular fractionation by chromatin immunoprecipitation is described in **Section 2.10**.

2.7.2. Protein isolation from tissues

1.5 ml Bioruptor Microtubes were filled with 200-250 mg of Protein Extraction Beads (Diagenode) and filled with RIPA buffer supplemented with protease and phosphatase inhibitors. 25 mg of tissue was added to the tubes and vortexed briefly. Tubes were sonicated on the Biorupter Pico for 5 cycles (30 sec ON/30 sec OFF) at 4°C. After every 2 cycles, tubes were vortexed. The supernatant was transferred to a new tube and

centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was transferred to a new tube. 80 µg of lysate was used for immunoblot analysis.

2.7.3. Protein quantification

Protein quantification was performed with the Qubit assay (Thermo Fisher Scientific) as per the manufacturer's protocol. A working solution was made by diluting the Qubit® Protein Reagent 1:200 in Qubit® Protein Buffer. 10 µL of each Qubit® standard and 1 µL of samples were added to 190 µL and 199 µL of Qubit® working solution, respectively, then mixed by vortexing for 2-3 seconds. Tubes were incubated for 15 minutes at room temperature, after which protein concentrations were measured on a Qubit® 2.0 Fluorometer.

2.7.4. SDS-PAGE

SDS-PAGE allows the separation of proteins according to their molecular weight. 10-20 µg of lysates were heated in 4 x loading buffer (**Section 2.13**) at 100°C for 5 minutes. Samples along with a full range rainbow molecular weight marker (RPN800; GE Healthcare) were resolved on a precast NUPAGE 4-12 % Bis-Tris gel (Invitrogen) in NuPAGE® MOPS SDS running buffer (Life technologies) for 1 hour at 160 V or until the blue dye ran to the bottom of the gel.

2.7.5. Protein transfer

The gel was transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Healthcare) by wet western blotting. PVDF membranes were briefly activated in methanol before transfer. A western blot sandwich was made by placing the gel containing resolved proteins on top of two pieces of transfer buffer-soaked filter paper, followed by the PVDF membrane and two pieces of buffer-soaked filter paper. The assembled sandwich was placed between two transfer buffer-soaked sponges in a transfer cassette. Air bubbles were

carefully removed by pressing the blot sandwich. The cassette was placed in a transfer tank filled with pre-cooled transfer buffer (**Section 2.13**) at 72 V for 2 hours, 4°C.

2.7.6. Western blotting

Following protein transfer, the membrane was incubated in blocking buffer (PBS, 0.1 % v/v Tween, 5 % w/v milk) for 1 hour at room temperature with gentle shaking. The block was removed and the membrane was incubated with primary antibodies (**Table 2.4**) diluted in PBS, 0.1 % v/v Tween, 2 % w/v BSA overnight with gentle shaking at 4°C. The next day, membranes were washed three times with PBS + 0.1 % Tween (PBS-T) with gentle shaking for 10 minutes between each wash step. Membranes were incubated with HRP-conjugated secondary antibody (**Table 2.4**) diluted in blocking solution for 1 hour at room temperature on a shaker. After three washes as described previous, the membrane was incubated with the chemiluminescent substrate solution ECL (GE Healthcare) according to the manufacturers protocols. Protein bands were visualised on X-ray film (Super RX; FujiFilm), and developed using an AGFA Cruis-60 automatic film processor (AGFA-Gaevert). When multiple protein detections of similar size were required on the same membrane, antibodies were stripped from the membrane using ReBlot Plus Strong Antibody Stripping Solution (Merck) as per the manufacturer's instructions.

Name	Catalog number	Company	Dilution
IRF5	ab21689	Abcam	1:1000
JNK2	ab76125	Abcam	1:500
Phosphoserine	ab9332	Abcam	1:200
IKK α	NB100-56704	Imgenex	1:1000
IKK β	NB100-56513	Imgenex	1:250
TBK1	108A429	Imgenex	1:500
Mekk3	5727	Cell Signaling Technology	1:1000

Phosphotyrosine	9411	Cell Signaling Technology	1:1000
Phospho-Pyk2 (Tyr402)	3291	Cell Signaling Technology	1:200
Pyk2	3292	Cell Signaling Technology	1:1000
β -actin	A5441	Sigma	1:5000
HIPK4	SAB1400812	Sigma	1:1000
Rabbit Trueblot®: Anti-Rabbit IgG HRP	18-8816-31	Rockland	1:5000
alpha-tubulin	3873	Cell Signaling Technology	1:1000
Histone H3	ab1791	Abcam	1:5000
Mouse Trueblot® ULTRA: Anti-Mouse Ig HRP	18-8817-30	Rockland	1:5000
ECL Rabbit IgG, HRP-linked whole Ab	NA934	GE Healthcare	1:5000
Polyclonal rabbit anti mouse immunoglobulins hrp	P0260	Dako	1:5000
GAPDH	ab8245	Abcam	1:5000

Table 2.4. List of antibodies used for western blotting

2.7.7. Measurement of cytokine production

Mouse serum cytokine concentrations were analysed by ELISA (Mouse IL12p70, #DY419-05), and Cytometric Bead Array (IL6 #558301, IL1 β #560232, BD Biosciences) as per manufacturer's instructions. IL-1 β , IL-6, and IL12p70 concentration in Human biopsy or cell culture supernatants was measured by Cytometric Bead Array (IL1 β #558279, IL6#558276, IL12p70#558283). IL-10 concentration in human intestinal biopsy supernatants was measured by ELISA (#DY217B-05, R&D systems). TNF α was measured in human monocyte-derived macrophage culture supernatants by ELISA (#DY210-05, R&D systems). All cytokine detection was performed according to manufacturer's instructions.

For ELISAs 96-well plates (Nunc) were coated with 50 μ l capture antibody diluted in PBS (final concentration of 4.00 μ g/ml), and incubated overnight at 4°C. The following day, plates were washed three times with Wash buffer (PBS + 0.05 % Tween-20). Remaining

wash buffer was removed by inverting the plate and blotting against clean paper towels. Plates were blocked with 100 μ l PBS + 1 % BSA for 1 hour at room temperature, before three washes with wash buffer. Standards ranging from 39.1 – 2500 pg/ml were prepared in PBS + 1 % BSA. 50 μ l of standards/samples in triplicates were applied to the capture antibody-coated plates and incubated overnight at 4°C.

After three washes with wash buffer, 50 μ l detection antibody to a final concentration of 400 ng/ml (in PBS + 1 % BSA) were added to each well, and incubated for 2 hours at room temperature. Following three washes, 50 μ l of Streptavidin-HRP in 1 % BSA in PBS were added to the wells. The plates were incubated for 20 minutes at room temperature in the dark, before a final three washes. The HRP substrate TMB (ThermoFisher Scientific) was added at a volume of 50 μ l per well. Plates were monitored for a blue colour change, before 50 μ l of Stop Solution (1 M H₂SO₄) were added to the wells to terminate the enzymatic reaction. The Stop Solution resulted in a colour change to yellow. Absorbance was read at 450 nm by a SPECTROstar Omega microplate reader (BMG Labtech). For analysis a standard curve was generated by plotting the mean absorbance for each standard against the concentration. The sample concentrations were interpolated from the standard curve.

2.8. IMMUNOPRECIPITATION

Immunoprecipitation was used to study protein-protein interactions. Proteins of interest are isolated from cell extracts by using specific antibodies. The antibody/antigen complex is then immunoprecipitated using Protein G- sepharose beads and the antigens are detected by western blotting.

2.8.1. Cell lysis

RAW 264.7 macrophages were seeded at 1×10^7 cells/10 mL on 10 cm plates (1 immunoprecipitation/plate) and left to attach overnight. The next day, media was replaced

with serum-free media for 30 minutes to reduce background levels. Cells were stimulated with LPS at indicated timepoints. Lysates were prepared as described (**Section 2.7.1**). 5 % lysate was boiled in 4 x loading buffer and kept as input material.

2.8.2. Preparing the beads

The Protein G Sepharose® 4 Fast Flow beads (GE Healthcare) were washed three times with lysis buffer (1% Tx-100) on a rotator for 10 minutes at 4°C. Between the washes the resin was centrifuged at 5,000 rpm for 30 seconds at 4°C and the supernatant discarded. A 50 % slurry of beads were prepared in lysis buffer.

2.8.3. Coupling the antigen to antibody

Lysates were immunoprecipitated with IgG isotype control antibodies (ab37415; Abcam) or anti-IRF5 antibodies (ab21689; Abcam) at a concentration of 5 µg/immunoprecipitation and incubated at 4°C for 2 hours on a rotator.

2.8.4. Precipitation of immune complexes

75 µl of a 50 % slurry of prewashed protein G Sepharose beads were added to each sample and incubated at 4°C for an additional 2 hours on a rotator. The samples were spun at 5,000 rpm for 30 seconds at 4°C and washed six times with ice-cold lysis buffer.

2.8.5. Dissociation and analysis

Immunoprecipitates were eluted by boiling the samples for 5 minutes in 50 µL of loading buffer (4 x concentrated). Eluates were collected from the beads by centrifugation at 5,000 rpm for 30 seconds and resolved on a NUPAGE 4-12 % Bis-Tris gel (Invitrogen). For large scale immunoprecipitation experiments the eluent was concentrated to a volume of 50 µl with Vivaspin 500 centrifugal concentrators (Sigma-Aldrich).

2.9. GENE EXPRESSION STUDIES

Changes in gene expression can be investigated by real time polymerase chain reaction (q-PCR) analyses. RNA is first isolated from cells, and transcribed into complementary DNA (cDNA) by reverse transcriptase. The cDNA is then used as template in qPCR reactions to detect and quantitate gene expression.

2.9.1. RNA isolation

Total RNA was extracted with the RNeasy Mini kit according to the manufacturer's instructions (Qiagen). RNA was quantified using the Nanodrop 1000 (Thermo Scientific), normalised to 100 ng/ μ l for RAW264.7 cells or the lowest sample concentration for BMDMs and stored at -80°C.

2.9.2. cDNA synthesis

The High Capacity cDNA Reverse Transcription kit (Life Technologies) was used to synthesise cDNA from total RNA. The following reaction mix was prepared:

2 μ l	10x RT buffer
0.8 μ l	25 x DNTP mix (100 nM)
2 μ l	10 x RT random primers
4.2 μ l	RNase/Dnase free H ₂ O (Ambion)
1 μ l	Reverse Transcriptase
10 μ l	total

Before the reverse transcriptase enzyme was added to the master mix, 9 μ l of mastermix was taken and added to 3 x 3 μ l random RNA samples and 2 μ l nuclease-free water as a 'no reverse-transcriptase' control to monitor genomic DNA contamination. 10 μ l of the complete master mix was added to 10 μ l RNA to give a total cDNA reaction volume of 20 μ L. Reverse transcription was performed in a thermal cycler using the following conditions:

1. 25°C for 10 min
2. 37°C for 120 min
3. 85°C for 5 min
4. 4°C ∞

When cDNA synthesis was completed, samples were diluted 1:4 in RNase/DNase free H₂O. Samples were stored at -20°C until needed for quantitative PCR (qPCR).

2.9.3. Quantitative real-time PCR (qPCR)

To determine mRNA expression levels using qPCR, the following mastermix was prepared and added to 2.4 μ l diluted cDNA:

- 3 μ l Fast Blue qPCR Master Mix + dTTP (Eurogentec)
- 0.3 μ l RNase/Dnase free H₂O (Ambion)
- 0.3 μ l Taqman Probe (**Table 2.5**)

Gene	Product code
CCL2	Mm00441242_m1
CCL4	Mm00443111_m1
CCL5	Mm01302427_m1
CXCL1	Mm04207460_m1
CXCL3	Mm01701838_m1
HPRT	Mm03024075_m1
IL-1a	Mm00439620_m1
IL-1b	Mm00434228_m1
IL-6	Mm00446190_m1
IL-10	Mm00439614_m1
IL-12b	Mm01288989_m1
IL-23a	Mm00518984_m1
TNF	Mm00443258_m1

Table 2.5. Taqman primer probes

Samples were run in duplicates on a 384 well plate (Life Technologies) with a 6 μ l total reaction volume. The qPCR reaction was performed on a ViiA7 system (Life Technologies) with the following programme:

1. 3 mins 95°C
2. 3 secs 95°C 40 cycles
3. 30 secs 60°C

mRNA levels were quantified by measuring the accumulation of a fluorescence signal at the exponential phase. The cycle threshold value (Ct) is the number of cycles at which the fluorescence signal crosses the threshold, as in exceed background levels. Gene expression was analysed using the comparative Ct ($\Delta\Delta\text{Ct}$) method where the Ct values of the samples are compared to those of a control. Here, control samples used were WT unstimulated cells. All Ct values were normalised to the endogenous housekeeping gene *Hprt* for murine samples and *RPLP0* for human samples.

2.9.4. mRNA sequencing (mRNA-seq)

mRNA-seq was performed on 1×10^6 GM-BMDMs from WT and IRF5^{-/-} mice (3 each), either unstimulated, DMSO/LPS treated, or defactinib /LPS treated. RNA was prepared using the RNeasy Mini kits (Qiagen), including an optional DNaseI digestion with RNase-Free DNase kit (Qiagen) as described above. Library preparation and sequencing was conducted by the High-Throughput Genomics Group at the Wellcome Trust Centre for Human Genetics, Oxford. mRNA-seq data were analysed using custom scripts by Dr Tariq Khoiratty (Udalova lab).

Libraries were sequenced on Illumina HiSeq4000 yielding $> 40 \times 10^6$ 150 b.p. paired end reads per sample. These were mapped to the mm10 genome using STAR (136) with the options: “--runMode alignReads --outFilterMismatchNmax 2.” Uniquely mapped read pairs were counted over annotated genes using featureCounts (137) with the options: “-T 18 -s 2 -Q 255.” Differential expression was then analysed with DESeq2 and genes with fold changes > 2 and false discovery rates (FDRs) < 0.05 were deemed to be differentially expressed. Variance stabilised (VST) counts for all DESeq2 differentially expressed genes were used for dimensionality reduction. For direct comparisons genes with fold changes > 2 and FDR < 0.05 were deemed to be differentially expressed. Gene set enrichment

analysis was performed using one-sided Fisher's exact tests (as implemented in the 'gsfisher' R package <https://github.com/sansomlab/gsfisher/>).

2.9.5. Luciferase gene reporter assays

Luciferase reporter assays were performed to study transcriptional activity in cells. All plasmids excluding tyrosine mutant constructs were provided and prepared by Dr. Grigory Ryzhakov. IRF5-tyrosine site mutants were prepared by VectorBuilder (**Table 2.6**).

Following overnight incubation with transfection medium (section 2.4.1), RAW264.7 and HEK-TLR4 cells were stimulated for 6 hours with LPS at 1 µg/ml and 100 ng/ml, respectively. For inhibitor experiments, RAW264.7 cells were pre-treated with vehicle (DMSO) solution or inhibitor for 1 hour, followed by LPS (1 µg/ml) for 6 hours. Reporter activities were measured using the Dual-Glo Luciferase system (Promega) according to the manufacturer's protocol. In brief, the supernatant was removed and replaced with 22.5 µl of PBS, and 22.5 µL of Dual-Glo Luciferase Reagent. The plate was placed on a shaker for 20 minutes at room temperature. Samples were transferred to a luminescent-plate (ThermoScientific) and luciferase activity measured using a luminometer (BMG FLUOstar). Dual-Glo Stop and Glo Reagent (22.5 µl) was then added to each well, and put on a shaker for 10 minutes. The plate was read for Renilla luciferase levels in the same manner. Results for firefly luciferase activity were normalised to Renilla luciferase activity. To account for plate variability including temperature, plate order and timing between experiments the ratio was normalised to the ratio from samples consisting of non-transfected cells. Each experiment was repeated three times. Data are expressed as mean +/-SEM of three replicates.

Construct name	Description	Vector
TNF 5"wt/3"wt	wt promoter and 3" region	pGL3
pRLTK	Renilla luciferase	pGL3
HA-IRF5		pBent2
pBent2-Empty	Empty pBent2 vector construct	pBent2
FLAG-IRF5	IRF5 v2 isoform	pBent2
FLAG-Y30F-IRF5	IRF5 v2 isoform with Y30F mutant	pBent2
FLAG-Y74F-IRF5	IRF5 v2 isoform with Y74F mutant	pBent2
FLAG-Y104F-IRF5	IRF5 v2 isoform with Y104F mutant	pBent2
FLAG-Y172F-IRF5	IRF5 v2 isoform with Y172F mutant	pBent2
FLAG-Y172/S173A-IRF5	IRF5 v2 isoform with Y172F/S173A mutant	pBent2
FLAG-Y329F-IRF5	IRF5 v2 isoform with Y329F mutant	pBent2
FLAG-Y351F-IRF5	IRF5 v2 isoform with Y351F mutant	pBent2
ARK5	Kinase construct	P8-Myc
CLK2	Kinase construct	P8-Myc
HIPK4	Kinase construct	P8-Myc
IKK α	Kinase construct	pBent2
IKK β	Kinase construct	pBent2
IKK ϵ	Kinase construct	pBent2
JNK2	Kinase construct	P8-Myc
MARK3	Kinase construct	P8-Myc
MEKK3	Kinase construct	P8-Myc
MST1	Kinase construct	P8-Myc
PYK2	Kinase construct	P8-Myc
RIP2	Kinase construct	P8-Myc
TAK1	Kinase construct	pBent2
TBK1	Kinase construct	pBent2
S130A-IRF5	human IRF5 isoform v3/v4 isoform with S130A mutant	pBent2
S137A-IRF5	human IRF5 isoform v3/v4 isoform with S137A mutant	pBent2
S457A-IRF5	human IRF5 isoform v3/v4 isoform with S457A mutants	pBent2
S1,2A-IRF5	human IRF5 isoform v3/v4 isoform with S421A and S425A mutants	pBent2
S3,4A-IRF5	human IRF5 isoform v3/v4 isoform with S427A and S430A mutants	pBent2
S4A-IRF5	human IRF5 isoform v3/v4 isoform with S421A, S425A, S427A and S430A mutants	pBent2
S5A-IRF5	human IRF5 isoform v3/v4 isoform with S421A, S425A, S427A S430A, and S436A mutants	pBent2
KKRR-IRF5	human IRF5 isoform v3/v4 isoform with K401R and K402R mutants	pBent2
N420-IRF5	human IRF5 isoform v3/v4 isoform with 420 amino acids (C-terminus missing)	pBent2

Table 2.6. Constructs used in this study

2.10. CHROMATIN IMMUNOPRECIPITATION

ChIP is used to determine whether a given protein binds to or is localised to specific DNA sequences. Cells are fixed to cross-link proteins to DNA, lysed and sheared by sonication to obtain small fragments. Following immunoprecipitation with a suitable antibody and Protein G beads, isolated chromatin fragments are heated to reverse the crosslinks and DNA purified for analysis.

2.10.1. Fixation of cells and cross-linking

1×10^7 cells were plated onto 10 cm plates with one plate per immunoprecipitation. For GM-BMDMS, cells were pre-treated with Defactinib inhibitor (3.5 μ M) or vehicle solution (DMSO) for 1 hour and stimulated with LPS (100 ng/ml) for a further 2 hours. RAW264.7 cells were stimulated with LPS (500 ng/ml) for 2 hours. Cells were crosslinked by the addition of formaldehyde (37.5 % v/v HCHO with 10 % v/v methanol stock solution; Sigma) to a final concentration of 1 %. Plates were mixed quickly by swirling and incubated for 10 minutes at room temperature. The reactions were quenched by the addition of a 1 M Tris pH 7.5 solution (Sigma) to a final concentration of 125 mM. Cells were collected using a cell scraper and washed three times with 10 ml cold PBS, centrifuging the cells at 1,500 rpm at 4°C for 5 minutes between each wash to obtain a cell pellet.

2.10.2. Cell lysis and sonication

ChIP cell lysis buffers (LB1, LB2 and LB3; **Section 2.13**) were supplemented with 4 μ l of protease inhibitors per 1 ml of buffer (P8340; Sigma) and kept on ice prior to use. Cells were first lysed for 10 minutes on ice in LB1 (500 μ l/ 10^7 cells). The nuclei was pelleted by centrifugation at 1,350 x g for 5 minutes at 4°C, and resuspended in LB2 (500 μ l / 10^7 cells). Lysates were gently rocked for 10 minutes at room temperature, then pelleted again at 1,350 x g for 5 minutes at 4°C. The nuclei was resuspended in LB3 (500 μ l/ 10^7 cells) and split into two 1.5 ml Bioruptor® Pico Microtubes (Diagenode). Nuclear lysates were

sonicated using a Biorupter (Diagenode) for 8 cycles (30 s on and 30 s off) for GM-BMDMS and 10 cycles (30 s on and 30 s off) for RAW264.7 cells to obtain fragment sizes that average 500 basepairs. The optimum sonication conditions for BMDMs has previously been identified by the Udalova lab (45). Sonication conditions for RAW264.7 cells were determined following sonication at multiple time points and observing the DNA bands on a 1 % w/v agarose gel by EtBr and UV excitation.

The sonicated fractions were pooled together (500 µl total volume) with the addition of 50 µl of 10% v/v Triton X-100, followed by centrifugation at maximum speed for 10 minutes at 4°C to pellet debris. The supernatants were transferred into new tubes and an aliquot of 50 µl was stored separately at -20°C as whole cell extract (WCE) for normalisation. The whole cell extract (input) corresponds to DNA extracted from the sample before immunoprecipitation. The remaining amount was used for the immunoprecipitation.

2.10.3. Antibody conjugation to Protein G magnetic beads

50 µl of Dynabeads™ Protein G (Invitrogen) per immunoprecipitation were prepared. The beads were washed in block solution (0.5 % BSA in PBS) three times, rotating for 10 minutes between the washes and collecting the beads by placing the tubes in a magnetic stand, and removing the supernatant by aspiration. On the last wash, the collected beads were resuspended in block solution (100 µl per 50 µl of beads) containing 4 µg of antibody (**Table 2.7**). The tubes were placed on a rotating wheel for 1 hour at room temperature. The tubes were placed on the magnetic rack and the supernatant removed. The antibody-bound beads were washed with block solution for a total of three washes to remove any unbound antibody. The beads were resuspended in 75 µl of block solution.

Antibody	Source	Product code	Supplier
IRF5	Rabbit	ab21689	Abcam
Pol II	Rabbit	904001	Biolegend
IgG	Rabbit	ab37415	Abcam

Table 2.7. Antibodies used for ChIP

2.10.4. Chromatin immunoprecipitation

The antibody-bound beads were added to the cell lysate sample (**Section 2.10.2**) and gently mixed overnight on a rotator at 4°C.

2.10.5. Washes, elution and reversal of crosslinks

The following day, the tubes were placed on the magnetic rack and supernatant removed. The beads were washed with 1 ml ice cold ChIP wash buffer (**Section 2.13**), and rotated for 5 minutes. The wash was repeated for a total of 6 times with the final wash in ice cold TE-containing 50mM NaCl. The beads were placed on the magnetic rack and the collected beads were then resuspended in 120 µl of Elution Buffer (1xTE buffer containing 2% SDS). Samples, along with the WCE prepared in **Section 2.10.2**, were reverse crosslinked by overnight incubation at 65°C.

2.10.6. Purification of DNA

The DNA was purified using the QIA quick PCR Purification Kit (Qiagen). DNA and WCE samples were eluted in 100 µl and 200 µl of TE buffer, respectively. WCE samples were purified separately to prevent cross-contamination. Samples were stored at -20°C.

2.10.7. ChIP-qPCR

The sequences of the primers used can be found in **Table 2.8**.

Primer pairs were designed with the following guidelines:

- Primer length of 22-27bp (optimal 25bp)
- GC content of 40-60% (optimal 50%)
- T_m 60-65°C (Optimal 63°C)
- T_m of forward and reverse primer must not largely differ (max. 1°C)
- Pairs should not have a complementary sequence of more than 2 bases at 3'
- Complementary sequence of more than 3 bases should not exist in the primer

Primer pairs were tested on serial dilution of sonicated genomic DNA to test for their efficiency. A standard curve was generated by plotting the Ct values against a logarithmic scale of the corresponding concentrations. The efficiency was calculated using the formula $E = -1 + 10^{(-1/\text{slope})}$. Amplification efficiencies ranging from 90-110 % and primer sets that gave a single peak on the melt curve indicated suitable primers.

Locus	Forward Primer (5'→3')	Reverse Primer (5'→3')
IL-1a	ACTTCTGGTGCTCATCTGTCATGTT	GCTCTATGGTTCCTGTGTCTGTAGG
IL-1b	GGATGTGCGGAACAAAGGTAGGCACG	ACTCCAAGTGCAGGCTCCCTCAGC
IL-6	GAGAGAGGAGTGTGAGGCAGAGAGC	GTTGTGTCACCAGCATCAGTCCCAAG
IL-12b	GCAAGGTAAGTTCTCTCCTCTTCCC	AATGACTATTTGAAGCCCCTGTCGT
TNF	GCTAAGTTCTTCCCCATGGATGTCCC	ACCCATTTCTTCTCTGTCTCCAGAGC

Table 2.8. ChIP primers used in this study

SYBR Green real-time PCR was performed in 384 well-plates (Applied Biosystems) using a ViiA7 real-time-PCR system (Life Technologies) with samples run in duplicate wells. The following reaction mix was prepared:

5 µl	Master mix
1 µl	RNase/Dnase free H ₂ O (Ambion)
1 µl	SYBR primers (1/20 dilution of 100 µM stock of both primers)
3 µl	ChIP/WCE DNA
10 µl	total

The reactions were performed with the following programme:

1. 3 mins 95°C (enzyme activation)
2. 15 secs 95°C (denaturation) 40 cycles
3. 35 secs 60°C (data collection)

A melt curve was performed at the end with the following programme:

Step 1. 95°C 15 seconds

Step 2. 60°C 1 minute

Step 3. 95°C 15 seconds (Dissociation)

Data was analysed by the percent input method which represents the amount of DNA pulled down by the antibody of interest in the ChIP reaction, relative to the input (WCE) sample.

The input fraction is 10% which is a dilution factor (DF) of 10 or 3.321 cycles (i.e $\log_2$ of 10).

The percent input method was calculated as follows:

1. Adjusted input = average Ct input – $\log_2 10^*$
2. Percent input = $100 * 2^{(\text{Adjusted input} - \text{average Ct (IP)})}$

2.11. PROTEOMIC TECHNIQUES

2.11.1. Sample preparation for IRF5 phosphorylation experiments

For IRF5 phosphorylation experiments WT and PYK2 KO cells were prepared and immunoprecipitated as described in **Section 2.8**. From 1×10^8 RAW264.7 cells 40 % of the eluent was run on colloidal blue gel (**Section 2.11.2**), 50 % was prepared for in-solution digestion (**Section 2.11.3**) and 10 % for western blot.

2.11.2. Colloidal blue staining of SDS-Page gels

Protein samples were resolved by electrophoresis (**Section 2.7.4**), and the gel fixed (50% v/v methanol, 10% v/v acetic acid) for 10 minutes at room temperature. The gel was incubated in Colloidal blue staining solution overnight per the manufacturer's protocol (Invitrogen, USA). Gels were washed for a minimum of 3 hours in distilled water to reduce background staining. All steps were carried out with gentle shaking at room temperature. Protein bands were visualised on a light box for excision. The protein band of interest was excised with a clean scalpel and cut into smaller pieces ($1\text{-}2 \text{ mm}^3$) and placed in a Protein LoBind tube (Sigma-Aldrich). The cut gel pieces were washed overnight in wash solution at room temperature while shaking.

2.11.3. Sample preparation

Sample preparation and MS run was carried out by Sarah Bonham at the Target Discovery Institute, Nuffield Department of Medicine (Oxford). Eluents were subjected to in-solution

digestion or in-gel digestion as described previously (138). In brief, samples were reduced and alkylated before double precipitation with Chloroform/Methanol as described (139). Protein pellets were resuspended in 50 μ l 6M urea for solubilisation. The samples were diluted to 1M Urea in 100 mM Tris buffer for tryptic digest. Trypsin selectively cleaves at the carboxyl side of the amino acids arginine and lysine except when either is bound to a C-terminal proline to generate peptide fragments.

Following overnight digestion, peptides were acidified with 3% Formic acid and desalted with solid phase extraction Sola cartridges (Thermo). Peptides were eluted with 600 μ L glycolic acid solution (1M glycolic acid, 80% acetonitrile, 5% trifluoroacetic acid). Phosphopeptide enrichment was performed using a TiO_2 protocol as described (140) with eluates from the Sola cartridges adjusted to 1 mL with 1M glycolic acid solution and incubated for 5 minutes with 50 μ L TiO_2 bead slurry solution. Bead washes (200 μ L) were carried out as previously described. In short, beads were sequentially washed with 200 μ L glycolic acid solution, ammonium acetate solution (100 mM ammonium acetate in 25% acetonitrile) and 10% acetonitrile solution repeated in triplicate. Phospho-peptides were eluted, following incubation for 5 minutes at room temperature with 50 μ L ammonia solution (5%) and centrifuged, this was repeated in triplicate. The three eluate fractions were combined and dried using a SpeedVac and pellets were stored at -80°C until analysis. For analysis by nano-liquid chromatography tandem mass spectrometry (nLC-MS/MS), a Dionex UHPLC system coupled to an Orbitrap Fusion Lumos mass spectrometer was used as described previously (141). Raw MS files were subjected to processing using PEAKS (version 8.5) software and searched against the UniProtSP Mus Musculus database. Searches included the data refine, denovo PEAKS and PEAKS PTM modes, the latter of which included phosphorylation on Ser (S), Thr (T) and Tyr (Y) residues. The proteomics data and MS raw files have been deposited into PRIDE Archive (<https://www.ebi.ac.uk/pride/archive/>).

2.11.4. Sample preparation for global phosphoproteome experiments

1×10^8 RAW264.7 cells were plated/cell line. The following day 5×10^7 cells were stimulated with LPS (500 ng/ml) for 1 hour. Cells were rinsed with PBS, pelleted and snap frozen in liquid nitrogen and stored at -80°C . A total of 8 cell pellets (4 cell lines +/- LPS) were prepared in this manner. Cell pellets were lysed in 400 μL ice cold RIPA buffer (**Section 2.13**) containing dissolved protease and phosphatase inhibitors, and benzonase nuclease to eliminate DNA-facilitated protein-protein interactions. Lysates were kept on ice for 30 minutes, followed by centrifugation at 13,000 rpm for 10 minutes to collect the supernatants. Protein concentration was measured using a BCA assay (ThermoPierce) per the manufacturers protocol. 300 μg lysate was made up to 175 μL in Milli-Q water for methanol/chloroform extraction and in-solution digestion (**Section 2.11.3**).

2.11.5. Peptide labelling

The Tandem Mass Tag™ (TMT™) 10-plex reagent set (Thermo Scientific) contains ten different isobaric compounds with the same mass and chemical structure composed of an amine-reactive NHS-ester group, a spacer arm and a mass reporter. The tags enables up to ten different peptide samples prepared from cells to be labelled in parallel and then combined for analysis. Each sample is assigned a unique reporter mass in the low-mass region of the high-resolution MS/MS spectrum and is used to measure relative protein expression levels during peptide fragmentation and tandem mass spectrometry (**Table 2.9**).

Peptides were labelled according the manufacturers protocol. Briefly, the dried samples were resuspended in 150 μL TEAB. The TMT reagent was resuspended in 41 μL ACN and transferred to 100 μL sample (200 μg protein digest). The reaction was incubated for 1 hour at room temperature. 8 μL of 5 % hydroxylamine were added to the samples and incubated for 15 minutes to quench the reaction. Samples were pooled in a new microcentrifuge tube and dried in a speedvac.

Cell line	LPS stimulation	TMT tag
WT	-	130C
WT	+	130N
MEKK3 KO	-	129C
MEKK3 KO	+	129N
PYK2 KO	-	128C
PYK2 KO	+	128N
TBK1 KO	-	127C
TBK1 KO	+	127N
Lysates pooled	Lysates pooled	126

Table 2.9. TMT labelled samples

2.11.6. Prefractionation

TMT-labelled peptides were fractionated on a HILIC column to increase the number of peptide identifications.

2.11.7. Immobilised metal affinity chromatography (IMAC)

20 µl of a 50 % slurry of PHOS-select iron affinity gel (Sigma) was added directly into (concatenated) HILIC fractions, and vortexed rapidly for 30 minutes. The supernatants were discarded, and the gel was washed with 500 µl acetic acid (250 mM) in 30 % acetonitrile, followed by 500 µl Milli-Q water. The gel was eluted with 100 µl ammonium hydroxide (400mM) with vortexing for 10 minutes. The eluent was dried in the speedvac and resuspended in MS buffer A (**Section 2.13**)

Labelled samples were run on a high resolution Orbitrap LC-MS/MS before data analysis to identify peptides and quantify reporter ion relative abundance. Data was analysed on MaxQuant software.

2.12. *HELICOBACTER HEPATICUS*-INDUCED COLITIS MODEL

Mice were free of known intestinal pathogens and negative for *Helicobacter* species. Animals from each experimental group were cohoused. On days 0, 1, and 2, mice were injected i.v. with 1 mg/kg defactinib or vehicle (5% DMSO, 2.5% Solutol HS (Sigma), 2.5% absolute ethanol, 90% Dulbecco's PBS). Daily, starting from day 3, mice were injected i.p. with 5 mg/kg defactinib or vehicle. 30 minutes after the initial i.v. injection, mice were infected with 1×10^8 colony forming units *Hh* on days 0 and 1 by oral gavage with a 22G curved, blunted needle (Popper & Sons). Mice were injected intraperitoneally once on day 0 with 1 mg anti-IL10R blocking antibody (clone 1B1.2). Infected mice were monitored daily for colitis symptoms. Mice were culled one week after day of infection, and organs were harvested for analysis.

2.13. HUMAN WORK

All work on human tissue was carried out by Dr. Alastair Corbin, a former post-doc of the *Udalova* lab.

2.13.1. Culture of UC patient colonic mucosal biopsies

Intestinal pinch biopsies were obtained from Ulcerative Colitis patients registered in the Oxford IBD Cohort, attending the John Radcliffe Hospital Gastroenterology Unit (Oxford, UK). Biopsies were collected during routine endoscopy. Informed, written consent was obtained from all donors. Human experimental protocols were approved by the NHS Research Ethics System (Reference numbers: 16/YH/0247). Biopsies were washed in PBS and transferred into wells containing RPMI + 10% FCS + 20 µg/mL G418 (Thermo Fisher) + 20 U/mL Pen/Strep.

Biopsies were fixed in 4% PFA in PBS (#30525-89-4, Santa Cruz) for 24 hrs at RT and transferred to 70% ethanol. Fixed biopsies were then dehydrated and embedded in paraffin

blocks, and 5 μ m sections were cut. Embedding and sectioning of tissues was carried out by the Kennedy Institute of Rheumatology Histology Facility (University of Oxford).

2.13.2. UC biopsy viability assessment

Viability of intestinal biopsies was measured by TACS® TdT in situ (Fluorescein) TUNEL assay (#4812-30-K, R&D systems) according to manufacturer's instructions. Sections were then mounted in Glycerol Mounting Medium with DAPI and DABCO (#ab188804, abcam) and coverslipped. Images of Three non-sequential sections per sample were acquired. Three images per section were acquired at 20x magnification using a BX51 microscope (Olympus). To generate the apoptotic index, the total cell number was enumerated by counting DAPI⁺, and TUNEL(FITC)⁺ cells in ImageJ, and calculating the percentage of total cells that were TUNEL⁺.

2.14. STATISTICAL ANALYSIS

Statistical analysis was carried out using GraphPad v7.0 (GraphPad Software) using two-way ANOVA with Bonferroni's correction (multiple comparisons) or Mann-Whitney tests (comparisons between two groups). p values < 0.05 were considered significant. Number of biological replicates is indicated in the respective figure legends.

2.15. PREPARATION OF BUFFERS

The following reagent buffers were prepared for experiments:

ChIP Lysis Buffer 1 (LB1):

50 mM HEPES-KOH, pH 7.5
140 mM NaCl
1 mM EDTA
10% v/v Glycerol
0.5% v/v NP-40
0.25% v/v Tx-100

ChIP Wash Buffer:

50 mM Hepes-KOH, pH 7.6
500 mM LiCl
1 mM EDTA
1% NP-40
0.7% Na-deoxycholate

4 x Loading Buffer:

200 mM Tris (pH6.8)
0.05 M DTT
8% w/v SDS (0.2 μ)
0.4% w/v Bromophenol Blue
40% v/v Glycerol

ChIP Lysis Buffer 2 (LB2):

10 mM Tris-HCl, pH 8.0
 200 mM NaCl
 1 mM EDTA
 0.5 mM EGTA

1% TX-100 Lysis Buffer:

1% v/v TX-100
 10% v/v Glycerol
 1 mM EDTA
 150 mM NaCl
 50 mM Tris (pH7.8)

Transfer Buffer:

25 mM Tris-base
 192 mM Glycine
 10% v/v Methanol

ChIP Lysis Buffer 3 (LB3):

10 mM Tris-HCl, pH 8.0
 100 mM NaCl
 1 mM EDTA
 0.5 mM EGTA
 0.1 % Na-Deoxycholate
 0.5 % Na-Lauroylsarcosine

1% NP-40 Lysis Buffer:

150 mM NaCl
 1% v/v NP-40
 50 mM Tris-HCl, pH 8.0

RIPA Lysis Buffer:

150 mM NaCl
 1 % NP-40
 0.5 % sodium deoxycholate
 0.1 % SDS
 50 mM Tris pH 8.0

TBST Wash Buffer:

100 mL 10x TBS
 900 mL Distilled H₂O
 0.1% tween

Blocking Buffer:

TBST
 5% dried milk powder

TBS (10x):

200 mM Tris (pH 7.5)
 1500 mM NaCl

Cytoplasmic Lysis Buffer

0.15 % NP-40
 10 mM Tris pH 7.5
 150 mM NaCl

Sucrose Buffer

10 mM Tris pH 7.5
 150 mM NaCl
 24% w/v sucrose

Cold buffer A

10 mM HEPES pH 7.9
 10 mM KCl
 0.1 mM EDTA
 0.1 mM EGTA
 1 mM DTT
 0.5 mM PMSF

Buffer B

20 mM HEPES pH 7.9
 0.4 M NaCl
 1 mM EDTA
 1 mM EGTA
 1 mM DTT
 1 mM PMSF

MS Buffer A

98 % MilliQ-H₂O
 2 % acetonitrile
 0.1 % formic acid

3.0. KINASE SCREEN AND VALIDATION

3.1. INTRODUCTION

Interferon regulatory factor 5 (IRF5) is a major factor defining inflammatory macrophages and is a multifunctional regulator of immune responses. Previous studies using IRF5-deficient mice have established a critical role of this transcription factor in macrophage and B cell biology and also pro-inflammatory and antiviral responses (30, 37). In humans, IRF5 gene polymorphisms are associated with many inflammatory and autoimmune diseases including inflammatory bowel disease (IBD), rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (25, 53, 142). Given the importance of IRF5 function in physiology and disease, blocking IRF5 may have therapeutic ramifications. One way of blocking IRF5 function is interfering with the molecular events that lead to its activation. IRF5 is proposed to exert its molecular function via a cascade of signaling events involving its phosphorylation, ubiquitination, dimerisation, nuclear translocation and selective binding to its target genes to enable their expression (81). Despite its known physiological role, the molecular mechanisms of IRF5 activation are still debated.

Several kinases including TBK1, RIP2, IKK ϵ , IRAK4, TAK1 and IKK β have been proposed to phosphorylate and activate IRF5 (31, 92, 95, 96, 143, 144), while IKK α inhibits IRF5 (111). LYN, a Src family kinase has been shown to negatively regulate IRF5 in the TLR-MyD88 pathway in a kinase independent manner via direct binding to IRF5 (38). Though distinct targeted serine sites towards the C-terminal region of IRF5 have been identified as essential contributors to IRF5 activation (89, 94), little is known about the functional contribution of phosphorylation on tyrosine sites. Given the importance of phosphorylation on IRF5 activation, I set out to identify novel IRF5 kinases as potential targets for specific control of IRF5 driven inflammatory conditions.

The initial goal of this investigation was to identify kinases involved in IRF5 activation. In this chapter I utilise a reporter-based screening of a kinase inhibitor library to identify candidate IRF5 kinases. The screen and validation rounds carried out in this part of the study has paved the way for the detailed investigation of lead kinases PYK2 and MEKK3 in IRF5 activation and are presented in Chapter 4.0 and 5.0 of this thesis.

3.2. RESULTS

3.2.1. Establishing an LPS/IRF5-driven firefly luciferase reporter

IRF5 is required for induction of a number of cytokines including TNF (46), and for this reason a luciferase gene reporter containing the 5' and 3' regulatory regions of *Tnf* upstream of the Firefly luciferase gene was tested as a readout of IRF5 activation. RAW264.7 cells a murine macrophage cell line were co-transfected with the TNF luciferase reporter, a Renilla luciferase expression plasmid a Strep-HA-IRF5 plasmid or an empty pBent vector expression plasmid (negative control). The IRF5 plasmid based on isoform v3/v4 was used as it is predominantly expressed in myeloid cells, and is one of most transcriptionally active and best studied isoform (26, 145). The Renilla expression plasmid was used as an internal control to which expression of the experimental firefly luciferase reporter gene was normalised to account for slight differences in transfection efficiency, toxicity and cell density between wells. As shown in **Figure 3.1**, ectopic expression of IRF5 induced TNF-reporter activity, which was further increased with LPS stimulation. LPS stimulation also induced luciferase activity in the absence of IRF5 plasmid, but to a lesser extent. This may be due to the activation/contribution of endogenous proteins including IRF5 and/or NF- κ B which are known to regulate *Tnf*-dependent transcription (46). As

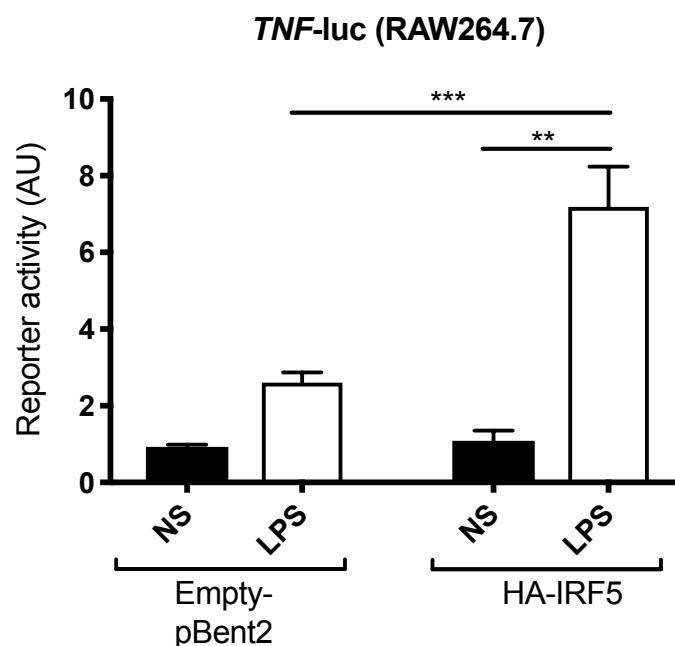


Figure 3.1. Induction of *Tnf* luciferase reporter activity by LPS stimulation and IRF5 transfection RAW264.7 cells (5×10^5) were co-transfected with TNF-luciferase constructs, HA-IRF5/pBent2 empty vector, and Renilla luciferase expression plasmid (for normalisation) in a 96-well poly-lysine coated plate. Following an overnight incubation at 37°C, cells were stimulated with LPS (1 µg/ml) for 6 hours. Firefly and Renilla luciferase activity was measured on the FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM from 3 independent experiments, with each experiment performed in triplicates. Statistical analysis by unpaired Student's t-test where **p≤0.01, ***p≤0.001.

RAW264.7 cells were synergistically activated by ectopic expression of IRF5 and LPS stimulation, the human TNF-promoter-driven firefly luciferase reporter assay was used as a readout of IRF5 activation for further experiments.

3.2.2. Shortlisting IRF5 candidate kinases with a kinase inhibitor library screen

This section of my results (**Figures 3.2-3.3**) was performed by Grigory Ryzhakov, a former post-doctoral scientist in the *Udalova* group, so these sections will be described in plural. To identify kinases that phosphorylate IRF5 in response to LPS stimulation, the *Udalova* lab utilised a kinase inhibitor library screen. The library from the GSK Published Kinase Inhibitor Set (PKIS) consisted of a panel of protein kinase inhibitors (365 total) for which inhibitory properties against a range of protein kinase have been established (146), including some which have been comprehensively characterised *in vitro* (147). The small molecule inhibitors were tested for induction of the human TNF promoter-driven firefly luciferase reporter in RAW264.7 cells, and DMSO the inhibitor diluent was used as a negative control for IRF5 inhibition (**Figure 3.2a**). We tested the effect of each kinase inhibitor in suppressing LPS/IRF5-driven TNF-luciferase reporter activation by comparing luciferase levels in the presence of inhibitor to those of normal TNF reporter activity. Among the kinase inhibitors tested in the screen, 57 inhibited IRF5 reporter activity by 30-50%, 34 inhibited by 50-70%, 8 inhibited by 70-80%, 5 inhibited by 80-90%, and 4 by >90%. Whereas, the remaining and majority of kinase inhibitors had little effect on suppressing LPS/IRF5-driven TNF reporter activity (<30% degree of reporter activation) (**Figure 3.2b**). As some of these compounds affected cell viability or general metabolism, they had an impact on the constitutive Renilla luciferase expression in the same cells, which was used

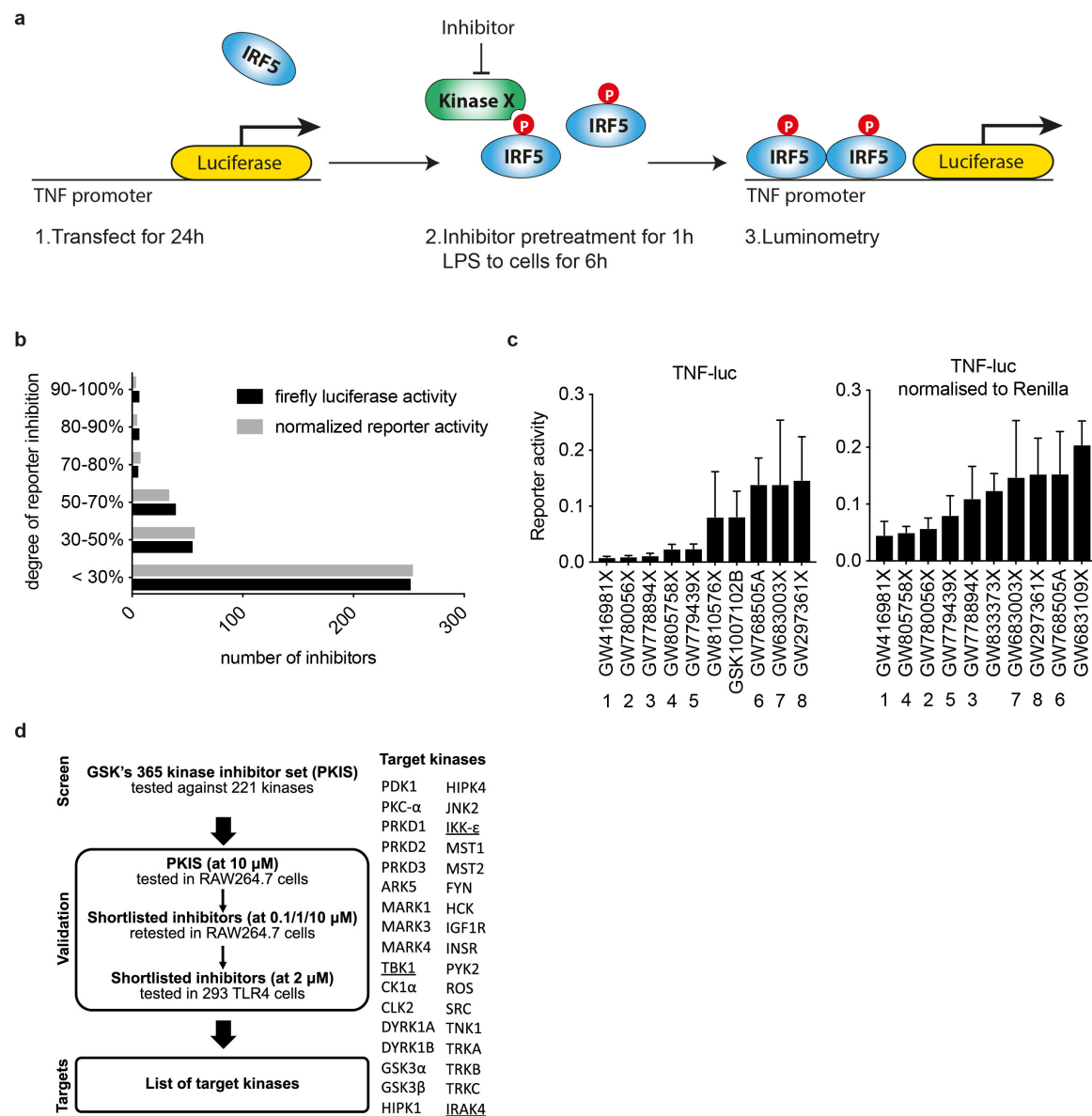


Figure 3.2. Kinase inhibitor library screen to identify candidate kinases involved in IRF5 activation (a) An overview of the kinase inhibitor screen. RAW264.7 cells were co-transfected with the IRF5 and TNF-luciferase reporter constructs. 24 hours post transfection, cells were incubated with kinase inhibitors or DMSO (control) for 1 hour followed by LPS for 6 hours. Luciferase activity was measured in a luminometer. (b) A summary result of the screening: all PKI inhibitors were grouped based on their suppressive effect on IRF5 activation. (c) Stratification of the kinase inhibitors used in the screen based on the degree of IRF5 reporter inhibition. The numbers are shown based on activities of the firefly luciferase reporter, raw values or normalised to Renilla luciferase activities to account for non-specific impact of cell viability. Activities of top 10 IRF5 reporter inhibitors are shown where the dataset was analysed based on raw firefly luciferase or normalised to Renilla values. The compounds indicated with numbers are in top 10 independently of normalisation. (d) The screening workflow showing the initial large screening in RAW264.7 cells, and the subsequent screens in RAW264.7 and 293 TLR4 cells. The top inhibitors were shortlisted based on their efficacy towards IRF5 reporter and low toxicity. Based on the known activities of these molecules against 221 kinases in the PKIS set, 34 kinases affected by the top inhibitors were shortlisted. The underlined kinases were previously proposed to target IRF5.

as an internal normalisation control. We compared the impacts of all compounds inhibiting IRF5 reporter activity prior to and after normalisation and found that most top inhibitors were similar in both settings (**Figure 3.2c**). Certain inhibitors of the PKIS set have previously been shown to block luciferase activity itself (146), so we cross-referenced those small molecules and found only one of our top screen hits (GW779439X) impacting on firefly luciferase.

To validate these compounds further, we have rescreened 59 of the shortlisted set of compounds by selecting those that affected IRF5 reporter the most (53% or less of the remaining reporter activity) and a random group (21 compounds) of those that did not suppress it (**Figure 3.2d**). The repeat screenings were carried out in RAW264.7 cells using three concentrations of the compounds (0.1, 1 and 10 μ M) and in 293 TLR4/CD14/MD-2 cells (at 2 μ M). The top inhibitors that suppressed IRF5 reporter were cross-referenced to a list of kinase targets for each of the small molecule inhibitors. In this way, we have identified 46 candidate kinases affected by those inhibitors. Activities of 12 those kinases (mainly of the CDK family) were also affected by several compounds that did not impact on IRF5 reporter activity, so those kinases were ruled out as potential candidates. The remaining list of 34 kinases included published IRF5 kinase candidates (TBK1, IKK ϵ , IRAK4), as well as previously unpublished candidates. In summary, primary and secondary kinase inhibitor screens resulted in the final list of 34 candidate IRF5 kinases which we have further taken into consideration for downstream validation assays.

3.2.3. Initial validation of candidate kinases in overexpression systems

3.2.3.1. Co-immunoprecipitation of candidate kinases with IRF5

Based on intensive literature search, we focused on 8 of the remaining 32 candidate IRF5 kinases for further validation by excluding kinases with functions more specific to non-immune organs and excluding transmembrane receptor kinases as they act further

upstream in signalling pathways. Poorly explored kinases or those with known links to inflammatory processes were selected. The kinases focused on included PYK2, HIPK4, MEKK3, ARK5, CLK2, MARK3, JNK2, MST1. Kinases TBK1, IKK ϵ and RIP2 were also included as published IRF5 kinases. To phosphorylate its substrates, kinases need to be in close proximity to its targets. To explore the shortlisted kinases further, we have cloned the kinases in a Myc-tagged mammalian expression vectors and screened for their ability to interact with IRF5. The candidates were overexpressed with IRF5 in 293 ET cells and we found that ectopically expressed IRF5 efficiently pulls down known IRF5 binding partners RIP2 and RelA (46, 92, 113), as well as candidates PYK2 and MEKK3. Candidate kinases HIPK4, ARK5 and JNK2 showed a weak association with IRF5, whereas MARK3, CLK2, and MST1 failed to co-immunoprecipitate with IRF5 (**Figure 3.3a**).

3.2.3.2. *Impact of kinases on IRF5-dependent TNF-luciferase reporter assay*

To determine if the candidate kinases could activate IRF5, the myc-tagged kinase expression plasmids were transfected into HEK cells together with HA-IRF5 and luciferase reporters to test the impact of these kinases on IRF5-dependent TNF-luciferase reporter activity (**Figure 3.3b**). Overexpression of all the candidate kinases with IRF5 excluding CLK2 in HEK293 cells potently induces TNF-reporter activity. MEKK3 and RIP2 had the strongest activity in this assay, though they could potently induce the reporter activity even in the absence of IRF5, which is in agreement with the known NF- κ B-activating property of RIP2 (148).

3.2.3.3. *In vitro kinase assay*

We have further tested the ability of these kinases to phosphorylate overexpressed IRF5 in HEK293/TLR4 cell lysates. We have employed a previously published method and carried out *in vitro* kinase assays in the presence of S- γ -ATP to enable the kinases to add thiophosphate groups to their targets and subsequently chemically modify those residues

using p- Nitrobenzyl mesylate (PNBM) into epitopes for high affinity pull-downs (**Figure 3.3c**). The known IRF5 kinase RIP2 was included as a positive control. As a result, we were able to detect phosphorylated IRF5 in the presence of ARK5, MEKK3, IKK ϵ , HIPK4, CLK2, JNK2, MST1 and PYK2. TAK1 and MARK3 failed to phosphorylate IRF5, however IRF5 input levels were lower in the immunoblot. It is also important to note that kinases may use S- γ -ATP poorly or not at all. Thus, in all three assays combined, MEKK3 and PYK2 along

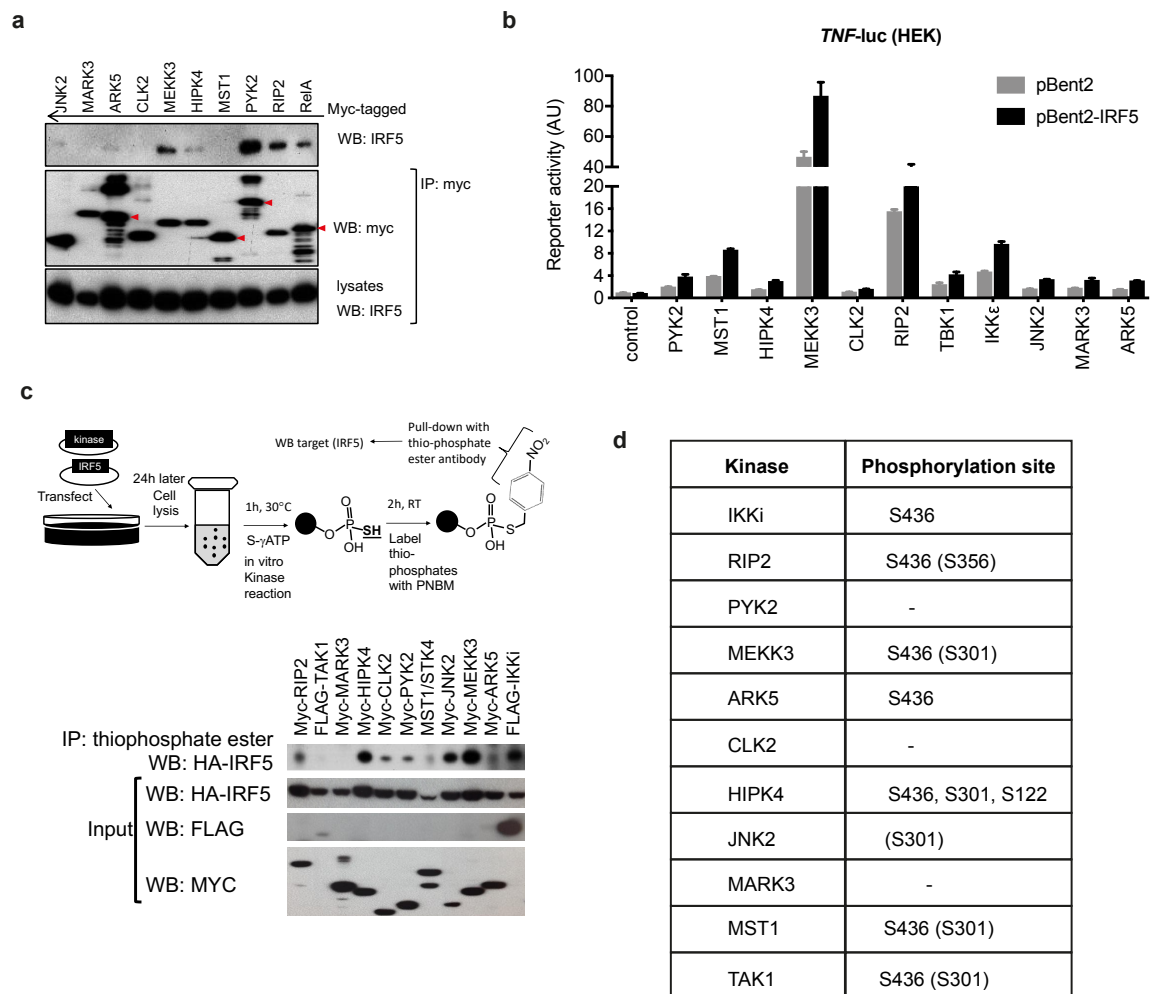


Figure 3.3. In vitro validation of shortlisted candidate IRF5 kinases in HEK293T cells (a) HEK cells were transfected with myc-tagged kinases and HA-tagged IRF5 by Lipofectamine 2000. Cell lysates were immunoprecipitated with c-myc antibody bound to protein G sepharose beads and immunoblotted for Myc or IRF5. Red arrows indicate weight of expected band (b) HEK cells were co-transfected with myc-tagged kinases, IRF5/pBent2, and the TNF reporter construct. Cells were stimulated with 1 μ g/ml LPS for 6 h and luciferase activity was measured. (c) Kinase assay overview (top). Kinase assay results (bottom). (d) Phosphoproteomics of IRF5. Candidate kinases were co-expressed with strep-tagged IRF5 and IRF5 was pulled down from streptactin beads. Eluates were subjected to SDS PAGE and gels were visualised with Coomassie Blue. IRF5 bands were excised, trypsinised and the peptides were analysed by mass spectrometry. The phosphorylation sites corresponds to each of the kinases are shown in the table, with low confidence sites shown in brackets.

with the previously proposed candidate IRF5 kinases IKK ϵ and RIP2 have demonstrated functional interactions with IRF5.

3.2.3.4. Phosphoproteomics of overexpressed IRF5

The short-listed candidate IRF5 kinases were co-expressed with IRF5 and the modified IRF5 protein was trypsinised and the peptides were analysed by mass spectrometry to identify sites of phosphorylation. PYK2, CLK2 and MARK3 failed to phosphorylate IRF5 *in vitro*. (**Figure 3.3d**). Serine 436 of IRF5 (numbered as in the human isoform v1) came up in 7 out of 11 kinases analysed, the same site was identified as RIP2 target residue (92). In addition, serines 122, 301 and 356 of IRF5 could be other phosphorylation sites for further analysis. It is possible that protein overexpression may produce false positive data, thus the endogenous system should be explored. Overall, we have combined literature data (86, 92, 118) with our phospho-proteomic and sequence conservation analyses to come up with the extended list of the candidate IRF5 phosphorylation sites: S122, S130, S137, S156, S158, S160, S301, S421, S425, S427, S430, S436 and S457.

3.2.3.5. Serine phosphorylation on IRF5 is important for activation

Phosphorylation on serine residues has previously been shown to play a critical role in IRF5 activation including serine sites 421, 425, 427, 430, 436 sites (numbered as in human isoform IRF5v3/v4) (89, 94). To verify these sites I evaluated the effect of serine phosphorylation on IRF5-driven *Tnf* reporter activity using expression plasmids encoding HA-tagged serine mutants of the IRF5 protein (cloned in the *Udalova* group by Grigory Ryzhakov). Single or multiple serine sites on IRF5 were mutated to alanine by site-directed mutagenesis to generate loss-of-function mutants (**Figure 3.4a**). Essential serine sites (421, 425, 427, 430, 436) are located within the serine rich region towards the C-terminus so a construct lacking the IRF5 C-terminal region (N430) was included. K63-linked ubiquitination on IRF5 lysine sites 401 and 402 is thought to play a role in IRF5 nuclear

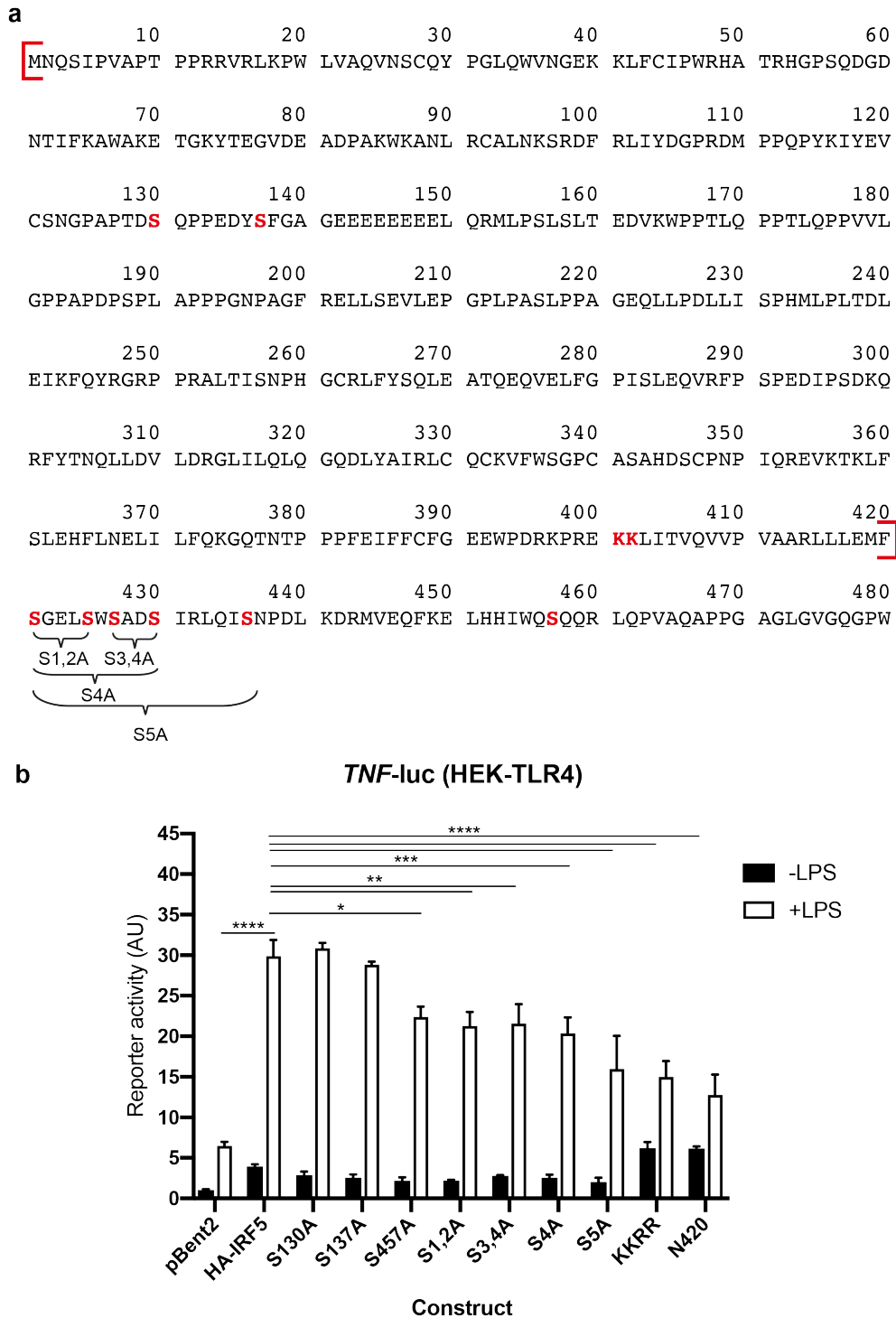


Figure 3.4. Serine phosphorylation sites involved in IRF5 activation (a) Schematic representation of the IRF5 mutants containing serine-alanine substitutions and lysine-arginine substitutions (human IRF5 isoform v3/v4) used in this study. N420 construct indicated in red brackets (b) HEK293-TLR4 cells (1×10^5) were co-transfected with pBent2-empty/HA-IRF5 (WT, S130A, S137A, S457A, S1,2A, S3,4A, S4A, S5A, KKRR, N420) along with TNF-firefly Luc and pRLTK-Renilla Luc by Lipofectamine 2000 to determine TNF reporter activity. Following 24 hours incubation at 37°C , cells were stimulated for 0/6 hours with 100 ng/ml LPS. Firefly and Renilla luciferase levels were measured by Dual Glo luciferase assay using a FLUOstar Omega microplate reader. Data are representative of three independent experiments each performed as triplicates. Bars show standard error of the mean, and are expressed as firefly luciferase relative to renilla luciferase levels. Statistical analysis by unpaired Student's t-test where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

translocation and target gene regulation (93), so a lysine to arginine double mutant construct (KKRR) was also included. Each mutant construct was transfected into HEK-TLR4 cells along with the TNF-luciferase reporter gene. After 24 h, cell extracts were prepared and assayed for luciferase activity. The results shown in **Figure 3.4b** indicate the requirement for sites K401, K402, S421, S425, S427, S430 and S436 in LPS induced activity of IRF5 in HEK cells. Furthermore, removal of the C-terminal region (N420) reduced the ability of IRF5 to activate the *Tnf*-luciferase reporter. Whereas, transcriptional activity was not altered with ectopic expression of mutants S130 and S137 compared to WT HA-IRF5. Together, these results confirm that the carboxyl terminal phosphorylation of IRF5 in the serine-rich region is the critical modification that determines IRF5 transcriptional activity, along with ubiquitination of sites K401 and K402.

3.2.3.6. Overexpression of IRF5 in RAW264.7 cells

To complement the functional assays carried out in HEK cells, I next validated the candidate kinases in RAW264.7 cells as it is a more biologically relevant system. I initially investigated whether the overexpression constructs can be transfected into RAW264.7 cells. The expression plasmids were loaded on an agarose gel to check they were in a supercoiled conformation (**Figure 3.5**). The supercoiled conformation of DNA has a higher transfection

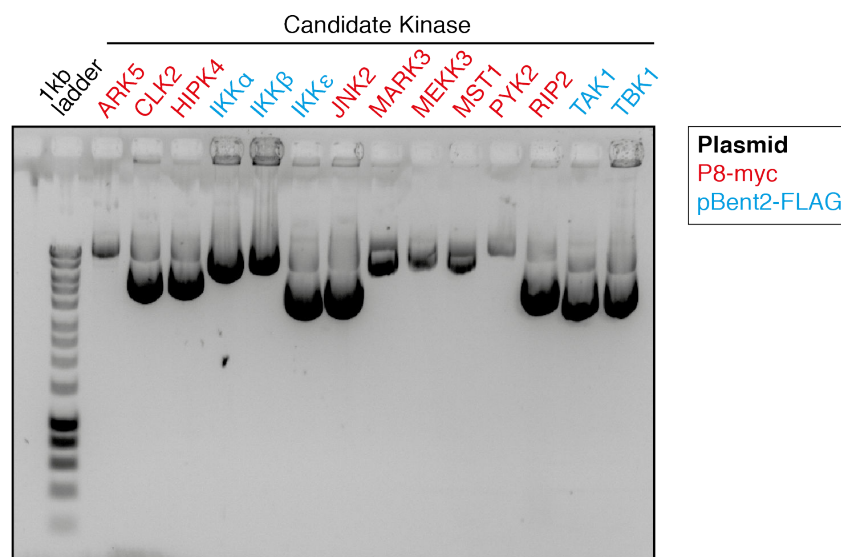


Figure 3.5. Plasmid conformation of candidate IRF5 kinases Candidate kinases cloned in a P8-myc (red) or pBent2-FLAG (blue) vector system were isolated, mixed with DNA loading buffer and loaded on a 1.5 % agarose gel (250 ng/ul of DNA per well) containing EtBr, along with a 1 kb ladder. Samples were separated by a 115 V for 1 h and visualised by UV excitation.

efficiency compared to the linear form. Due to its compact conformation supercoiled DNA migrates at a faster rate than linear DNA through the gel. Plasmids CLK2, HIPK4, IKK α , IKK β , IKK ϵ , JNK2, RIP2, TAK1 and TBK1 run as a predominantly single thick band corresponding to a supercoiled conformation so these plasmids were kept for transfection experiments. Plasmids for ARK5, MARK3, MEKK3, MST1 and PYK2 kinases showed smearing of DNA bands and the appearance of additional DNA conformations with a faint band for the supercoiled conformation. Plasmids for these kinases exist mainly in the linear conformation and are no longer intact. This is probably due to degradation over time from continuous freeze/thaw cycles. I then proceeded to re-prepare plasmids for ARK5, MARK3, MEKK3, MST1 and PYK2 from frozen bacterial glycerol stocks. **Figure 3.6** shows that the re-prepared plasmids for these kinases were properly folded in the supercoiled conformation with a single thick band, thus are suitable for transfection experiments.

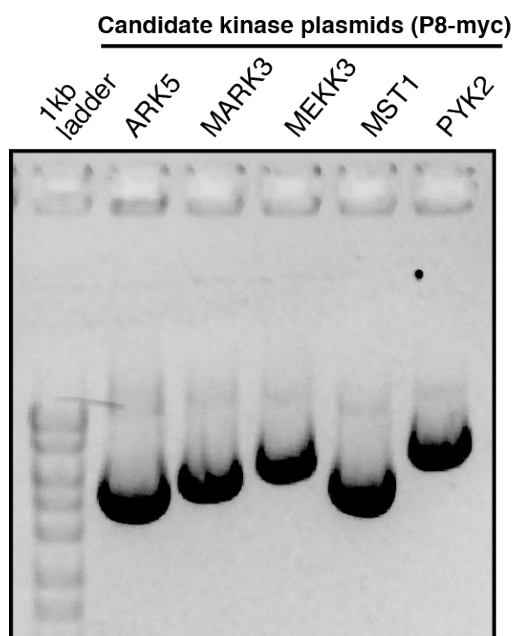


Figure 3.6. Plasmid conformation of re-prepared plasmids Candidate kinases ARK5, MARK3, MEKK3, MST1 and PYK2 were re-prepared from frozen bacterial glycerol stocks and isolated by midi-prep. Analysis by agarose gel electrophoresis of plasmid DNA of the candidate kinases cloned in a P8-myc expression vector. Plasmids were mixed with DNA loading buffer and loaded on a 1.5 % agarose gel (250 ng/ul of DNA per well) containing EtBr, along with a 1 kb ladder. Samples were separated by a 115 V for 1 h and viewed by UV excitation.

The candidate kinase plasmids were initially transfected in RAW264.7 cells with 4, 6, 8, and 10 μ l of Lipofectamine LTX reagent (transfection reagent). Transfection efficiency was assessed by immunoblotting for c-MYC or FLAG as plasmids were cloned in either tagged vectors (**Figure 3.7**). Candidate IRF5 kinases MST1, IKK ϵ and TAK1 were successfully overexpressed in cells. Likewise, ARK5, MEKK3, PYK2, and IKK β were ectopically expressed but to a lesser extent with weak expression. Candidates MARK3, IKK α and TBK1 failed to overexpress in RAW264.7 cells at all concentrations of transfection reagent used. As an alternative to lipid based transfections, I then used the electroporation technique, to see if this this change in method would further improve ectopic kinase expression. Candidate kinases IKK α , IKK β , IKK ϵ and TAK1 were co-transfected with a GFP vector by nucleofection and immunoblotted for FLAG. Similar to the lipid-based method IKK β and IKK ϵ was successful overexpressed, whereas IKK α and TAK1 failed to overexpress (**Figure 3.8**). Possible reasons for this could be due to the cDNA used or an issue with protein instability when overexpressed. RAW264.7 cells are also considered a difficult-to-transfect cell line, so it is likely that overexpression of candidate kinases was inefficient due to cell type specific reasons.

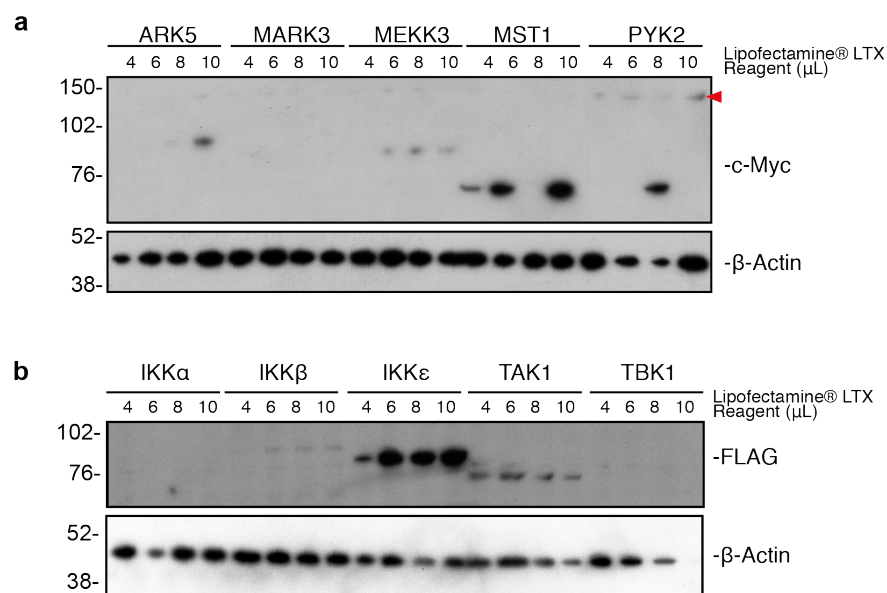


Figure 3.7. Overexpression of candidate kinases in RAW264.7 cells by lipid transfection Candidate kinases cloned in a (a) Myc or (b) Flag expression vector were transfected into RAW264.7 cells by lipofectamine LTX/plus reagent. 4,6,8,10 μ l of Lipofectamine LTX reagent was used to transfect cells. 24 hours post transfection, lysates were prepared, loaded on an SDS-PAGE gel and transferred to PVDF membrane for immunoblotting for c-Myc, FLAG and B-actin (loading control).

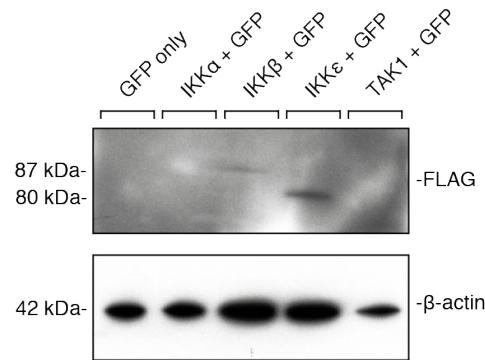


Figure 3.8. Overexpression of candidate kinases in RAW264.7 cells by nucleofection Candidate kinases IKK α , IKK β , IKK ϵ and TAK1 cloned in a Flag expression vector were co-transfected with a GFP vector into RAW264.7 cells by nucleofection. 24 hours post transfection, lysates were prepared, loaded on an SDS-PAGE gel and transferred to PVDF membrane for immunoblotting for FLAG and levels of B-actin served as a loading control.

3.2.4. Endogenous expression of IRF5 and candidate kinases in macrophages

To validate the candidate kinases I next focused on the endogenous system specifically in macrophages. Endogenous expression of IRF5 in RAW264.7 cells and in mouse bone marrow derived macrophages (BMDMs) stimulated with GM-CSF or M-CSF to induce the M1 and M2 macrophage phenotypes, respectively was explored. Macrophages were challenged with the TLR4 ligand, lipopolysaccharide (LPS) for 2 hours and IRF5 protein levels were analysed by immunoblotting. We have previously reported higher expression of IRF5 in GM-CSF treated macrophages compared to M-CSF treated macrophages (24, 47). In agreement with this, expression of IRF5 was higher in GM-CSF compared to M-CSF BMDMs but to similar levels with and without LPS (**Figure 3.9**). In addition, expression of

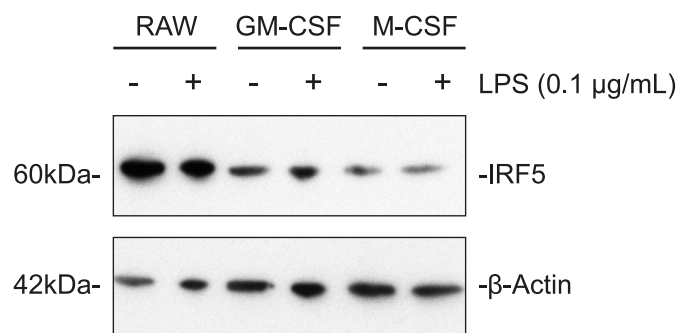


Figure 3.9. Endogenous expression of IRF5 in macrophages RAW264.7 cells or mouse bone-marrow derived macrophages stimulated with GM-CSF or M-CSF were stimulated with LPS for 0/2 hour at 0.1 $\mu\text{g/ml}$ then lysed on ice for 30 minutes with total cell lysis buffer with added protease inhibitors. Lysates were centrifuged to pellet cellular debris, and then the protein content quantified using a Qubit assay. 10 μg protein was prepared by heating to 100°C in loading buffer, then loaded onto SDS-PAGE gels for electrophoresis followed by transfer to PVDF membrane for immunoblotting with IRF5 and B-actin specific antibodies.

IRF5 was highly expressed in RAW264.7 cells. However, IRF5 protein levels were not further induced upon LPS stimulation.

I next aimed to assess the endogenous expression of shortlisted candidate kinases including IKK α , HIPK4, IKK β , TBK1, MEKK3, RIP2, JNK2 and PYK2 in macrophages including RAW264.7 cells, GM-BMDMs, and M-BMDMs (**Figure 3.10**). With the exception of MEKK3, all the candidate kinases tested were endogenously expressed in GM-BMDMs and M-BMDMs. Furthermore, all the candidate kinases were strongly expressed in RAW264.7 cells regardless of LPS stimulation. I decided to focus on the RAW264.7 cell line for functional assays to study IRF5 regulation, as they are often used as an appropriate monocyte/macrophage model and have been shown to recapitulate multiple aspects of macrophage physiology (149, 150).

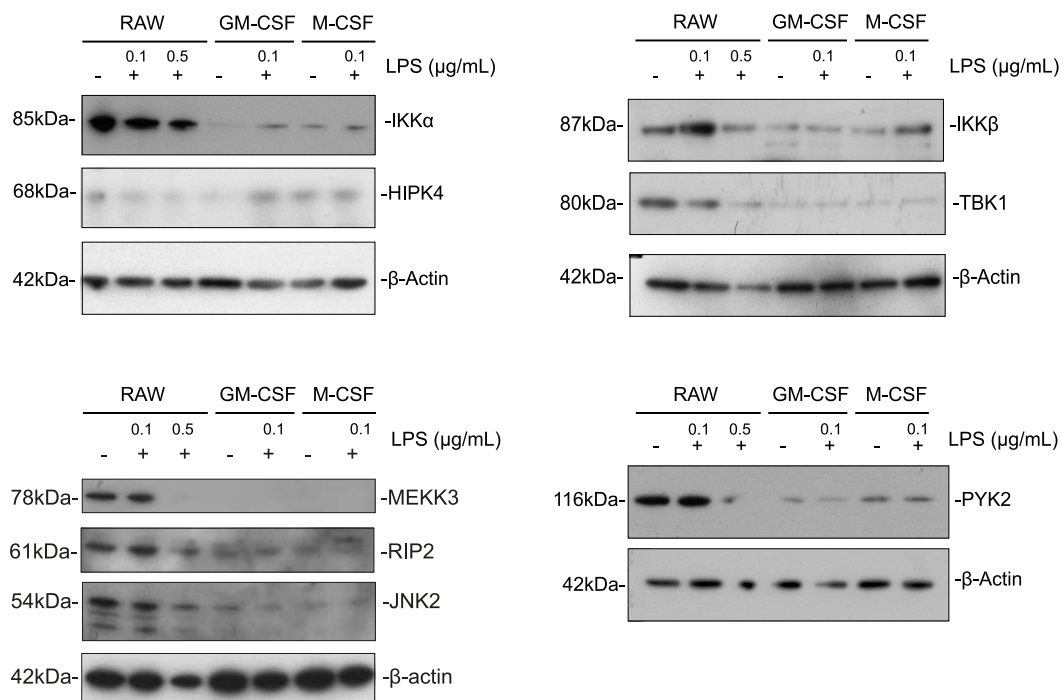


Figure 3.10. Endogenous expression of candidate kinases in macrophages RAW264.7 cells were stimulated with LPS for 0/2 hour at 0.1 μ g/ml or 0.5 μ g/ml. Mouse GM-BMDMs and M-BMDMs were stimulated with LPS for 0/2 hour at 0.1 μ g/ml. Cells were lysed on ice with total cell lysis buffer, before being cleared by centrifugation. 10 μ g protein (determined by Qubit analysis) was loaded per lane of an SDS-PAGE gel, resolved by electrophoresis then transferred to PVDF membrane for Western blotting with IKK α , HIPK4, MEKK3, RIP2, JNK2, IKK β , TBK1 and PYK2 antibodies. Levels of B-actin served as a loading control.

3.2.5. IRF5 association with candidate kinases

The endogenous expression of the candidate kinases in macrophages prompted me to investigate whether IRF5 and candidates interact physically at the endogenous level. Endogenous co-immunoprecipitations with IRF5 antibody were performed to determine the interaction of IRF5 with candidate kinases in RAW264.7 cells unstimulated or stimulated with LPS (**Figure 3.11**). IRF5 which is a 60 kDa protein runs at a similar size to the immunoprecipitating IgG heavy chain (55 kDa). To mitigate the issue of interference from the heavy chain, a secondary antibody that preferentially detects the non-reduced form of rabbit IgG over the reduced immunoprecipitating IgG was used. Western blot analysis of IRF5-immunoprecipitates from RAW264.7 cells revealed that IRF5 interacts

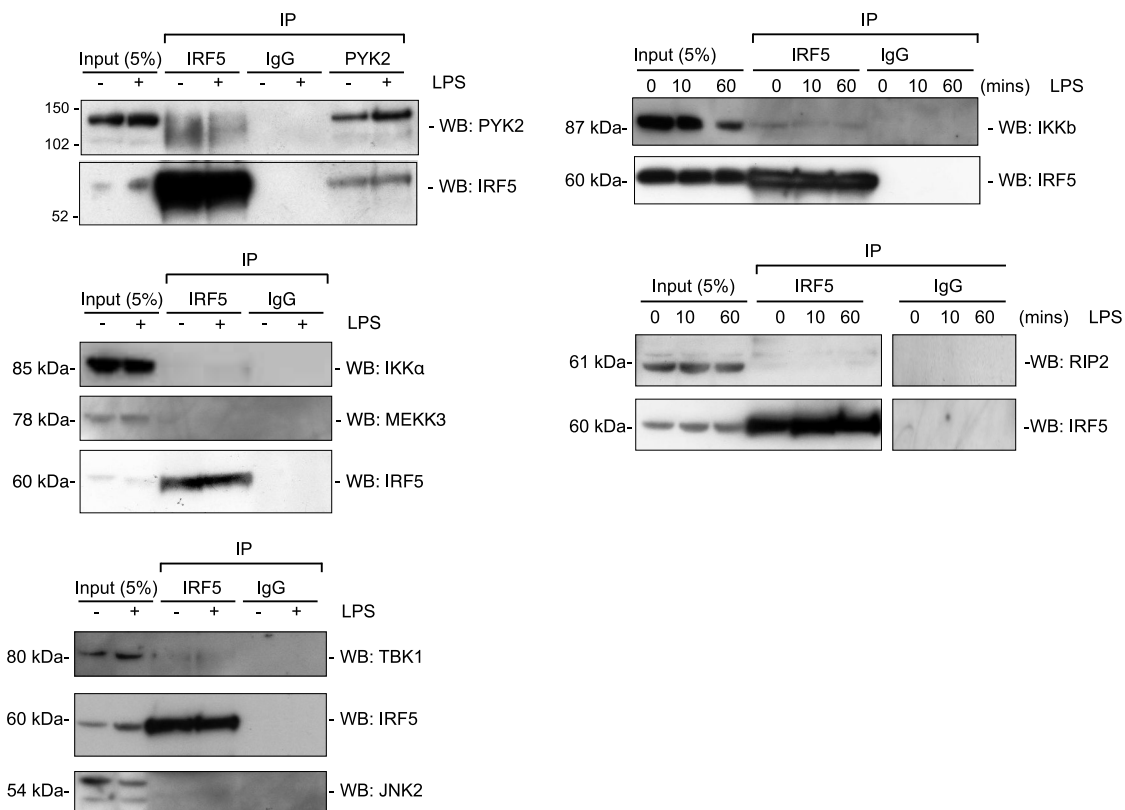


Figure 3.11. Association of IRF5 with candidate kinases 1×10^7 RAW264.7 cells were stimulated with LPS (500 ng/ml) for 0/0.5/1 hours as indicated. The cells were lysed on ice for 30 minutes with 500 μ l of chilled whole cell lysis buffer with added protease inhibitors. Lysates were cleared by centrifugation to remove cellular debris before being incubated with 2 μ g IRF5 antibody or Rabbit IgG isotype control for 2 hours rotating at 4°C. Prewashed trueblot IP beads were added and incubated overnight. Immuno-complexes were washed 3x with chilled lysis buffer by rotating for 5 minutes, pelleting the beads by centrifugation and removing the supernatant. Prey and bait protein complexes were eluted from the column by heating to 100°C in 50 μ l of Laemmli buffer. 10 % of non-immunoprecipitated lysate was kept for input control. Samples were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting with IRF5 and antibodies against the kinases. WB = Western blot, IP = immunoprecipitation

strongly with kinase PYK2 and weakly with IKK β , RIP2, TBK1 in the absence and presence of LPS stimulation. There may be a weak interaction with MEKK3, however the input levels are weak and the blot should be repeated with new MEKK3 primary antibodies. No direct interaction between endogenous IRF5 with kinases IKK α , or JNK2 regardless of LPS stimulation could be detected by co-immunoprecipitations. No interaction was detected in the control isotype IgG lane. Similar results were obtained in reciprocal experiments, in which IRF5 immunoprecipitates with PYK2 in the anti-PYK2 pulldown with and without LPS treatment. For the immunoblots showing weak or no interactions it may be the case that the time point used for LPS stimulation is not capturing IRF5 – kinase interactions. Phosphorylation events are transient and reversible where the extent of protein phosphorylation could be relatively high at one time point but below detectable levels at other time points. Taken together, these results indicate that endogenous IRF5 physically interacts with PYK2, IKK β , RIP2, TBK1, and possibly MEKK3 independently of LPS activation, but not with IKK α or JNK2. A kinase-substrate interaction suggests that these kinases may possibly phosphorylate IRF5 or may just act as scaffolds for protein complex assemblies.

3.2.6. CRISPR/CAS9 knockouts of IRF5 and candidate kinases

Due to the poor ectopic expression of candidate kinases in macrophages (Section 3.2.3.6), I used a loss-of-function approach to gain further insight into the function of IRF5 and candidate kinases. Stable cell lines were generated in which endogenous expression of IRF5 and candidate kinases had been ablated. To do this, genome editing of RAW264.7 cells was carried out using the CRISPR/Cas9 system. PYK2 and MEKK3 were prioritised as candidates for follow-up studies as they were the lead hits from the initial validation round and represent novel, interesting kinases. IKK β and TBK1 were also selected for loss-of-function knockouts as reported IRF5 kinases (92, 95, 96). As RAW264.7 cells are difficult to transfect, a lentivirus-mediated transfection system was used. This system consists of a

lentivirus vector that expresses several genes including Cas9 nuclease to generate double stranded DNA breaks, guide RNA (gRNA) to target the gene of interest, puromycin resistance to enrich for cells with the deletion, and GFP that serves as a visual marker (**Figure 3.12a**). Following selection and enriching the number of transduced cells via FACS sorting GFP-positive cells, I then assayed knockout efficiency at the protein level by western

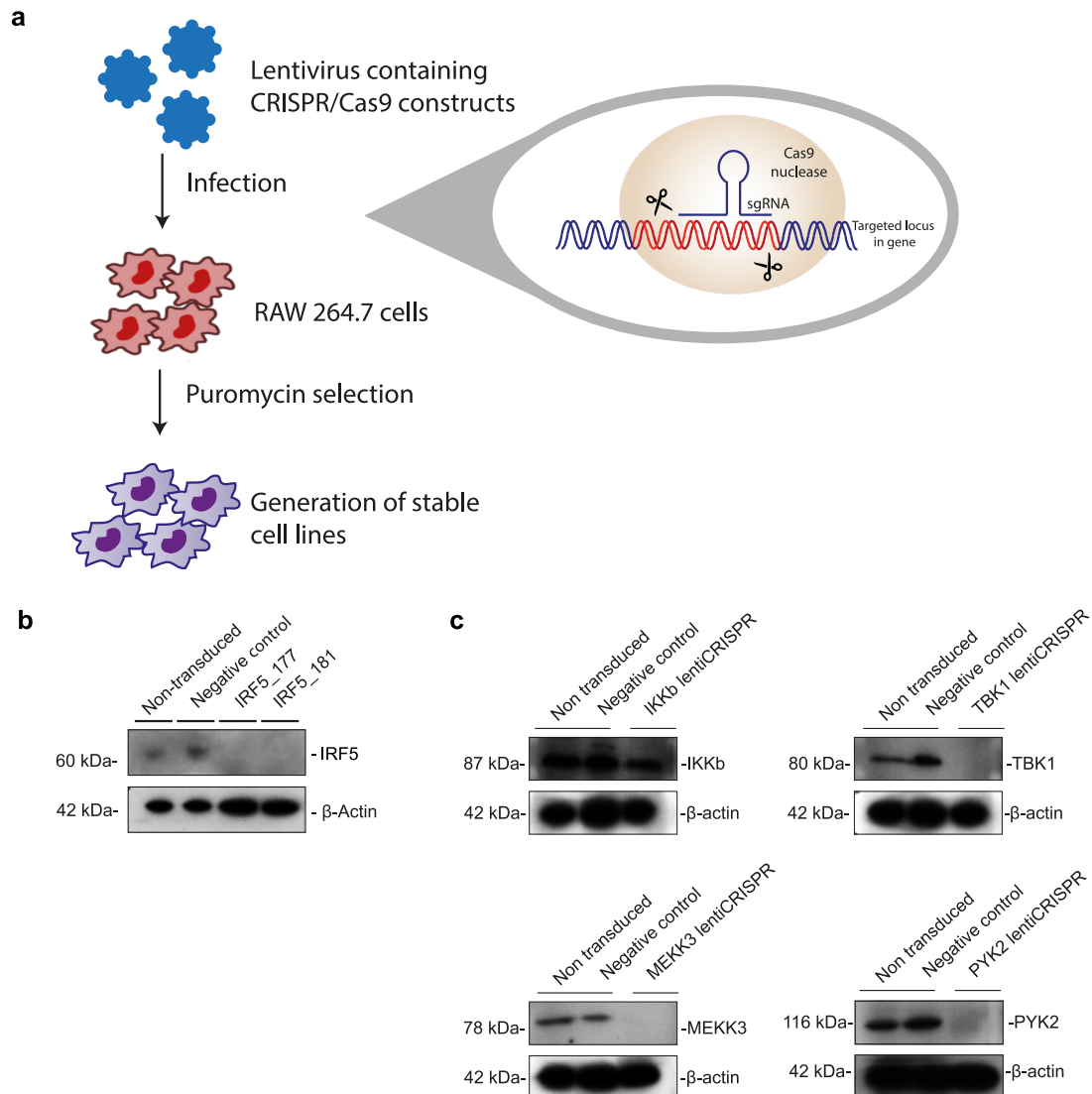


Figure 3.12. CRISPR/Cas9 lentiviral transduction of RAW264.7 cells to generate knockouts (a) Overview of lentiviral CRISPR/Cas9 system. RAW264.7 cells were infected with lentiviral particles containing the CRISPR/Cas9 construct and selected for deletion by puromycin. **(b)** CRISPR/Cas9 constructs targeting IRF5 (target ID 177 and 181), **(c)** IKK β , TBK1, MEK3, PYK2, or a non-targeting control were infected into cells by lentiviral particles (pLV_U6g_EPCG) at a MOI of 10 by spin infection at 1,500 rpm for 1 hour. Cells are then selected with puromycin (4 μ g/ml) every 2-3 days over a 2 week period to enrich for cells with gene insertion. 10 μ g of total protein was collected from both CRISPR-edited cells and unedited cells by diluting cell lysates in Laemmli sample buffer and boiling at 100°C for 10 minutes. Samples were then loaded onto a 4-20% precast gel followed by SDS-PAGE. Verification of gene knockout was performed by immunoblotting for target proteins and β -actin as a loading control. Negative control samples included non-transduced cells and cells infected with a non-targeting construct.

blotting. A non-transduced sample and a non-targeting lentiviral construct was used as negative controls. As shown in **Figures 3.12b** and **3.12c**, mock-transduced cells expressed similar protein levels to non-transduced cells. The lentiviral CRISPR knockout infections were successful for RAW264.7 cells infected with two different IRF5 gRNAs (labelled IRF5_177 and IRF5_181) (**Figure 3.12b**). Likewise, lentiviral infections of PYK2, TBK1, and MEKK3- containing CRISPR constructs resulted in successful knockouts of these kinases (**Figure 3.12c**). The IKK β CRISPR construct failed to induce an IKK β knockout cell line, probably due to off-target effects. Additionally, the indel mutation generated by Cas9 may keep the protein in frame, allowing the protein to be detected by western blot.

To help rule out any false positives and confirm that the cells are genetically deficient, the CRISPR knockout lines were DNA sequenced. Primers were designed to sequence roughly 500 basepairs (bps) around the protospacer adjacent motif (PAM) sequence of the gRNA. I aligned the DNA sequences to uninfected wildtype cells and wildtype cells infected with a non-targeting control. **Figure 3.13** shows that as expected both negative controls showed identical DNA sequences. Furthermore, all the knockout lines displayed deletions preceding the PAM sequence in the alignments. These deletions could lead to frameshift



Figure 3.13. DNA sequencing alignment of WT and KO cells DNA from WT, non-targeting control, IRF5 KO (lines 177 and 181), MEKK3 KO, PYK2 KO, TBK1 KO cell lines were isolated and sent for sequencing. Forward and reverse primers were designed to sequence the region around and including the gRNA/PAM site. Genomic sequences from the knockout lines were aligned to WT (non-infected and non-targeting) sequences by ClustalW analysis. gRNA and PAM sequences are indicated in red and blue colours, respectively. Hyphens indicate deleted sites/regions.

mutations and introduce premature stop codons leading to loss-of-function mutation within the targeted gene. These results demonstrate that introducing the CRISPR/Cas9 system by means of a lentiviral vector effectively and specifically produced knockouts of IRF5, PYK2, MEKK3 and TBK1 in RAW264.7 cells.

3.2.7. Kinase KO lines display reduced IRF5-driven TNF reporter activity

I next examined whether IRF5-dependent gene expression of *Tnf* is regulated by the candidate kinases. To this end, the IRF5/TNF-dependent luciferase reporter assay was carried out in wildtype and kinase knock out cells (PYK2, MEKK3 and TBK1) co-transfected with the reporter plasmids. Wildtype cells expressing IRF5 and stimulated with LPS exhibit an approximate six-fold increase in TNF luciferase activity (**Figure 3.14**). However,

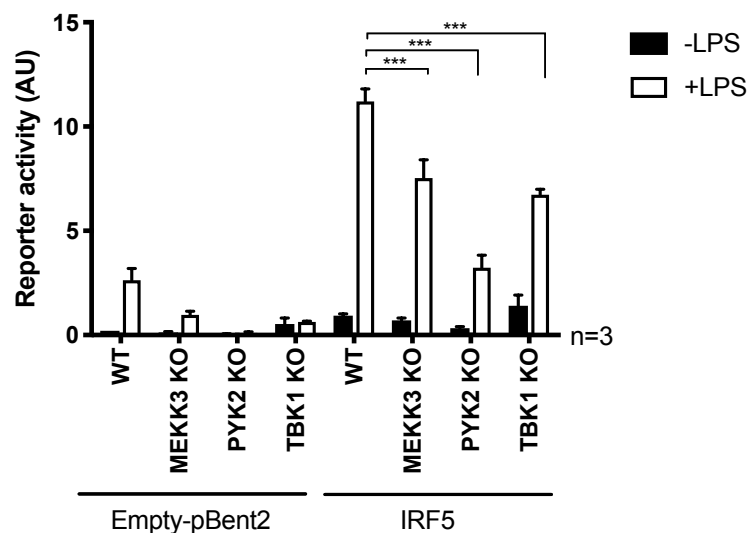


Figure 3.14. Reduced IRF5 driven TNF-reporter activity in kinase KO lines TNF-reporter in WT, MEKK3 KOs, PYK2 KOs and TBK1 KOs. RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were stimulated with LPS (1 µg/ml) for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of n=3, each performed in triplicates. Statistical analysis by unpaired Student's t-test where *** $p \leq 0.001$.

RAW264.7 cells lacking PYK2, MEKK3 or TBK1 failed to induce luciferase activity to the same levels as WT cells. Even in the absence of IRF5 plasmid, LPS stimulation induced reporter activity which was reduced in all the kinase knockout lines and could be due to endogenous levels of IRF5. Overall, these results suggests that all three kinases have an effect on TNF-gene expression that is delivered via IRF5.

3.2.8. Kinetics of IRF5 phosphorylation

Upon TLR4 activation, IRF5 is phosphorylated and activated in myeloid cell lines. However, the kinetics of LPS-induced IRF5 phosphorylation in RAW264.7 cells has not been previously explored. I sought to determine the optimal length of incubation with LPS to achieve maximal IRF5 phosphorylation in RAW264.7 cells. Phosphorylation of IRF5 was determined using a site specific anti-phospho S437 IRF5 antibody in response to LPS at several timepoints. However, under my experimental conditions I could not detect phospho-IRF5 (S437) even at higher antibody concentrations and with higher concentrations of lysates loaded (data not shown). In the absence of a working phospho-IRF5 antibody, Phostag-gels were used to determine the kinetics of IRF5 phosphorylation. As samples run through the Phostag gel, two distinct bands are generated; the retarded phosphorylated species and the faster migrating non-phosphorylated species. However, I failed to detect a separation of phospho-IRF5 from IRF5 and only a single band was detected by Phos-tag immunoblot analysis (**Supplementary Figure S1**). This could be due to inefficient transfer of phosphoproteins compared to their non-phosphorylated counterparts from the Phos-tag gel to the membrane as phosphorylated proteins interact strongly with the Phos-tag gel.

To circumvent the issues of studying IRF5 activation I examined IRF5 phosphorylation in untreated and LPS-stimulated cells by immunoprecipitating IRF5 and immunoblotting for total phosphoserine or phosphotyrosine. RAW264.7 cells were stimulated with LPS at 0, 5, 10, 15, 30, 60 and 120 minutes. Results in **Figure 3.15** show that LPS induced IRF5

phosphorylation on both serine and tyrosine- residues in a time-dependent manner. The presence of a distinct higher molecular weight band above IRF5 (just above 60 kDa) most likely represents IRF5 serine and tyrosine phosphorylated species as phosphorylation modifications are heavier than their non-phosphorylated counterparts. The presence of a high molecular weight smear above 76 kDa could be indicative of IRF5 ubiquitination.

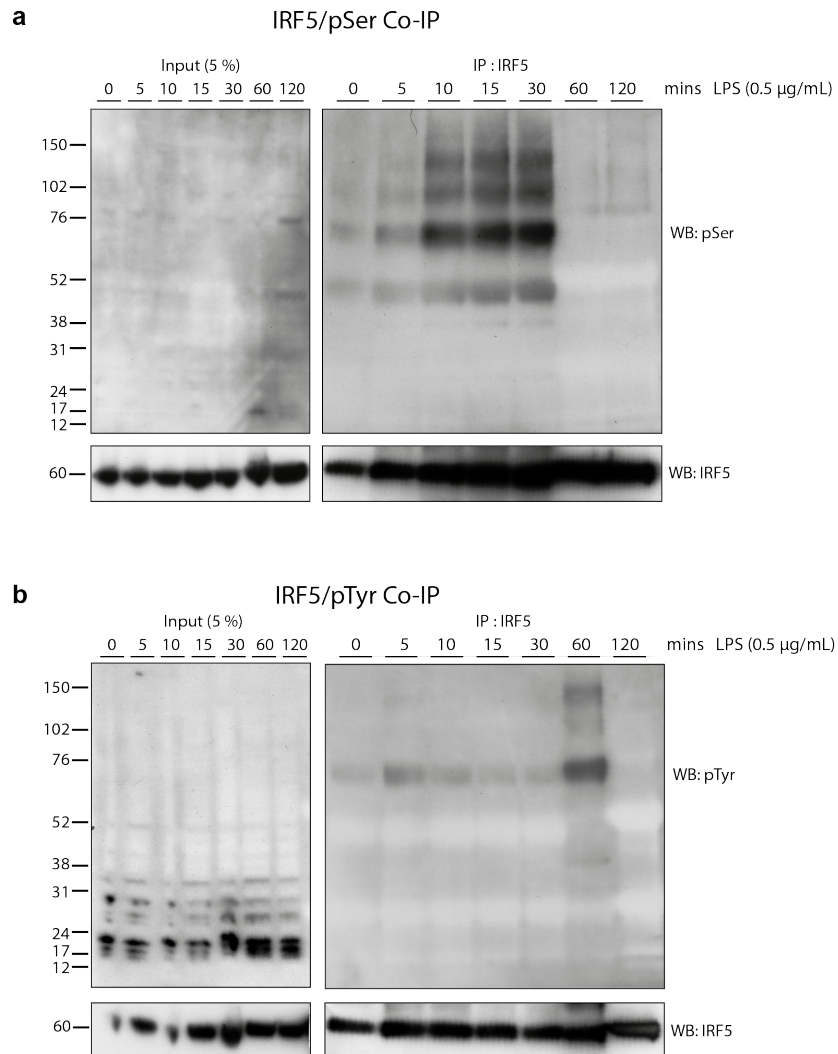


Figure 3.15. Kinetics of LPS-induced IRF5 phosphorylation (a) IRF5 phosphoserine levels (b) IRF5 phosphotyrosine levels. RAW264.7 cells were stimulated with LPS (500 ng/ml) at indicated timepoints. Cells were lysed on ice with whole cell lysis buffer (with added protease and phosphatase inhibitors) for 30 minutes and centrifuged to remove cellular debris. A fraction (5 %) of cell lysate was reserved for input control. Lysates were incubated with 2 µg IRF5 antibody for 2 hours rotating at 4°C. Pre-washed trueblot IP beads were added and incubated overnight. Immuno-complexes were washed 3x with chilled lysis buffer by rotating for 5 minutes, pelleting the beads by centrifugation and removing the supernatant. Immunocomplexes were eluted from the column by heating to 100°C in 50 µl of Laemeli buffer. Samples were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting with phosphoserine, phosphotyrosine and IRF5 antibodies. WB = Western blot, IP = immunoprecipitation.

Low levels of constitutively phosphorylated IRF5 were detected in unstimulated cells on serine and tyrosine residues. Maximal phosphorylation on IRF5 serine (**Figure 3.15a**) and tyrosine sites (**Figure 3.15b**) occurred 10-30 minutes and 60 minutes post LPS stimulation, respectively. Of interest, these slower migrating bands were completely abrogated by 1-2 hours post treatment with LPS on serine residues, and 2 hours post LPS stimulation on tyrosine residues. Two hypotheses emerged as potential possibilities to explain phospho-IRF5's disappearance: (1) Phosphorylation is a transient event and at these timepoints IRF5 is no longer active and (2) Subcellular localisation of IRF5 has altered where it has already translocated to the nucleus and bound to chromatin.

I investigated the latter hypothesis as to whether the disappearance of IRF5 phosphorylated bands at later timepoints after TLR4 triggering was due to IRF5 being bound to DNA. To further dissect the phosphorylation pattern, the immunoprecipitation was repeated but with enzymatic treatment. The enzyme benzonase nuclease was added to the lysis buffer to facilitate digestion of chromatin bound IRF5. The results show at later timepoints DNA-bound IRF5 is released by the addition of benzonase nuclease and can be detected by immunoblotting with phospho-serine/tyrosine antibodies (**Figure 3.16**). A strong band for phosphoserine on IRF5 was expressed 60 minutes post LPS-stimulation and a fainter band at 120 minutes (**Figure 3.16a**). Tyrosine phosphorylation was also expressed 120 minutes after LPS stimulation (**Figure 3.16b**). Thus, 60 minutes LPS stimulation (with benzonase added to the lysis buffer) is the optimum timepoint to see maximum phosphorylation on IRF5 for both serine and tyrosine sites. These results indicate that IRF5 is phosphorylated on both serine and tyrosine residues in a temporal manner and these phosphorylated residues may be involved in the activation of IRF5.

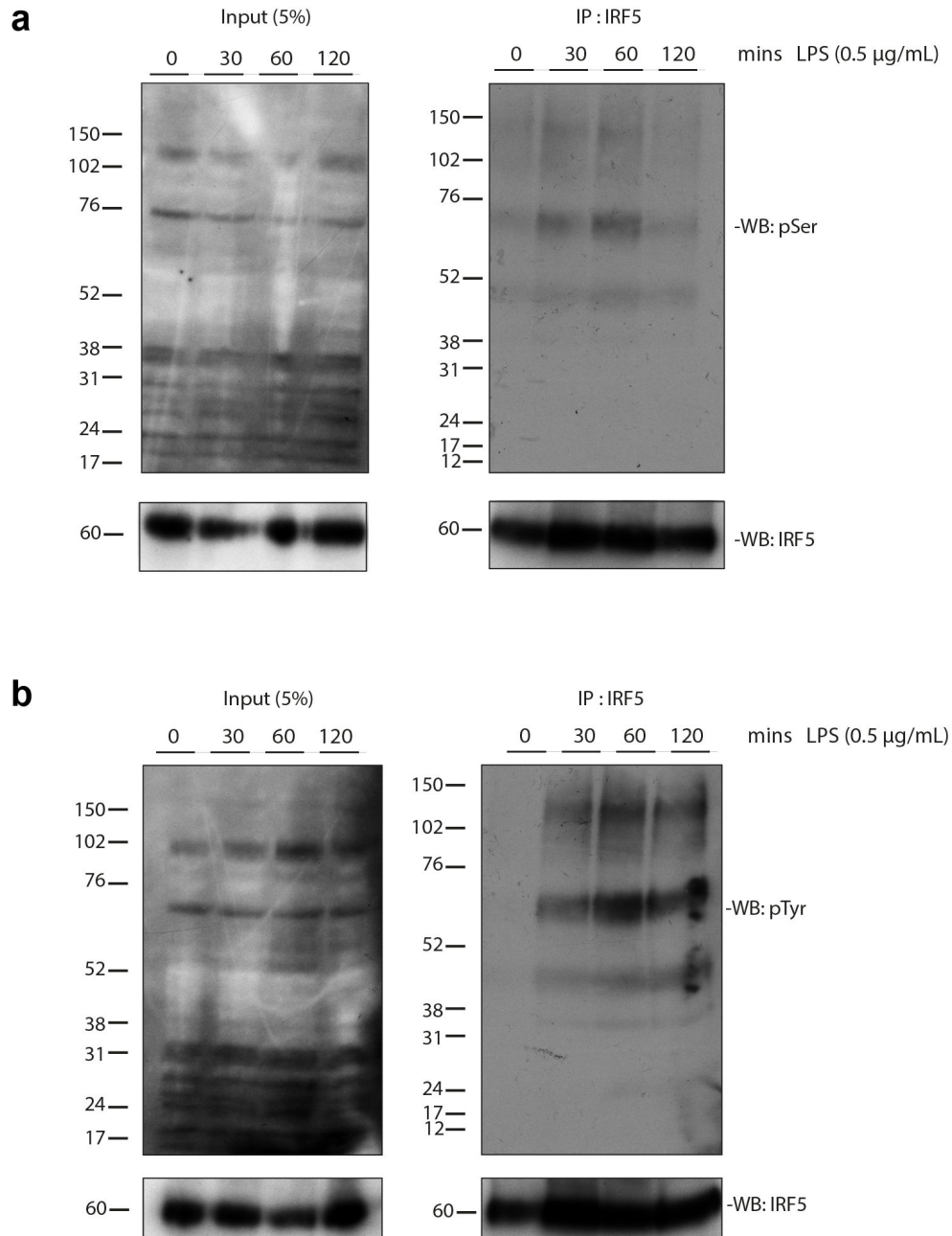


Figure 3.16. IRF5 phosphorylation kinetics with release of chromatin-bound IRF5 (a) IRF5 phosphoserine levels (b) IRF5 phosphotyrosine levels. RAW264.7 cells were stimulation with LPS (500 ng/ml) at indicated timepoints. Cells were lysed on ice with whole cell lysis buffer (with added protease and phosphatase inhibitors and benzonase nuclease) for 30 minutes and centrifuged to remove cellular debris. A fraction (5 %) of cell lysate was reserved for input control. Lysates were incubated with 2 μ g IRF5 antibody for 2 hours rotating at 4°C. Prewashed trueblot IP beads were added and incubated overnight. Immuno-complexes were washed 3x with chilled lysis buffer by rotating for 5 minutes, pelleting the beads by centrifugation and removing the supernatant. Immunocomplexes were eluted from the column by heating to 100°C in 50 μ l of laemeli buffer. Samples were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting with phosphoserine, phosphotyrosine and IRF5 antibodies. WB = Western blot, IP = immunoprecipitation.

3.2.9. IRF5 phosphorylation in kinase deficient lines

To test whether MEKK3, PYK2 and TBK1 are important in IRF5 signalling, I analysed the phosphorylation profile of IRF5 in CRISPR knockout cell lines. As MEKK3 and TBK1 are serine/threonine kinases I looked at phosphoserine levels 60 minutes post LPS stimulation in IRF5-immunoprecipitates. As previously shown, LPS stimulation leads to an increase in phosphoserine levels. However, LPS stimulation in the CRISPR MEKK3, and TBK1 cell lines also increased phosphoserine levels (**Figure 3.17a**). Likewise, KO in the tyrosine kinase PYK2 did not impair LPS-induced tyrosine phosphorylation at 60 minutes (**Figure 3.17b**). Although phosphorylation levels were not further affected in the kinase KO lines,

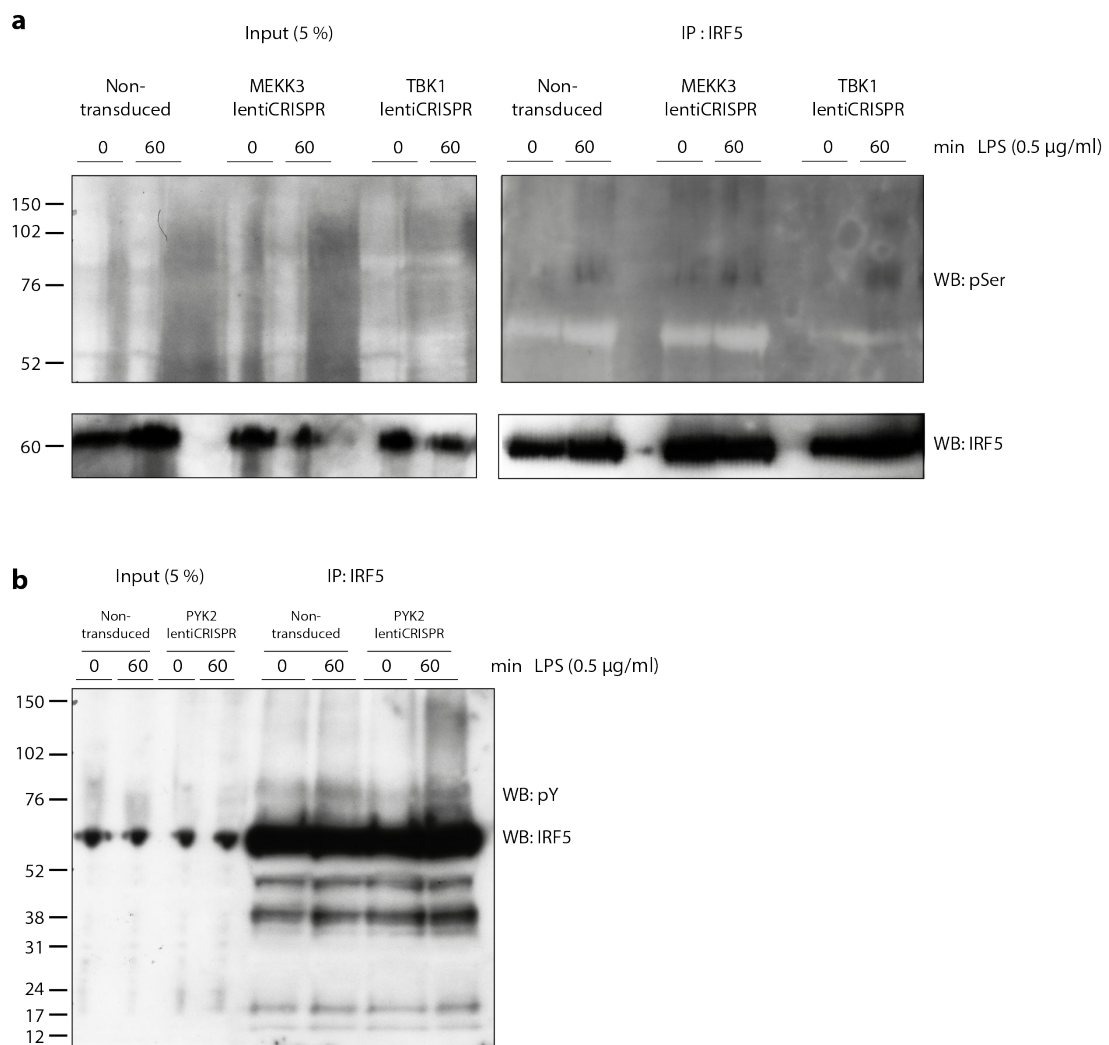


Figure 3.17. IRF5 phosphorylation in kinase deficient lines IRF5 was immunoprecipitated from cell lysates (non-transduced, MEKK3 KO, TBK1 KO, and PYK2 KO) after 60 min LPS treatment. IRF5 serine and tyrosine phosphorylation was assessed by immunoblotting for total serine in **(a)** and total tyrosine in **(b)**. A fraction (5 %) of cell lysate was used for input control. WB = Western blot, IP = immunoprecipitation.

the possibility that the kinases phosphorylate IRF5 cannot be excluded at this stage. Firstly, the kinases may phosphorylate IRF5 at a specific time point, which I may not be capturing. Secondly, other kinases such as IKK β (95, 96) and other unknown kinases may contribute to IRF5 phosphorylation in the absence of MEKK3, PYK2 and TBK1 and cannot be excluded as a possibility. Finally, the kinases may target IRF5 on a single specific site, and western blot may not be sensitive enough to detect any differences in phosphorylation on a single site. The phospho-bands are not as distinct as previous, and it may be the case that a new antibody solution should be used for a clearer blot. Future investigations should include a range of time points and use of mass spectrometry would be the best way to provide a more selective and sensitive approach for phosphopeptide characterisation.

3.2.10. Optimisation of lysis conditions for subcellular fractionation of IRF5

To regulate gene transcription IRF5 needs to translocate from the cytoplasm to the nucleus. I next assessed IRF5 localisation in non-stimulated and LPS-stimulated cells to be used as a good readout of IRF5 activation for further functional studies. In order to optimise assay conditions, various methods of cell lysate preparations were prepared for RAW264.7 cells. These included a (1) high salt and low salt lysis buffer, (2) chromatin immunoprecipitation buffers, and (3) a sucrose buffer. In method (1) cells are lysed in a high salt buffer to separate cytoplasmic extracts, followed by a low salt buffer to extract nuclear proteins (**Figure 3.18**). Histone H3, a nuclear marker and alpha-tubulin a cytosolic marker was detected in both compartments suggesting nuclear and cytosolic lysis occurs in both buffers. IRF5 was only expressed in the 'nuclear' compartment and to similar levels in the absence and presence of LPS. As IRF5 was only expressed in the nuclear fraction and nuclear and cytosolic proteins contaminated into the 'cytoplasmic' and 'nuclear' fractions this method was excluded for future experiments.

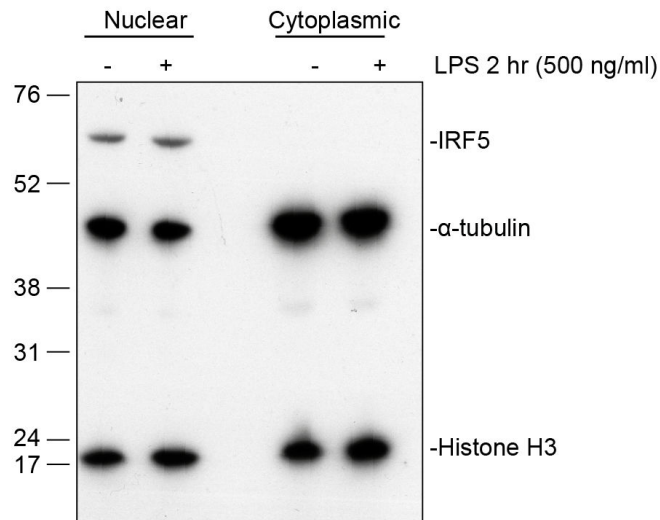


Figure 3.18. Subcellular fractionation of IRF5 with salt buffers RAW264.7 cells were unstimulated (-) or stimulated (+) with LPS (500 ng/ml) for 2 hours. 1×10^7 RAW264.7 cells were fractionated following lysis with a high salt buffer for cytosolic extracts and a low salt buffer for nuclear extracts. 60 μ g and 20 μ g of cytoplasmic lysate and nuclear lysate, respectively was resolved on an SDS-PAGE gel. Proteins were transferred to PVDF membrane for immunoblotting with Histone H3 (nuclear loading control), alpha-tubulin (cytosolic loading control) and IRF5 antibodies. WB = Western blot

I then tested methods (2) and (3) for subcellular fractionation in LPS-stimulated cells (**Figure 3.19**). Both methods, were able to prevent cytoplasmic protein contamination into the nuclear fractions as alpha-tubulin was only expressed in the cytoplasm. Moreover, histone H3 was predominantly expressed in the nuclear fraction in method 2 and only found in the nuclear fraction in method 3. Stimulation of TLR4 with LPS resulted in the nuclear accumulation of IRF5 in the sucrose buffer method (Method 3). As protocol (3) satisfied

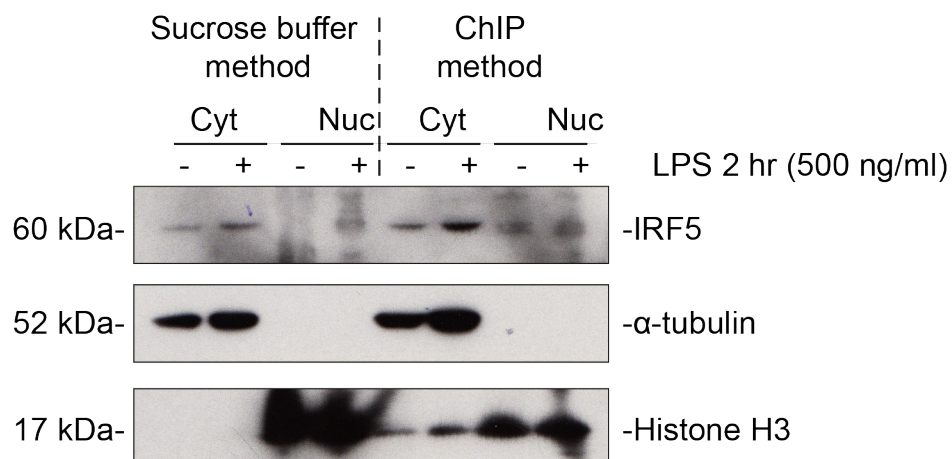


Figure 3.19. Subcellular fractionation of IRF5 with sucrose buffer or ChIP buffers RAW264.7 cells were unstimulated (-) or stimulated (+) with LPS (500 ng/ml) for 2 hours. 1×10^7 RAW264.7 cells were fractionated following lysis with sucrose buffers or ChIP buffer. 60 μ g and 20 μ g of cytoplasmic lysate and nuclear lysate, respectively was resolved on an SDS-PAGE gel. Proteins were transferred to PVDF membrane for immunoblotting with Histone H3 (nuclear loading control), alpha-tubulin (cytosolic loading control) and IRF5 antibodies. WB = Western blot

both criteria of LPS-stimulated nuclear translocation of IRF5 and good fractionation of cytosolic and nuclear compartments this method was used for the remainder of the project.

3.3. CONCLUSION

IRF5 is a genetic risk factor for several inflammatory and autoimmune conditions. Better understanding the mechanisms regulating IRF5 in inflammatory macrophages during inflammatory disease is now paramount, as it could lead to new therapies and promote lifelong health and wellbeing. In the present study, I have utilised a kinase inhibitor library screen for identification of candidate IRF5 kinases, validated the candidates in overexpression and endogenous systems, and explored the kinetics of IRF5 phosphorylation.

To identify IRF5 kinases, a screen with the GSK kinase inhibitor library was performed by selecting those that were suppressed by several IRF5 inhibitors, while excluding kinases not affected by inhibitors with no effect on IRF5 activation. It is important to note that half of the kinases in the human genome are missing in the screen so potential IRF5 kinases may be missed out as the library targets 221 kinases and the human genome consists of 518 kinases. Nonetheless, the identification of IRF5 reported kinases including IKK β , TBK1, RIP2 and TAK1 (92, 95, 96, 143) in the screen is encouraging. Of all the candidate kinases identified, PYK2 also known as non-receptor protein tyrosine kinase 2 beta and MEKK3 also known as mitogen-activated protein kinase kinase kinase 3 piqued my interest after coming up as the lead hits in most of the validation assays. Both kinases are expressed in macrophages, are capable of binding to IRF5, phosphorylating IRF5 and had an effect on IRF5-driven TNF reporter activity. These candidate kinases may phosphorylate IRF5 directly or may activate an upstream IRF5 kinase. The former option predicts that the candidate kinases interact with IRF5 directly, or as part of a complex.

Immunoprecipitation experiments with overexpressed IRF5 and candidate kinases showed IRF5 bound to PYK2 and MEKK3. Binding assays with over-expressed proteins are useful for detecting interactions between low-affinity binding partners, however ectopic expression

of proteins is usually considered an artificial system and might lead to detection of interactions that are not physiologically relevant. I then focused on the endogenous system in RAW264.7 cells as it is a more physiologically relevant system. PYK2 also displayed a strong endogenous interaction with IRF5 whereas MEKK3 displayed a possible weak interaction that needs repeating. However, this strategy has considerable limitations as the nature of kinase-substrate interactions are weak and transient and its possible that I am not capturing the right timepoint. Moreover, the concentration of MEKK3 antibody may require further optimisation.

Through my studies, I demonstrate that IRF5 is phosphorylated on both serine and tyrosine residues with the TLR4 ligand LPS, and stays phosphorylated when bound to DNA. This is evidenced by the fact that phospho-IRF5 peaks 30 minutes post stimulation and completely disappears at later timepoints. Importantly, this 'disappearance' can be resolved by enzymatic digestion of chromatin-bound IRF5. Although serine phosphorylation is essential for IRF5 activation, thus far relatively little is known about phosphorylation sites on tyrosine residues on IRF5 and their function. Tyrosine phosphorylation on IRF5 has been shown in untreated p53^{-/-} tumour cells which disappeared with CPT-11 treatment (90). Previously, phosphorylation on site Tyr-104 (human/mouse) by BCR-ABL kinase in chronic myeloid leukaemia cells has been linked to negative regulation of IRF5 transcriptional activity (69). Lyn kinase has also been demonstrated to phosphorylate IRF5 at sites Tyr-313 and Tyr-335 (human, isoform v1) but its kinase activity is dispensable for IRF5 inhibition (38). In this chapter, I confirm that IRF5 carboxyl-terminal serine sites are essential for IRF5 transactivation, as well as ubiquitination. However, one caveat of such mutagenesis experiments is that mutations may alter IRF5 conformation in such a way that is unrelated to the loss of phosphorylation. MEKK3 is a serine/threonine kinase, so may target these IRF5 serine site(s). Further research should also focus on the role of tyrosine sites on IRF5 activation as PYK2 a tyrosine kinase may target IRF5 tyrosine site(s). Alternatively, these

kinases may act via an intermediate substrate to activate IRF5, and further work is required to determine whether this is the case.

After optimisation, I have identified suitable lysis condition for subcellular fractionation, where I was able to find a good separation of nuclear and cytoplasmic fraction and found IRF5 within nuclear fractions after LPS stimulation. This will serve as a good readout of IRF5 activation in future experiments. Overall, I now have an idea of the kinetics of LPS-IRF5 signalling in RAW264.7 cells which is longer than usual phosphorylation events. After 60 minutes LPS stimulation IRF5 is phosphorylated on both serine and tyrosine sites, and IRF5 translocates to the nucleus at 120 minutes post-stimulation.

In summary, this section has provided a platform for future experiments in subsequent chapters. I have identified the kinetics of LPS-stimulated IRF5 phosphorylation and optimised the protocol for nuclear subcellular fractionation. Further interrogation of PYK2 and MEKK3 *in vitro* and in *ex vivo* experiments with knockouts and inhibitors will be necessary for determining whether these kinases are regulators of IRF5. Such studies are described in Chapters 4.0 and 5.0 of this thesis.

4.0. PYK2 KINASE VALIDATION

4.1. INTRODUCTION

In the previous chapter PYK2 came out as one of the top IRF5 candidate kinase hits. This data lead to the question of whether PYK2 regulates IRF5 activity. Given that PYK2 and IRF5 are both genetic risk factors for IBD (53, 151), I sought to investigate the role of PYK2 kinase in regulating IRF5 activation during inflammation.

Proline-rich tyrosine kinase 2 (PYK2, also known as PTK2B, FAK2, CAK beta and RAFTK) is a 116 kDa cytoplasmic, non-receptor tyrosine kinase, and is a member of the focal adhesion kinase family, which includes the homologous FAK (focal adhesion kinase). Both kinases share structural and sequence similarity with 48% sequence identity of amino acids, 60% sequence similarity of the central kinase domain, and identical positions of 4 phosphorylation sites. Both kinases comprise of an N-terminal FERM domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain. While FAK is ubiquitously expressed, PYK2 expression is restricted to cells of the central nervous system, epithelial cells and haematopoietic cells.

PYK2 KO mice have previously been described- they are viable and fertile, are phenotypically normal under homeostatic conditions and display no overt abnormalities (152, 153). They have a high bone mass resulting from increased bone formation due to enhanced osteogenesis (154). Several studies have demonstrated an association between PYK2 expression and increased activation in several cancers including lung, breast, gastrointestinal tract, prostate and multiple myeloma (155–160). The role of PYK2 in other conditions including obesity, diabetes, asthma, vasculitis and osteoporosis have also been highlighted (154, 161–163). For instance, PYK2 regulates allergic airway

inflammation, Th2 cytokine secretion, and airway hyperresponsiveness in a mouse model of asthma (161).

Several studies have characterised the cellular roles of PYK2 and have reported the involvement of PYK2 downstream of the integrin family of receptors including cell migration, proliferation and survival via interaction with and/or phosphorylation of key cellular proteins. In macrophages, PYK2 is involved in complement mediated phagocytosis, but is dispensable for Fcγ receptor (FcγR)-mediated uptake (164). PYK2 is involved in macrophage polarisation, membrane ruffling and migration towards sites of inflammation. (152, 165). PYK2 is also involved in the secretion of pro-inflammatory cytokines in macrophages. LPS primed macrophages secrete IL-1β and IL-18 which is blocked upon PYK2 inhibitor treatment (165). PYK2 was later shown to directly phosphorylate the inflammasome adaptor protein ASC leading to caspase-1 activation and secretion of IL-1β and IL-18 (166). In neutrophils, PYK2 plays a role in neutrophil degranulation and migration (167). Several PYK2 binding partners and substrates have been identified and characterised including Src, ASC, MyD88 and paxillin (166, 168–170).

As with other kinases, PYK2 undergoes self-activation by autophosphorylation. Following activation, PYK2 autophosphorylation on tyrosine 402 (pY402) creates a binding sites for Src family kinase (SFK). Recruited SFK can then phosphorylate additional tyrosine sites within PYK2, to further enhance PYK2 enzymatic activity and provide additional docking sites for Src Homology 2 (SH2) domain containing proteins. Thus, PYK2 autophosphorylation at Y402 is a reporter of PYK2 activity.

Small molecule inhibitors of PYK2 including PF-431396 and Defactinib (VS-6063, PF-04554878), have been developed. PF-431396 displays dual inhibitory activity for both PYK2 and FAK with a 50% inhibitory concentration (IC₅₀) of 11nM and 2nM, respectively. PF-431396 is not absolutely selective with inhibition of other inflammatory kinases (JAK3,

TrkA, Aur2) at higher concentrations (171). Defactinib is an oral small molecule, potent ATP competitive, reversible inhibitor of PYK2 and FAK with an IC_{50} of 0.6 nM for each kinase and more than 100-fold greater selectivity than other non-target kinases.

Defactinib has extensively been tested and met all the necessary drug inhibitor criteria including potency, selectivity within target family, selectivity outside target family, suitable pharmacokinetic profile and *in vivo* efficacy. In mice ovarian cancer models, oral administration of defactinib in combination with paclitaxel (PTX) reduced tumour weight (172). Defactinib is currently under investigation in phase I and II clinical trials (Clinical trial numbers; NCT01870609, NCT01778803, and NCT02004028). For example, oral administration of defactinib in a phase I study, was shown to have an acceptable safety profile and mild reversible adverse events in humans with advanced solid tumour malignancies (173, 174).

4.2. RESULTS

4.2.1. PYK2 knockouts reduce TNF-reporter activity

Based on the validation rounds in Chapter 3.0, PYK2 came up as a positive hit and may be involved in IRF5 activation. To address the hypothesis that PYK2 is required for IRF5 dependent signalling, I performed the IRF5 driven TNF-luciferase reporter assay in WT and PYK2 knockout RAW264.7 cells. Cells were transfected with IRF5/empty pBent2 vectors and luciferase reporters and stimulated the following day with LPS for 6 hours. Upon ectopic expression of IRF5 and activation by LPS, WT cells exhibit increased TNF reporter activity, whereas TNF-reporter activation was significantly reduced in IRF5 expressing cells lacking PYK2 (**Figure 4.1**). No differences were observed in cells expressing the empty control vector suggesting that activation of the TNF-reporter is due to the presence of IRF5. Overall, these results highlights the importance of PYK2 in targeting IRF5 to control TNF-transactivation.

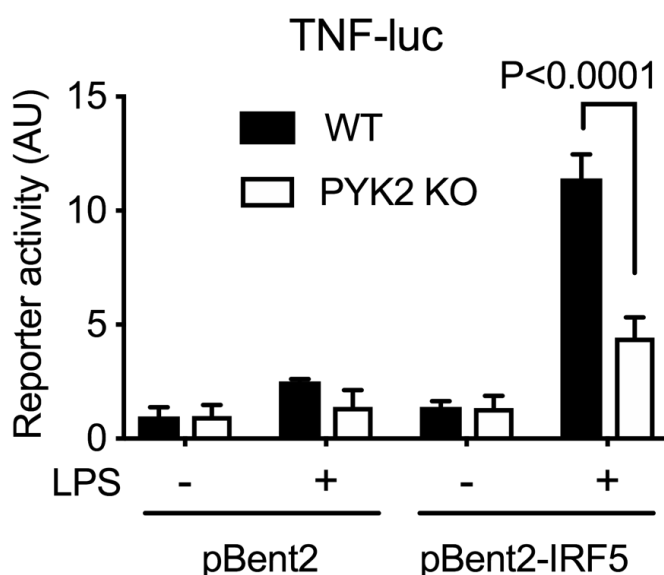


Figure 4.1. IRF5 TNF-reporter of PYK2 KOs WT and PYK2 KO RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation) in a 96-well polylysine coated plate. Following an overnight incubation at 37°C, cells were stimulated with LPS (1 µg/ml) for 6 hours. Firefly and Renilla luciferase activity was measured on the FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of n=3 independent experiments, with each experiment performed in triplicates. Statistical analysis by unpaired Student's t-test.

4.2.2. PYK2 deletion reduces pro-inflammatory cytokine gene expression

IRF5 is involved in the production of pro-inflammatory cytokines and chemokines by binding to the promoter regions of target genes. To determine whether PYK2 must be present for macrophages to produce pro-inflammatory cytokines/chemokines, I next investigated the effect of knocked-out IRF5 or PYK2 levels on gene expression of several IRF5-regulated cytokines and chemokines after the addition of LPS at several timepoints. IRF5 directly induces expression of the pro-inflammatory cytokines *Il1a*, *Il1b*, *Il6* and *Tnf*, whilst suppressing the anti-inflammatory cytokine *Il10* (24, 46). Additionally, *CCL4*, *CCL5*, *CCL2* and *CXCL1* are chemokines all known to be regulated by IRF5 (50). While the expression of pro-inflammatory cytokines (*Il1a*, *Il1b*, *Il6*) and chemokines (*CCL4*, *CCL5*) in response to TLR4 agonist at 4 hours were high in WT cells, levels were significantly reduced in IRF5^{-/-} and PYK2^{-/-} RAW264.7 cells at this timepoint (**Figure 4.2**). Interestingly, expression of these genes was depleted further in PYK2 KO compared to IRF5 KO. This highlights its role as a kinase that may target other pathways besides IRF5. *Tnf* expression was also reduced in

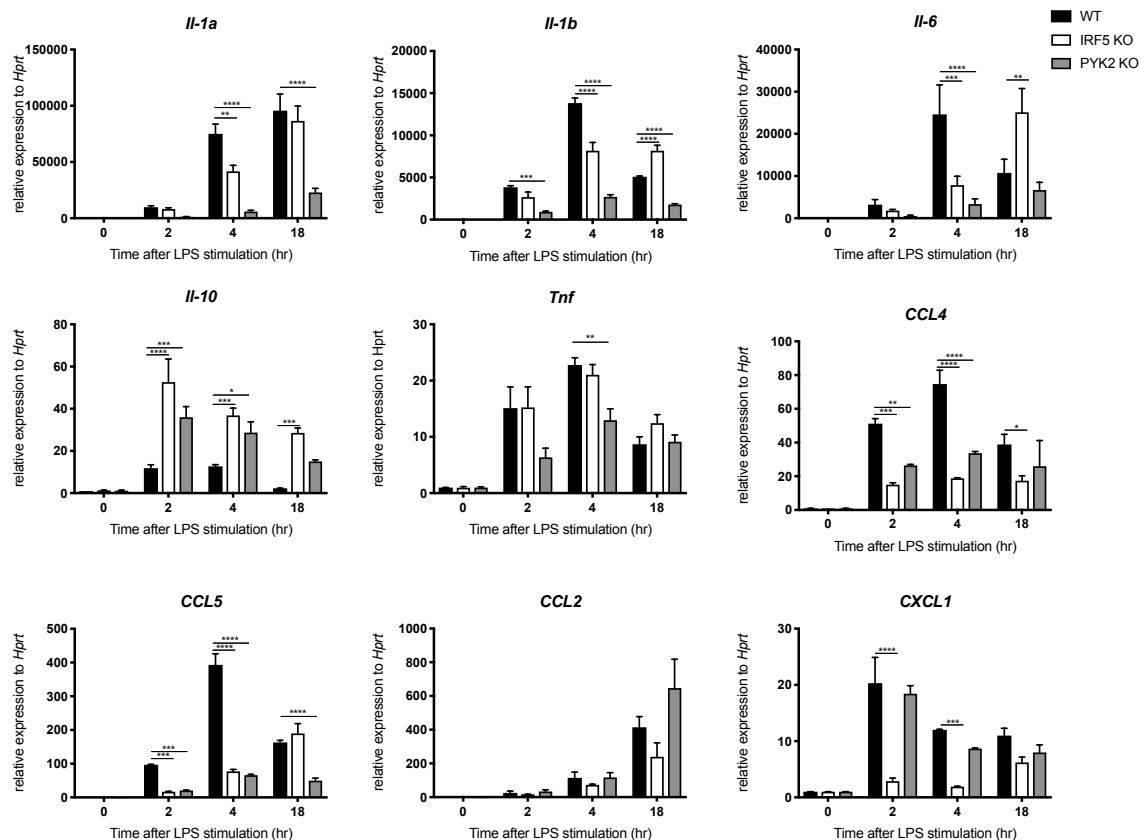


Figure 4.2. Cytokine and chemokine profiles of LPS-stimulated RAW264.7 cells WT, IRF5 knockout, and PYK2 knockout RAW264.7 cells (1×10^6) were stimulated with LPS (500 ng/ml) for 2, 4, and 18 hours. RNA was extracted and 1 μ g (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il1a*, *Il1b*, *Il6*, *Il10*, *Tnf*, *CCL4*, *CCL5*, *CCL2*, *CXCL1* and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data shown as means $\pm$ SEM of three independent experiments conducted in duplicates. Statistical analysis was performed by 2-way ANOVA and Bonferroni's multiple comparison $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, $****p \leq 0.0001$.

PYK2 KOs at 4 hours LPS stimulation, with no differences in WT and IRF5 KOs. Conversely, LPS-induced levels of the anti-inflammatory cytokine *Il10* was increased in both KO cells compared to WT at 2 and 4 hours. Compared to WT, LPS-induced *CXCL1* expression levels was reduced in IRF5 KOs at 2 and 4 hours, but LPS induced responses were similar between WT and PYK2 KO cells at all timepoints. Although, *CCL2* expression was consistently reduced in LPS-stimulated IRF5 KO cells, the difference was not significant and levels were similar between WT and PYK2 KO cells. Differences at later overnight timepoints may reflect death of cells as they appeared unhealthy with longer LPS treatment. Overall, these results highlight that PYK2 plays an important role in LPS-stimulated *Il1a*, *Il1b*, *Il6*, *Il10*, *Tnf*, *CCL4* and *CCL5* gene expression in macrophage RAW264.7 cells where PYK2^{-/-} cells phenocopies IRF5^{-/-} cells. In contrast, *CCL2* and *CXCL1* may not require PYK2, and the loss of PYK2 may be compensated for by other pathways.

IRF5 plays a role in immunopathology during *Helicobacter hepaticus* (*Hh.*)-induced colitis, as well as fungal infection (55, 65). I then assessed the involvement of PYK2 downstream of other agonists including the *Hh*-bacterial pathogen, or the Dectin-1 only agonist whole glycan particles (WGP) in WT, IRF5^{-/-} and PYK2^{-/-} RAW264.7 cells over a time course. *Hh.* stimulation induced levels of *Il1a* at all timepoints, whereas levels of *Il6* and *Il10* were only induced at overnight timepoints (**Figure 4.3**). *Il1a* levels were impaired in IRF5^{-/-} and PYK2^{-/-} cells, whereas expression levels of *Il6* and *Il10* were not further affected in the KO cell lines. In response to WGP, WT cells produce high level of *Il10*, which was further increased in IRF5 and PYK2 knockout cells at 4 and 18 hours post stimulation (**Figure 4.4**). Levels

of *Il6* and *Tnf* were similar in all cell lines at all timepoints. Overall, LPS stimulation seemed to have the most profound effect on cytokine and chemokine induction, whereas *Hh*. and WGP stimulation affects cytokine levels less robustly.

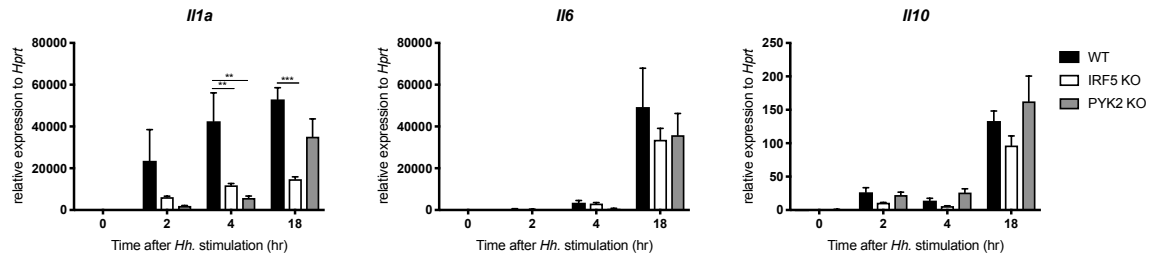


Figure 4.3. Cytokine profile of Hh-stimulated RAW264.7 cells WT, IRF5 knockout, and PYK2 knockout RAW264.7 cells (1×10^6) were stimulated with Hh (ng/ml) for 1, 2, 4, and 18 hours. RNA was extracted and 1 μ g (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il1a*, *Il6*, *Il10* and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data shown as means $\pm$ SEM of three independent experiments conducted in duplicates. Statistical analysis was performed by 2-way ANOVA and Bonferroni's multiple comparison ** $p \leq 0.01$, *** $p \leq 0.001$.

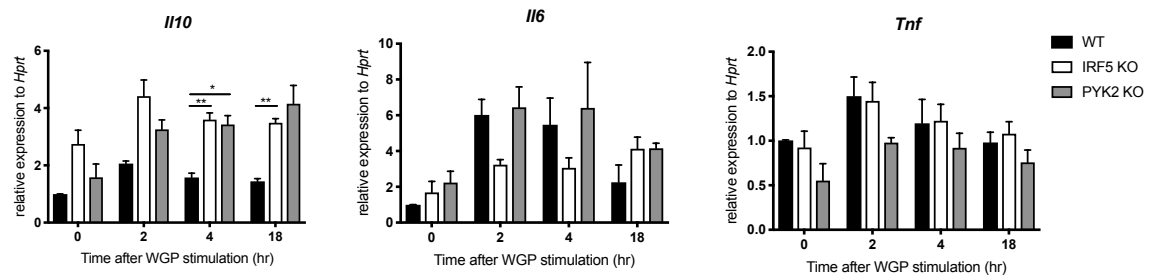


Figure 4.4. Cytokine profile of WGP-stimulated RAW264.7 cells WT, IRF5 knockout, and PYK2 knockout RAW264.7 cells (1×10^6) were stimulated with WGP (100 μ g/ml) for 1, 2, 4, and 18 hours. RNA was extracted and 1 μ g (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il10*, *Il6*, *Tnf* and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data shown as means $\pm$ SEM of three independent experiments conducted in duplicates. Statistical analysis was performed by 2-way ANOVA and Bonferroni's multiple comparison * $p \leq 0.05$, ** $p \leq 0.01$.

4.2.3. PYK2 deficiency impacts IRF5 recruitment to target gene promoters

As the TNF reporter activity relies on other transcription factors including NF- κ B, I next investigated whether PYK2 deficiency would directly impact IRF5 function. The *Udalova* lab have previously shown that upon LPS stimulation IRF5 is recruited directly to target gene promoters and enhancer regions in GM-BMDMs (24, 45). I examined the binding of IRF5 to the promoters of IRF5 target genes (*Il6*, *Il1a* and *Tnf*) in WT and PYK2 KO cells by

chromatin immunoprecipitation (ChIP) assays as a readout of PYK2-dependent IRF5 activation. An RNA polymerase II (Pol II) antibody and IgG isotype antibody was also included in the ChIP assay. As expected, the ChIP-quantitative PCR (ChIP-qPCR) results show increased IRF5 binding to the *Il6*, *Il1a* and *Tnf* gene promoters in WT cells following 2 hours LPS stimulation (**Figure 4.5**). Likewise, recruitment of Pol II to these gene loci was

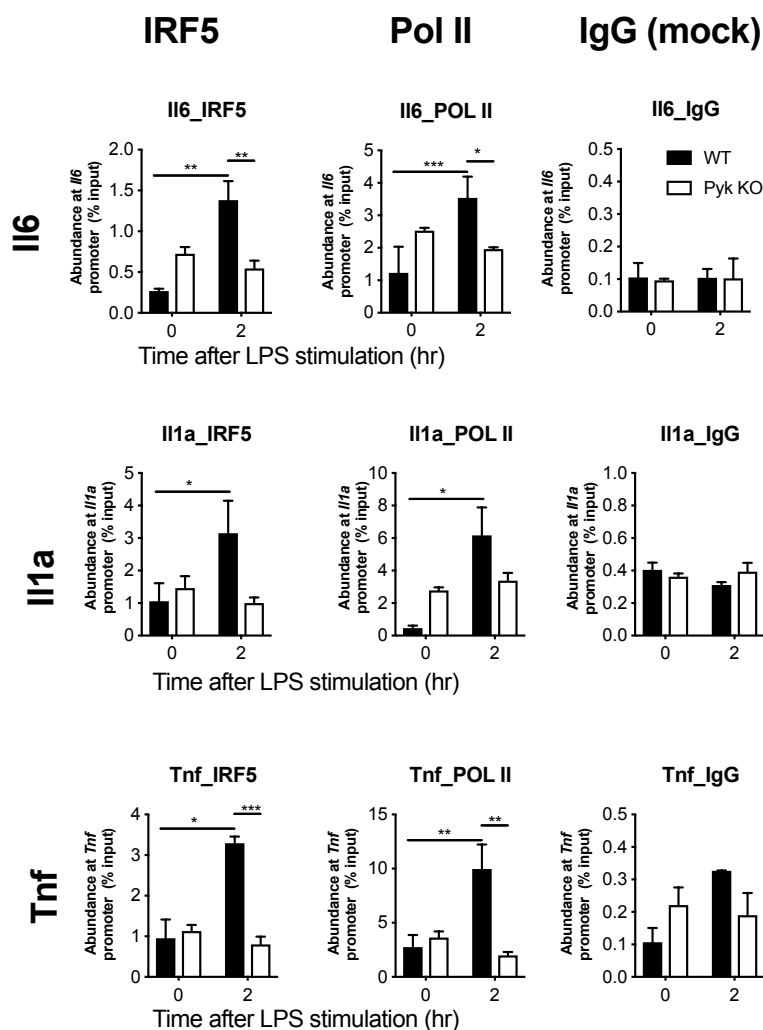


Figure 4.5. Recruitment of IRF5 to *Il6*, *Il1a* and *Tnf* promoters in WT and PYK2 KO cells Resting or LPS-treated (500 ng/ml, 2 hours) wild type PYK2 KO RAW264.7 cells were fixed in formaldehyde. Cells were lysed with cytoplasmic lysis buffers LB1 then LB2, followed by nuclear lysis buffer LB3 with added protease inhibitors. The nuclear lysate was sonicated for 10 minutes (30s on/30s off cycle) at 4°C to obtain chromatin fragment size of 500 bp. Lysates were incubated at 4°C with protein G magnetic beads that had been pre-incubated with antibodies specific to IRF5, Pol II or IgG isotype control. After an overnight incubation protein-DNA complexes were washed 6 times with ice-cold ChIP wash buffer and complexes eluted with 120 µl ChIP elution buffer. Samples were reversed cross-linked overnight at 65°C, along with non-immunoprecipitated lysate for 'whole cell extract' input control, and the DNA was purified from the samples. ChIP-qPCR reactions were performed in duplicates using primers designed against the *Il6*, *Il1a* and *Tnf* gene promoter using SyBR green master mix. Data was normalised against chromatin amount in lysates and expressed as percentage of input for each gene. Data shown as mean values +/- SEM from n=3 experiments. Comparison by two-way ANOVA *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

observed at this timepoint. Whereas, IRF5 recruitment to these gene promoters was impaired in PYK2 deficient cells. Combined with the reporter assay, this data suggests that PYK2 acts as a critical regulator of IRF5-dependent transcription and inflammatory responses induced by LPS.

4.2.4. PYK2 phosphorylates IRF5 at tyrosine site 171 (mouse)

I then sought to clarify the mechanism by which PYK2 suppresses the transcriptional activity of IRF5. PYK2 belongs to a family of non-receptor tyrosine kinases (175), but the role of tyrosine phosphorylation on IRF5 activity has not yet been fully elucidated. Although in Chapter 3, overexpressed PYK2 was capable of phosphorylating IRF5, I aimed to test if this was the case in an endogenous setting. It is well known that the overexpression of kinases in cells can force the phosphorylation of proteins that are not physiological substrates so it is important to test in the endogenous system. PYK2 is expected to target tyrosine residues but no phospho-tyrosine IRF5 specific antibodies have been generated. To circumvent this, I used phospho-proteomics to characterise potential PYK2 dependent target sites on IRF5. IRF5 was immunoprecipitated from the lysates of LPS-stimulated WT and PYK2 knockout RAW264.7 macrophages. The eluents were separated by gel electrophoresis, stained by colloidal blue staining, and 10% eluent was reserved for western blot analysis (**Figure 4.6a**). The colloidal blue stain shows a distinct band (indicated in red) just above the immunoprecipitating antibody heavy chain band (52kDa) in WT and PYK2 knockout cells immunoprecipitated with IRF5 (**Figure 4.6b**). This band was absent in cells immunoprecipitated with the isotype antibody control and likely corresponds to IRF5. Likewise, the immunoblot shows that IRF5 and phospho-tyrosine was detected in lysates immunoprecipitated with IRF5 cells but absent in the IgG isotype control lane (**Figure 4.6c**). Although the levels of phosphotyrosine on IRF5 are similar in LPS-stimulated WT and KOs, PYK2 cannot be excluded as a kinase that phosphorylates IRF5. It is possible that PYK2

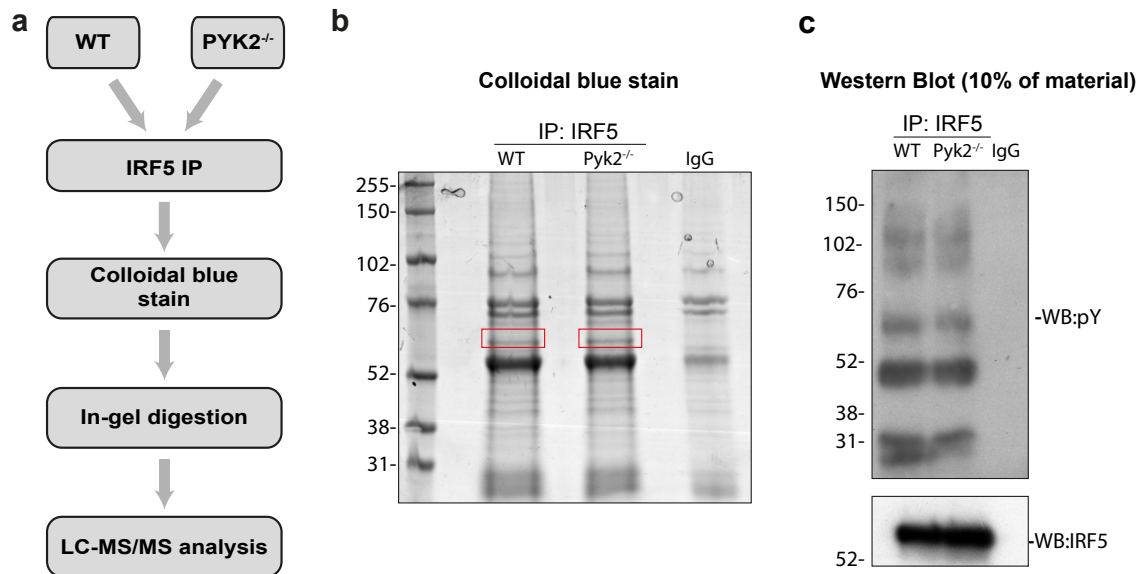


Figure 4.6. IRF5 immunoprecipitation in WT and PYK2 KO cells (a) In-gel digestion of IRF5 overview (b) 1×10^8 RAW264.7 cells were plated and incubated overnight. Media was replaced with serum-free media for 30 minutes to reduce background phosphorylation levels, then stimulated with LPS (500 ng/ml) for 2 hours. Cells were lysed on ice with whole cell lysis buffer (with added protease and phosphatase inhibitors and benzonase nuclease). Lysates were precleared by centrifugation prior to incubation with IRF5 antibody or Rabbit IgG isotype control for 2 hours rotating at 4°C. Pre-washed rabbit trueblot IP beads were added and incubated overnight. Immunocomplexes were washed 3x with chilled lysis buffer by rotating for 5 minutes, pelleting the beads by centrifugation and removing the supernatant. Immunocomplexes were eluted from the column by heating to 100°C in Laemmli buffer and concentrated by Vivaspin column. 90 % was resolved by SDS-PAGE and subjected to colloidal blue staining to visualised protein of the samples. Bands were excised, trypsinised, and the resulting peptide identified by LC-MS/MS at NDM. (c) 10 % was resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting with IRF5 and phospho-tyrosine antibodies. WB = Western blot, IP = immunoprecipitation.

may phosphorylate on a single site which is too crude to detect any differences by WB. Alternatively, the absence of PYK2 may be compensated by other kinases.

The indicated bands in the colloidal stain were excised, subjected to in-gel digestion and peptide masses and quantities were analysed by ultra high pressure liquid chromatography coupled with mass spectrometry (TDI, Oxford). Proteins corresponding to the peptides were search against the MASCOT database. Although, IRF5 came out as the top protein hit with a high sequence coverage of over 90%, no phosphorylation sites were identified (**Supplementary Figures S2-S3**). One explanation for this is that during excision of gel bands, phospho-IRF5 may have been excluded as it runs as a heavier slower migrating

band. Although the risk of other protein contaminants is likely, excising a bigger gel band area would overcome this issue. Another explanation for the absence of phosphorylation PTMs is that the low stoichiometry of phosphopeptides may be difficult to detect by mass spectrometry. To circumvent these issues, the experiment was repeated, but instead of loading the eluents on an SDS-PAGE gel for colloidal blue staining, the eluents were directly processed for in-solution digestion. This was followed by a phosphopeptide enrichment step on titanium dioxide columns (TiO₂) to improve phosphorylation detection. Peptide masses and quantities were analysed by nano ultra high pressure liquid chromatography coupled with mass spectrometry (nULPC-MS/MS) (**Figure 4.7a**). The mass spectrometry for the in-solution digestion displayed a lower sequence coverage than the previous in-gel digestion with 27% and 31% for WT and PYK2^{-/-} cells, respectively suggesting lower recovery of material (**Figure 4.7b**). WT LPS-stimulated cells displayed 5 phosphorylated sites on Y171, S172, S300, Y334, S445 (mouse IRF5), and PYK2 KO LPS-stimulated cells displayed 6 phosphorylated sites including S56, S172, S300, Y312, Y334, S445 (**Figure 4.7b**, **Supplementary Figure S4**).

Figure 4.7c shows a comparison of the phosphorylated sites on IRF5 in WT and PYK2^{-/-} LPS-treated cells. In line with previously published reports, the IKK β -dependent site S445 (mouse) was identified in both WT and PYK2 KO cells (95, 96). Residue Y171 was only found in LPS-stimulated WT cells, whereas residues S56 and Y312 were only found in LPS-treated PYK2^{-/-} cells. A possible explanation for novel sites appearing in LPS stimulated cells in the absence of PYK2 is that PYK2 phosphorylation of IRF5 may affect IRF5 modifications by other enzymes. This would also explain why the previous immunoblot (**Figure 4.6c**) showed similar levels of IRF5 phosphotyrosine in WT and PYK2 KOs. In fact, Src tyrosine kinase Lyn was capable of phosphorylating IRF5 at orthologues of Y312 and Y334 sites in *in vitro* co-expression system (38). Interestingly, Tyr-171 phosphorylation was only detected in WT cells (**Figure 4.7d**), thus PYK2 is likely to target this tyrosine site on IRF5.

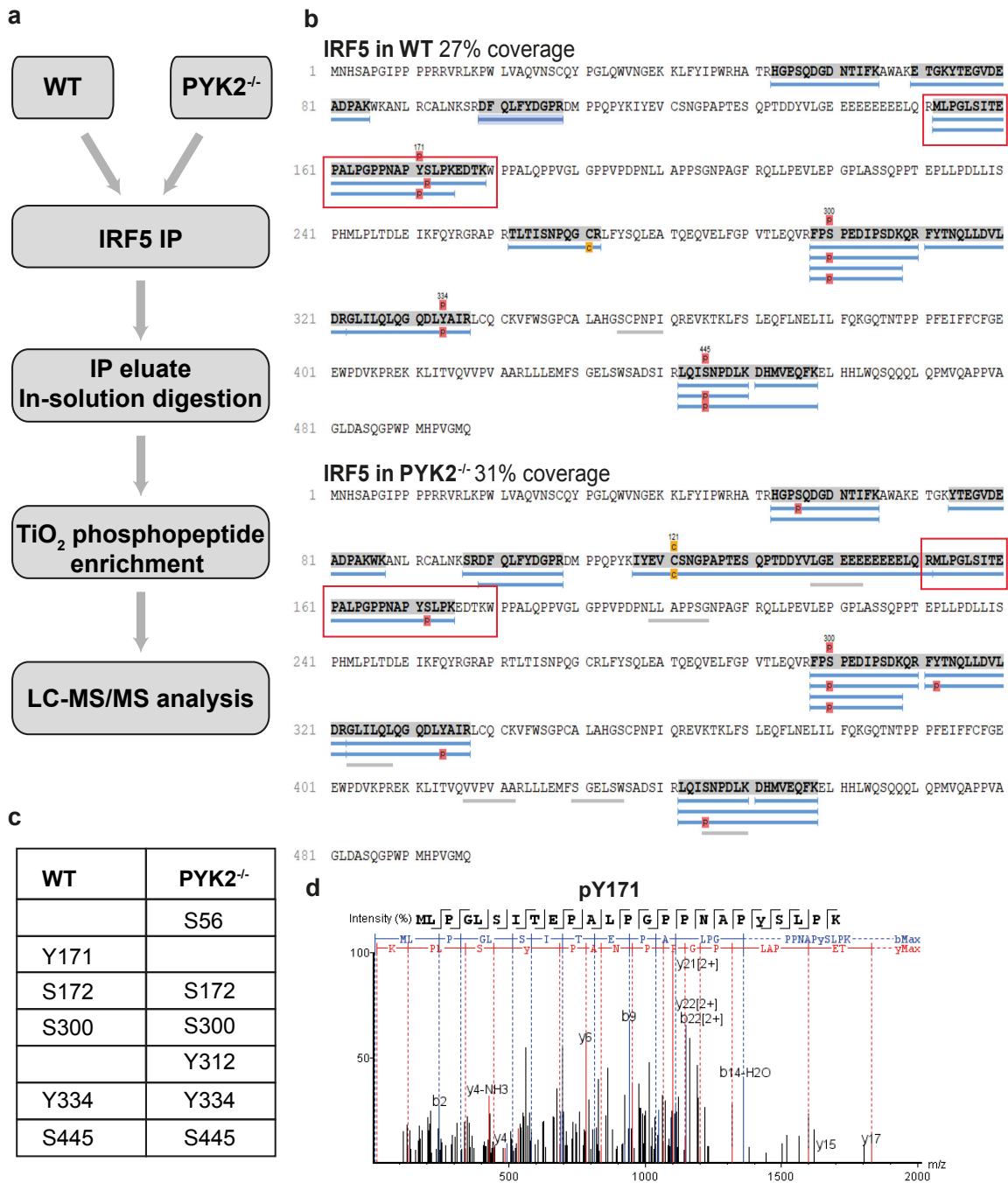


Figure 4.7. IRF5 phosphorylation in WT and PYK2 KO RAW264.7 cells (a) In-solution digestion of IRF5 overview. Lysates from LPS-stimulated WT and PYK2 KO cells were immunoprecipitated with IRF5 antibody. The eluate was sampled for in-solution digestion, phosphopeptide enriched on TiO₂ columns and processed by LC-MS/MS. (b) PEAKS SPIDER search analysis of peptides and post-translational modifications of mouse IRF5 from WT (top panel) and PYK2 KO cells (lower panel) in which the peptides identified by LC-MS/MS are underlined by a blue line. Each line corresponds to a unique MS/MS spectrum. Post-translational modifications including cysteine carbamidomethylation (orange box) and phosphorylation (red box) are indicated. The location of pTyr-171 and pSer-172 observed in WT IRF5 are marked in a red box. (c) Table comparison of phosphorylation sites in WT and PYK2 knockouts (d) MS/MS spectrum of the IRF5 derived tryptic peptide 152-179 indicating phosphorylation sites at position Y171. Fragmentation ions of the b- and y- series are indicated in blue and red, respectively. Spectra of other sites in **Supplementary Figure S4**.

4.2.5. Site Y171 important for IRF5 transactivation

Phosphorylation is thought to be the critical modification for IRF5-dependent activation, but little is known about tyrosine phosphorylation sites on IRF5. To address this question, I generated loss-of-function mutations. IRF5 tyrosine residues identified in the MS experiment (mouse IRF5 Tyr-171, Tyr-312, Tyr-334) as well as the published Tyr-104 site (69) were mutated to phenylalanine, and I explored the consequence of these mutations in the TNF-reporter assay in HEK293-TLR4 cells (mouse and human IRF5 site comparisons in **Figure 4.8a**). A double mutant Y172F, S173A (Y171, S172 of mouse IRF5) was also

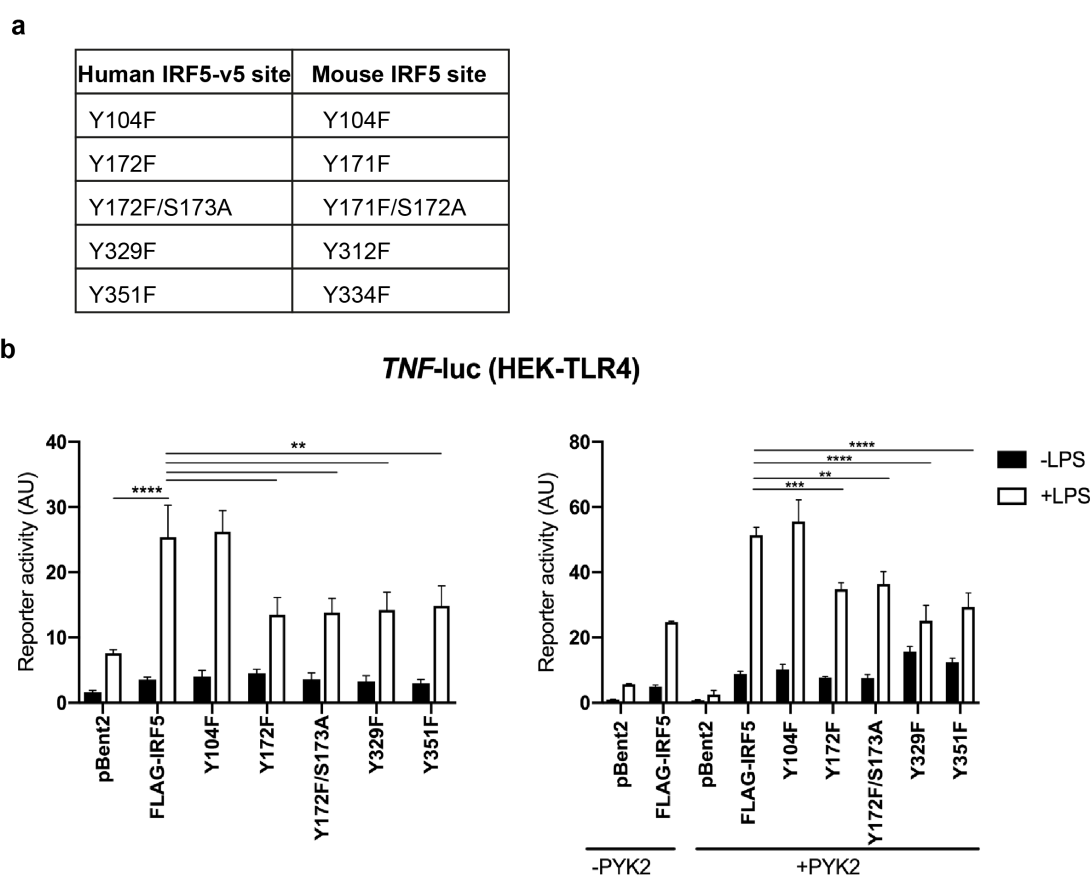


Figure 4.8. Tyrosine sites involved in IRF5 activation (a) IRF5 point mutants, containing tyrosine-phenylalanine and serine-alanine substitutions (human IRF5 isoform v5) used in this study and equivalent mouse site. (b) The activity of WT IRF5 or mutant IRF5 (Y104F, Y172F, Y172F/S173A, Y329F, Y351F) on the TNF-luciferase reporter assay was evaluated in the presence or absence of PYK2 in HEK-TLR4 cells. Following 24 hours-post transfection, cells were stimulated for 0/6 hours with 100 ng/ml LPS. Firefly and Renilla luciferase levels were measured by Dual Glo luciferase assay using a FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Data are representative of three independent experiments each performed as triplicates. Bars show standard error of the mean, and are expressed as firefly luciferase relative to renilla luciferase levels where ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

included as residue S173 (human v5 IRF5) was identified as a phosphorylation site in the mass spectrometry experiment. IRF5-dependent activation of the TNF-reporter gene correlated with a requirement for tyrosine at position 172, 329 and 351 of human v5 IRF5 (171, 312, and 334 of mouse IRF5), whereas the Y104F mutant induced reporter activity to similar levels as the FLAG-IRF5 control (**Figure 4.8b**). In addition, the transcriptional activation of the IRF5 Y172, S173A double mutant was reduced to similar levels to the single Y172F mutant.

To further elucidate whether IRF5 tyrosine sites are targeted by PYK2, a myc-tagged PYK2 expression plasmid was co-expressed with WT IRF5/mutant IRF5/empty vectors and luciferase constructs (**Figure 4.8b**). PYK2 co-expression increased reporter activity two-fold. Transfection with mutants Y172, Y329, and Y351 (mouse IRF5 Tyr-171, Tyr-312, Tyr-334) again reduced reporter activity whereas Y104 had no further effect. Again, the double mutant Y172F, S173A diminished ability to activate the TNF-luciferase reporter to similar levels as the single Y172F mutant. Together, these results indicate that PYK2 mediates LPS-induced activation of IRF5 by phosphorylating the tyrosine site Y171 of mouse IRF5 and possibly indirectly affecting sites Y312 and Y334.

4.2.6. PYK2 inhibitors

PYK2 has been considered an attractive drug target due to its physiological relevance in cancer and neurological disorders amongst many other diseases (176–178). Recently, specific inhibitors of PYK2 and a related kinase FAK have been developed (179). One of them called PF-431396 has dual inhibitory activity for both PYK2 and FAK but not absolutely selective with inhibition of other inflammatory kinases (JAK3, TrkA, Aur2) at higher concentrations (171). Another compound called defactinib (also known as VS-6063 or PF-04554878) has been successfully used to tackle cancer in a mouse model and is currently being tested in clinical trials (180, 181). In this section, I explore the efficiency of

inhibitors to PYK2 and their effect on IRF5-dependent pro-inflammatory gene expression in macrophages.

4.2.6.1. RAW264.7 cell viability

The PYK2 inhibitors defactinib (Def) and PF-431396 (PF) were titrated from 0.1-10 μM and the cell viability of RAW264.7 cells were tested by CTG viability assay to establish the concentration range well tolerated by macrophages. Cells were pre-treated with either PYK2 inhibitors or DMSO vehicle control for 1 hour, followed by LPS for a further 6 hours. The concentration to inhibit 50% of cell viability (IC_{50}) was determined based on the curve of the slope. Cells were viable for DMSO vehicle control, whereas defactinib and PF-431396 displayed IC_{50} values of 2.8 and 4.2 μM , respectively (**Figure 4.9**). Based on this data, I chose to treat RAW64.7 cells at an inhibitor concentration of $\leq 1\mu\text{M}$ as an initial starting point to study the IRF5-dependent response of cells to PYK2 inhibition.

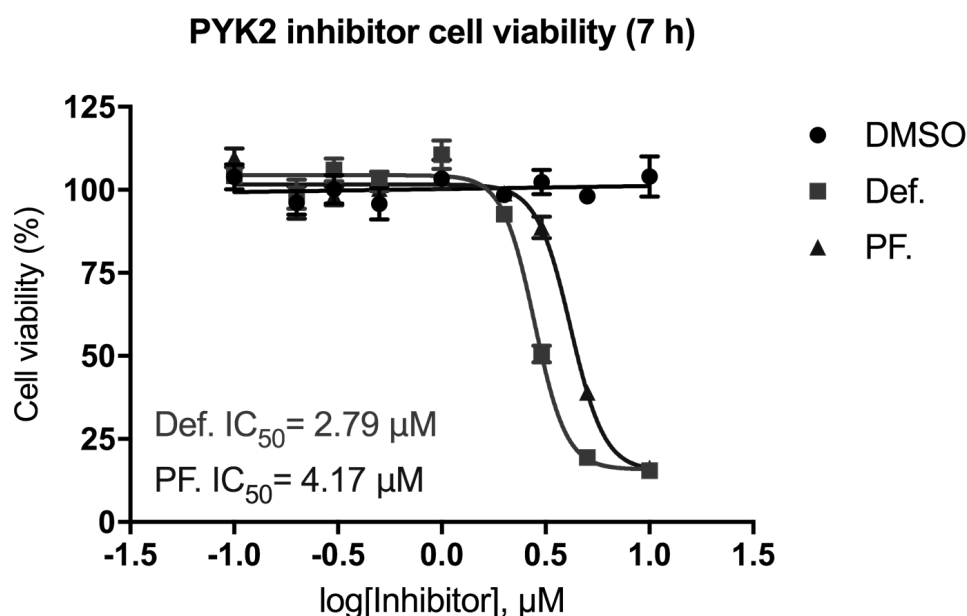


Figure 4.9. Cell viability of RAW264.7 cells with PYK2 inhibitor treatment RAW264.7 cells (5×10^5) plated in triplicates were stimulated with Defactinib (Def), PF (PF-431396) or DMSO vehicle control at indicated concentrations ($\log_{10}$ transformed) for 7 hours. Cell viability was assessed with the CTG viability assay. Luminescence signals were measured on the FLUOstar Omega microplate reader. Cell viability (%) was measured by normalising luminescence signals from treated cells to untreated cells. Cell viability IC_{50} values for each probe are indicated on the graphs and calculated from the curve of the slope. $n=3$.

4.2.6.2. PYK2 inhibition affects TNF-reporter activity

I explored the effect of both PYK2 inhibitors on TNF-reporter activity in WT, IRF5 KO, and PYK2 KO RAW264.7 cells. Cells were pre-incubated with either inhibitors or the carrier DMSO for 1 hour, then stimulated with LPS for a further 6 hours. An inhibitor concentration of 1 μ M was selected as it was well tolerated by RAW264.7 cells. At this dose both inhibitors effectively inhibited TNF-reporter activity in LPS stimulated WT cells and IRF5 KO cells, in which IRF5 expression was restored via ectopic expression of IRF5 (**Figure 4.10**). This suggests that both inhibitors act in an IRF5-specific manner. To test whether the effects shown by the inhibitors on gene expression was specifically related to PYK2 inhibition and not to a non-specific effect on other pathways, the same experiment was carried out in PYK2 KO cells. Both inhibitors had no further effect on TNF-reporter activity, which suggests that these inhibitors act in a PYK2-specific manner. However, it may be the case that the inhibitors have no effect because reporter activity is low to begin with.

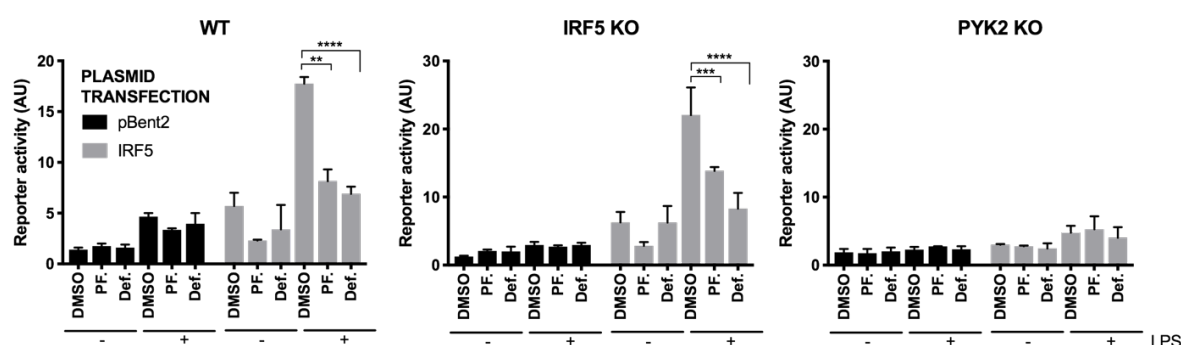


Figure 4.10. TNF luciferase reporter with PYK2 inhibitors WT, IRF5 KOs and PYK2 KOs RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pre-treated with 1 μ M Defactinib (Def), PF (PF-431396) or DMSO vehicle control for 1 hour, followed by LPS (1 μ g/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay using a FLUOstar Omega microplate reader to measure luminescence levels. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of $n=3$, each performed in triplicates. Statistical analysis by unpaired Student's t-test where ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

4.2.6.3. GM-BMDMs cell viability

I wanted to perform my assays in a more relevant biological setting and to do this I decided to focus on primary macrophages (GM-BMDMs). The inhibitors along with a DMSO vehicle

control were titrated in GM-BMDMs at a range of concentrations (0.1-10 μ M) to determine the optimal dose of inhibitor well tolerated by macrophages at 3 hr, 8 hr, and 18 hr (**Figure 4.11**). GM-BMDMs were pre-incubated with either PYK2 inhibitors or DMSO carrier for 1 hour, followed by LPS for a further 2, 7 and 17 hours. No effect on cell viability was observed with DMSO control vehicles at all time points. At 3 hours incubation GM-BMDMs were viable at all concentrations tested for both inhibitors. At 8 hours incubation, both inhibitors displayed toxicity to cells at higher concentrations with cell viability IC_{50} values of 3.56 and 5.12 for defactinib and PF-431396, respectively. Similar IC_{50} values were also obtained at a longer inhibitor incubation period of 18h.

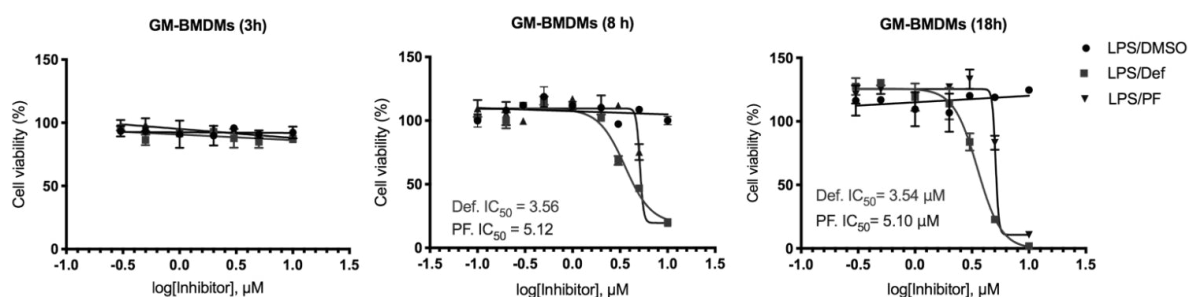


Figure 4.11. Cell viability of GM-BMDMs with PYK2 inhibitor treatment GM-CSF BMDMs (5×10^5 cells) plated in triplicates were stimulated with Defactinib (Def), PF (PF-431396) or DMSO vehicle control at indicated concentrations ($\log_{10}$ transformed) for 1 hour, followed by LPS (100 ng/ml) for a further 2, 7 or 17 hours. Cell viability was assessed with the CTG viability assay. Luminescence levels were measured on the FLUOstar Omega microplate reader. Cell viability (%) was measured by normalising luminescence signals from treated cells to untreated cells. Cell viability IC_{50} values for each probe are indicated on the graphs and calculated from the curve of the slope.

4.2.6.4. PYK2 inhibition reduces cytokine and chemokine levels in macrophages

To assess the effects of PYK2 inhibition on activated macrophages, mRNA expression of IRF5-induced proinflammatory cytokines and chemokines in GM-BMDMs was analysed. To this end, I isolated primary BMDMs from WT mice and cultured GM-CSF macrophages over an 8-day period. GM-BMDMs were treated with PF-431396 or defactinib or DMSO vehicle control for 1 hour at concentrations below the amounts at which the inhibitors start affecting cell viability, then stimulated with LPS for a further 2 hours. Then I extracted the mRNA and evaluated the effect of the inhibitors on LPS-induced gene expression by qPCR.

Consistent with the *Udalova* labs previous findings LPS treatment of GM-BMDMs induced *Il1a*, *Il1b*, *Il6*, *Il10*, *Il12b*, *Il23a*, *Tnf*, *CCL2*, *CCL4*, *CCL5*, and *CXCL1* levels (**Figure 4.12**). The expression of proinflammatory cytokines and chemokines in response to LPS was downregulated in inhibitor treated macrophages when compared to that in LPS-stimulated only cells. Interestingly, *Il10* levels were reduced in PYK2-inhibitor treated cells, whereas I

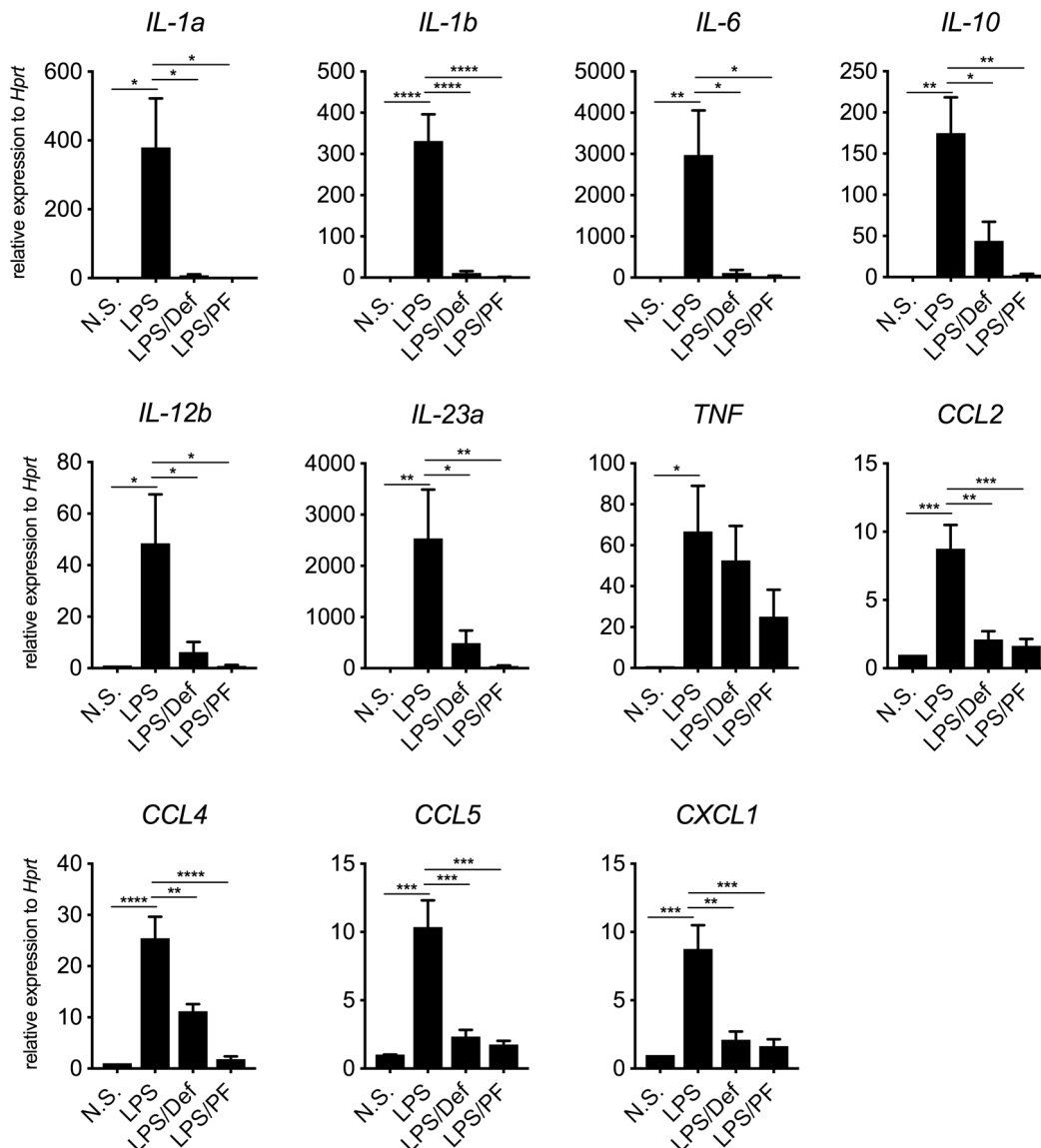


Figure 4.12. Gene expression of PYK2 inhibitor treated macrophages in response to LPS Cytokine and chemokine levels profiles of GM-BMDMs pre-treated with Defactinib (Def) at 3.5 μ M or PF-431396 (PF.) at 6 μ M for 1 hour, followed by LPS (100 ng/ml) stimulation for 2 hours. RNA was extracted and 150 ng (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il1a*, *Il1b*, *Il6*, *Il10*, *Il12b*, *Il23a*, *Tnf*, *CCL2*, *CCL4*, *CCL5*, and *CXCL1* and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta$ Ct-method. Data are shown as means $\pm$ SEM for n=4 individual mice (triplicate wells per mouse) and analysed by one-way ANOVA * P <0.05, ** P <0.01 *** P <0.001, **** P <0.0001

have previously shown (**Figure 4.2**) that PYK2 KO cells display increased levels of *Il10*. It is likely that both inhibitors target other pathways that may affect *Il10* levels.

I next studied the effect of the inhibitor on gene expression in response to other agonists including the *Hh*- bacterial pathogen, and the dectin-1 only agonist WGP in GM-BMDMs. *Hh*. stimulation induced expression of pro-inflammatory cytokines *Il1a*, *Il1b*, *Il6*, *Il23a* and *Tnf*, whereas inhibitor pre-incubation impaired *Hh*-stimulated gene responses (**Figure 4.13a**). Likewise, WGP induced levels of *Il6*, *Il12b*, *Il23a*, *CXCL3* and *Tnf* were reduced in inhibitor treated macrophages compared to WGP only stimulated cells (**Figure 4.13b**). Overall, these results show that PYK2 inhibition effects cytokine and chemokine gene expression in response to IRF5 activating agonists.

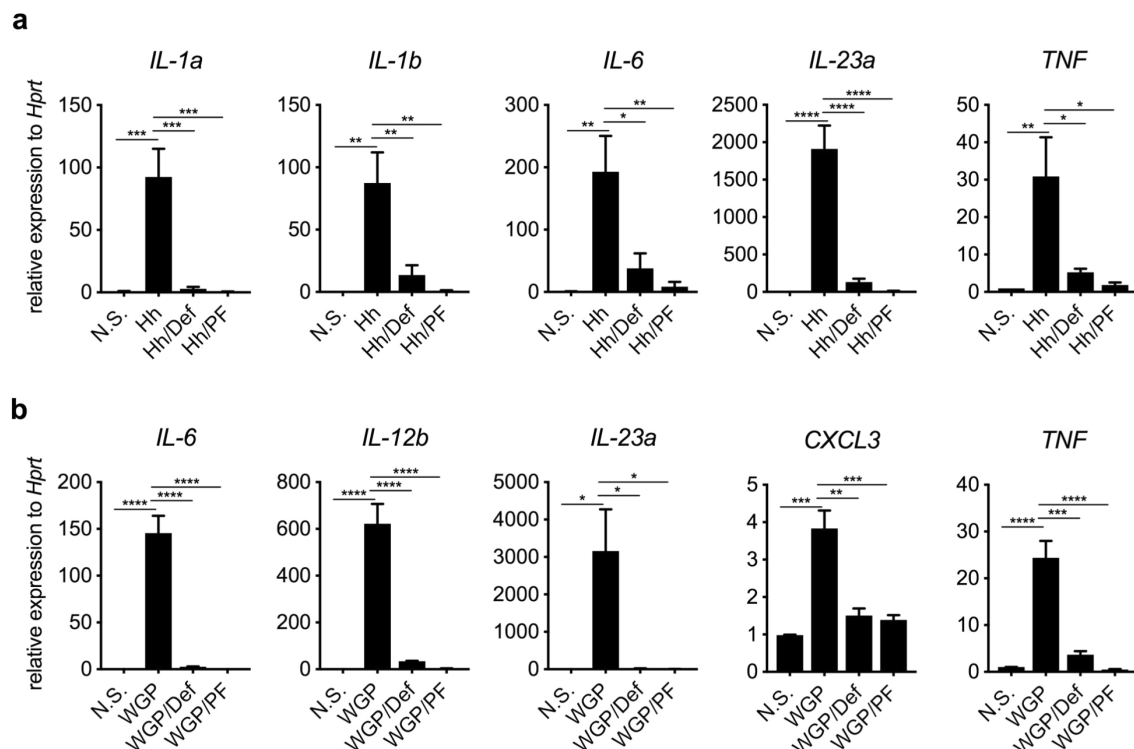


Figure 4.13. Gene expression of PYK2 inhibitor treated macrophages in response to *Hh* or WGP Cytokine and chemokine levels profiles of GM-BMDMs pre-treated with Defactinib (Def) or PF-431396 (PF.) for 1 hour, followed by (a) *Hh* or (b) WGP (100 µg/ml) stimulation for 2 hours. RNA was extracted and 150 ng (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il1a*, *Il1b*, *Il6*, *Il23a*, *Tnf*, *Il12b*, *CXCL3* and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data are shown as means $\pm$ SEM for n=4 individual mice (triplicate wells per mouse) and analyzed by one-way ANOVA * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

To confirm the role of PYK2 in cytokine production, GM-BMDMs were pre-incubated with defactinib, PF-431396 or DMSO vehicle carrier for 1 hour, stimulated for a further 7 hours with LPS, and levels of IL12p70 in the cell supernatants were assessed by ELISA method. Defactinib inhibitor concentrations of 1 and 2 μ M, and PF-431396 concentrations of 2 and 4 μ M were selected as these doses did not affect cell viability (**Section 4.2.6.3**). As anticipated LPS stimulation significantly increased secretion of IL12p70 (**Figure 4.14**). Consistent with the gene expression data results, pre-incubation with either PYK2 inhibitors reduced levels of IL12p70 at both concentrations tested. Together, these results demonstrate that PYK2 is critical for production of IRF5-dependent cytokines induced by LPS.

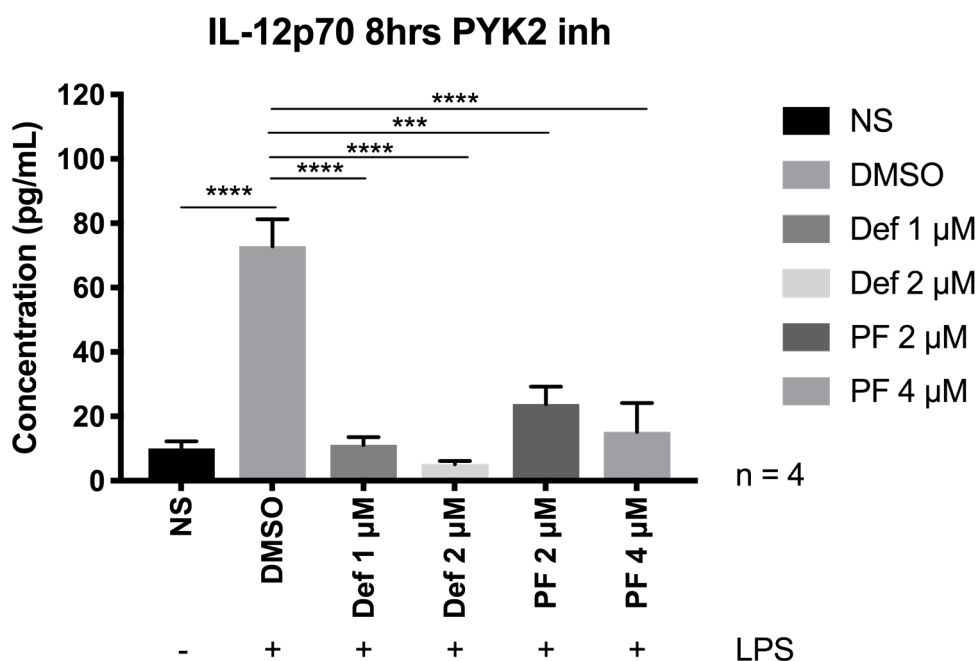


Figure 4.14. Effect of PYK2 inhibitors on IL12p70 protein secretion by GM-BMDMs Mouse GM-CSF BMDMs (1.5×10^5) were seeded into 96-well plates. Cells were pre-treated with inhibitor at indicated concentrations, then stimulated with 100 ng/ml LPS for a further 7 hours. Supernatants were collected and IL12p70 protein secretions measured by IL12p70 ELISA. Absorbance was read at 450nm by a SPECTROstar Omega microplate reader (BMG Labtech). Data represents mean $\pm$ SEM for n=4 individual mice, each performed in duplicates. Comparison by one-way ANOVA ***P<0.001, ****P<0.0001.

4.2.6.5. Defactinib specifically regulates IRF5-mediated transcripts

Although the qPCR inhibitor studies provided insight into the role of PYK2 in regulating TLR-stimulated gene expression, it was still unclear whether defactinib regulated macrophage gene expression profile was IRF5-dependent. To this end, I analysed the gene expression profile of GM-CSF BMDMs taken from WT and *Irf5*-deficient mice where the IRF5 KOs act as a control for IRF5 non-specific inhibitory effects. RNA from GM-BMDMs pre-incubated with defactinib for 1 hour, followed by LPS stimulation for a further 2 hours was collected for qPCR analysis and RNA sequencing. The expression of pro-inflammatory cytokines *Il1a*, *Il1b*, *Il6*, *Il12b* and *Tnf* was induced with LPS stimulation in WT GM-BMDMs, whereas *Irf5*^{-/-} GM-BMDMs generally show minimal expression of proinflammatory cytokines (**Figure 4.15**). As anticipated, the expression levels of all

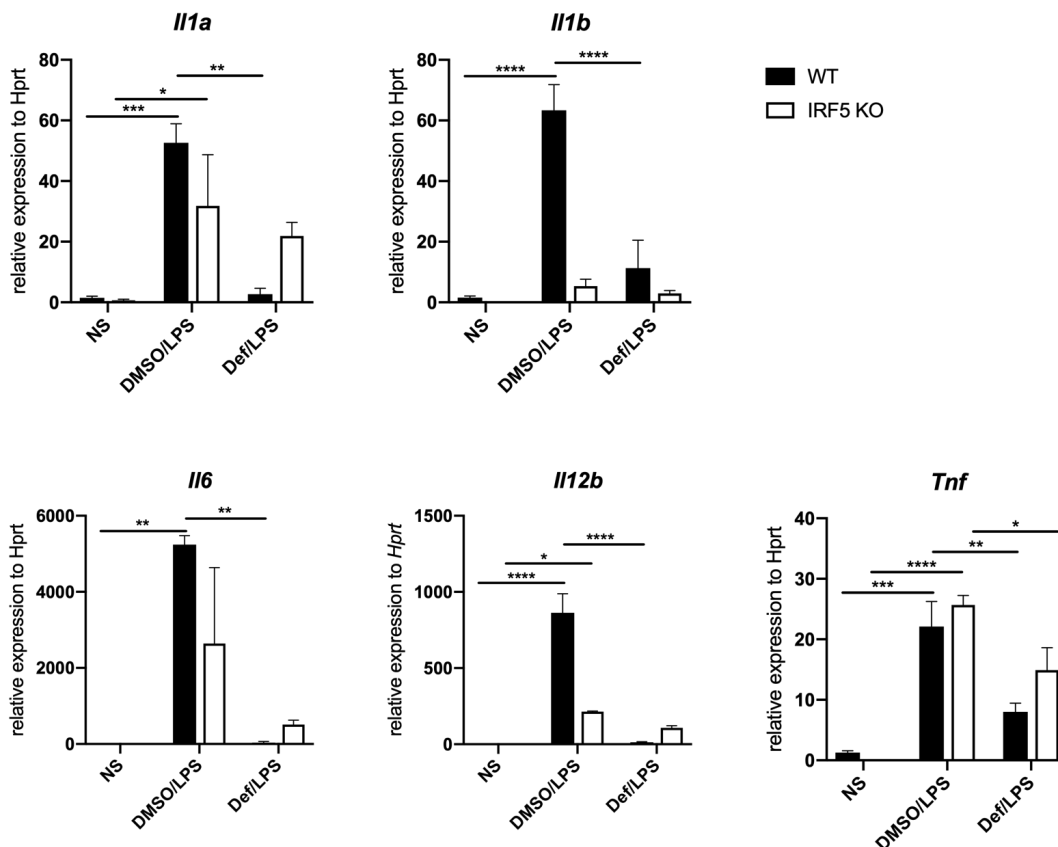


Figure 4.15. Gene expression of PYK2 inhibitor treated WT and *Irf5*^{-/-} macrophages Cytokine level profiles of GM-BMDMs from WT and *Irf5*^{-/-} mice pre-treated with Defactinib (Def) at 3.5 μ M for 1 hour, followed by LPS (100 ng/ml) stimulation for 2 hours. RNA was extracted and 150 ng (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il1a*, *Il1b*, *Il6*, *Il12b*, *Tnf*, and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data are shown as means $\pm$ SEM for n=3 individual mice and analysed by two-way ANOVA * P <0.05, **<0.01 ***<0.001, **** P <0.0001.

proinflammatory cytokines tested were downregulated in defactinib treated WT GM-BMDMs. However, *Irf5*-deficient GM-BMDMs displayed similar expression levels of *Il1a*, *Il1b*, *Il6*, and *Il12b* between DMSO/LPS and defactinib/LPS stimulated, and only *Tnf* levels were further affected by defactinib treatment in *Irf5*^{-/-} GM-BMDMs. These results indicate that defactinib has a selective, suppressive effect on IRF5 dependent signalling in innate immune responses by acting in an IRF5-dependent manner.

I next compared the transcriptome of these cells and conditions by RNA-sequencing (analysis carried out by Tariq Khoiratty, Udalova lab). Principal component analysis (PCA) of differentially expressed genes ($p < 0.05$) created a map of the clusters of cells (**Figure 4.16**). As expected PCA clearly separated WT and IRF5 KOs, as well as untreated and LPS-treated sample groups. LPS stimulated WT samples treated with defactinib or vehicle were also clearly separated, with the defactinib treated samples grouping closely with the IRF5 KOs. Consistent with the previous results, defactinib had no further effect on gene

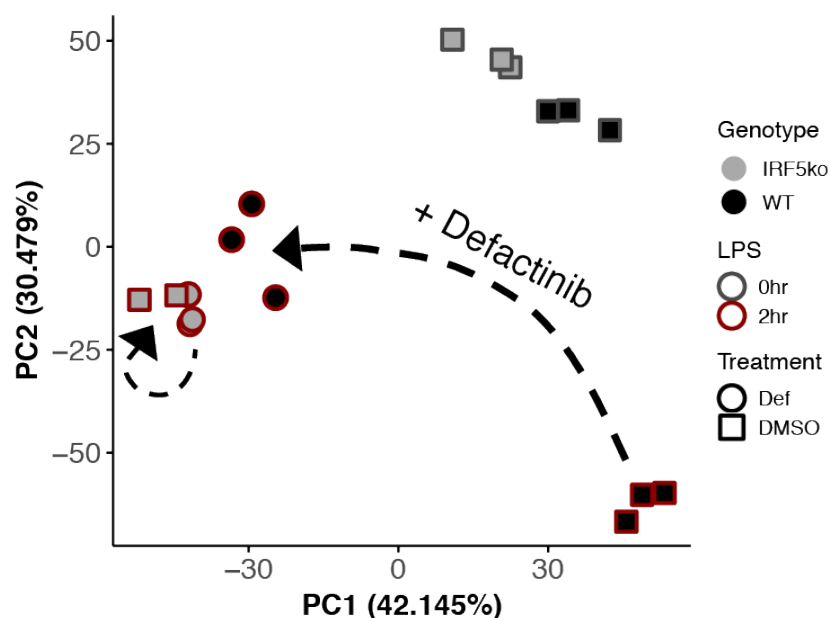


Figure 4.16. Transcriptome analysis of defactinib treated WT and IRF5 KO macrophages PCA analysis of RNA-seq data from WT (black filled shapes) and *IRF5*^{-/-} (grey filled shapes) GM-BMDMs (n=3) pre-treated with 3.5 μ M defactinib (circle) or DMSO control (square) for 1 hr and further stimulated with LPS for 0 (grey outline) or 2 hrs (red outline). Dashed arrows indicate the addition of defactinib.

expression in IRF5 KOs where LPS/DMSO and LPS/Defactinib macrophages cluster together, indicating overall similar transcriptome profiles. This was reflected in the number of differentially expressed genes. WT BMDMs treated with defactinib have 4,026 differentially expressed genes at the 2 hr time point, 2,750 of which are down-regulated compared to the DMSO control, whereas only 217 genes were differentially expressed between defactinib or vehicle treated LPS stimulated IRF5 KO BMDMs (**Figure 4.17a-4.17b**).

Gene ontology (GO) analysis for defactinib down-regulated genes revealed that they are predominantly pro-inflammatory in nature (e.g. cellular response to interferon-beta, regulation of inflammatory response, cytokine activity etc.) (**Figure 4.17c**). These GO terms were also enriched in LPS induced genes, and in IRF5 up-regulated genes, suggesting that defactinib is highly specific for IRF5 target genes. As defactinib has a profound effect on the LPS response in WT macrophages but not in IRF5 KO BMDMs, I investigated the correlation between IRF5 regulated genes and defactinib target genes. The majority of IRF5 up-regulated genes were strongly repressed by defactinib and there was a high degree of overlap between IRF5 up- and defactinib down-regulated genes, including *Il1a*, *Il1b*, *Il6*, *Il12a*, *Il12b*, *Il23a*, *Ccl3*, *Ccl4* etc (**Figure 4.17d**). Interestingly, there was also a smaller overlap between IRF5 down-regulated genes and defactinib up-regulated genes, suggesting that the actions of IRF5 and defactinib are in direct opposition to each other. Overall, IRF5 deficiency and PYK2 inhibition with defactinib have comparable impact on LPS-induced transcriptomic signature in macrophages.

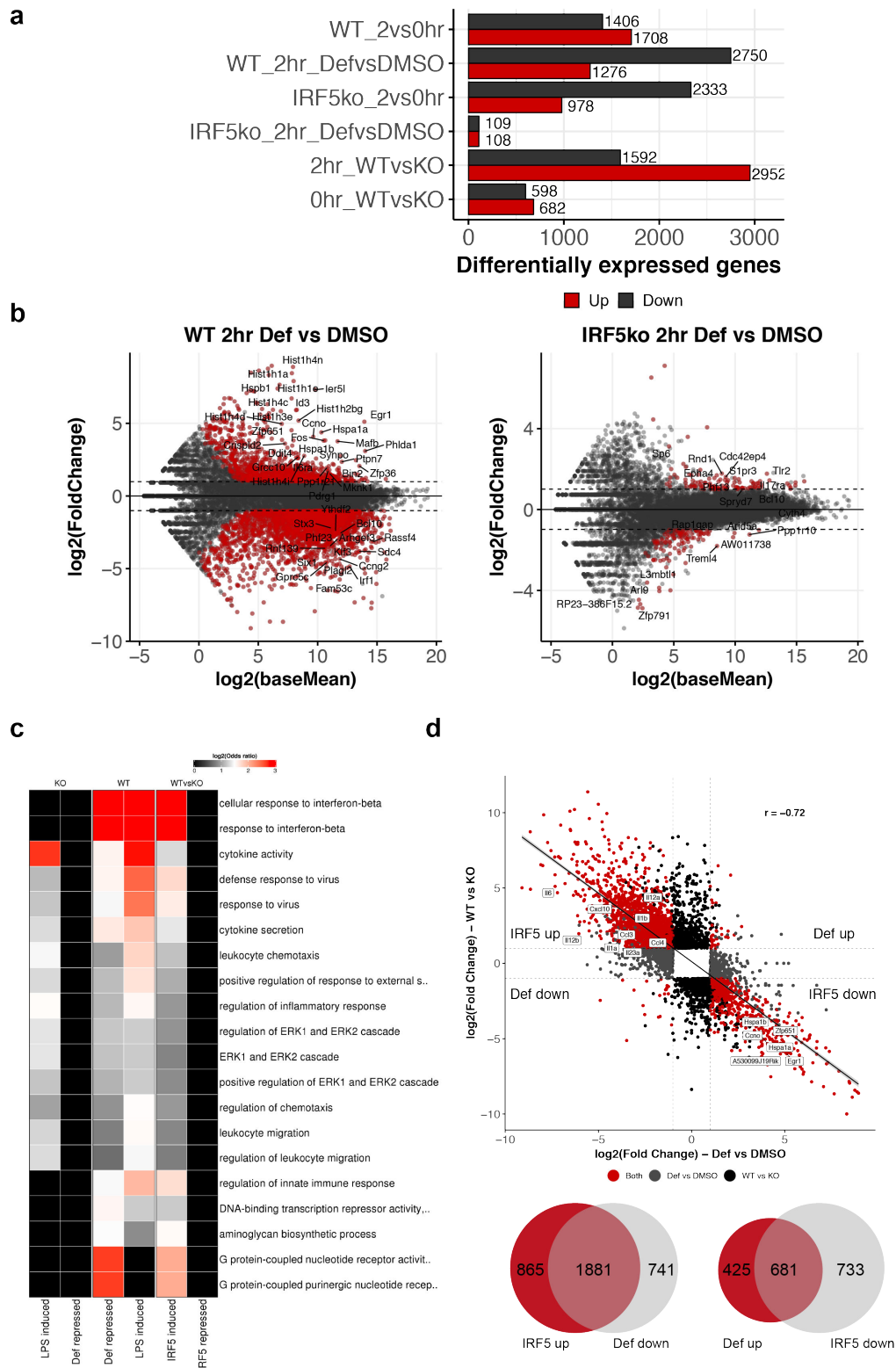


Figure 4.17. RNA-seq transcriptome analysis of WT and IRF5 KO macrophages (a) Summary plot of the number of upregulated (red) and downregulated (grey) differentially expressed genes from RNAseq in indicated cells/conditions. (b) MA plots depicting effect of defactinib on LPS stimulated GM-BMDMs from WT or *IRF5*^{-/-} mice. Differentially expressed genes (defactinib vs DMSO, fold change >1 and padj<0.05) are highlighted in red. (c) Gene ontology enrichment analysis for differentially expressed genes as in (b). (d) Correlation analysis of IRF5 and defactinib regulated genes after 2 hrs LPS stimulation. Red indicates genes are differentially expressed (significance as in b) in comparisons, genes regulated by IRF5 only (black), genes regulated by defactinib only (grey). Venn diagrams demonstrate overlap between IRF5 and defactinib regulated genes.

4.2.6.6. PYK2 inhibition reduces IRF5-mediated transcription

To complement the ChIP-qPCR assay carried out in PYK2 deficient RAW264.7 cells (**Section 4.2.3**), I tested the recruitment of IRF5 and Pol II to IRF5-dependent cytokine promoters in defactinib treated macrophages. GM-BMDMs were pre-treated for 1 hour with defactinib, followed by 2 hour LPS stimulation. ChIP-qPCR analyses confirmed increased IRF5 and Pol II binding to the upstream promoter regions of *Il-6*, *Il-1b* and *Il-12b* upon LPS stimulation with no changes in the IgG isotype control (**Figure 4.18**). In contrast, LPS stimulation did not significantly increase recruitment of IRF5 and Pol II to the *Il-1a* promoter and may be due to the wrong time frame. Mirroring the PYK2 knock out data, LPS-induced IRF5 and Pol II recruitment to its target gene promoters (*Il-6*, *Il-1b* and *Il-12b*) were

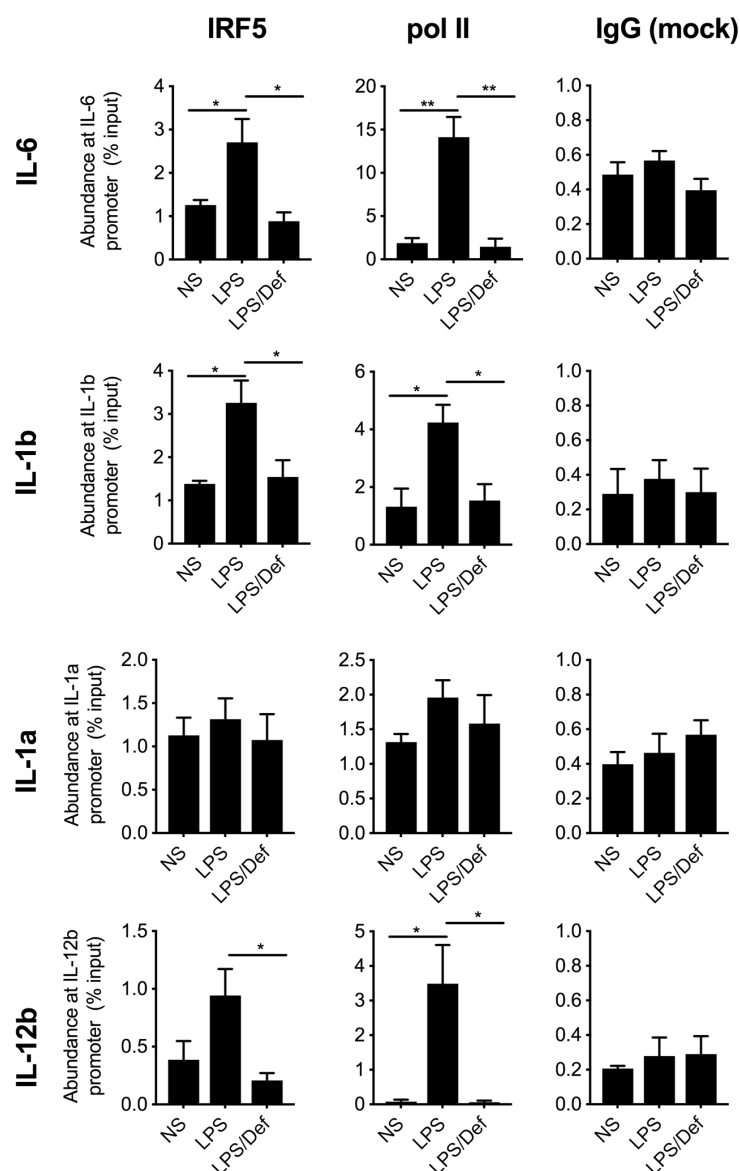


Figure 4.18. Recruitment of IRF5 in GM-BMDMs with defactinib treatment GM-CSF differentiated mouse BMDMs were pre-treated with 3.5 μ M defactinib (def) or DMSO control and further stimulated with LPS (100 ng/ml) for 2 hours. Cells were lysed with cytoplasmic lysis buffers LB1 and LB2, followed by nuclear lysis buffer LB3 with added protease inhibitors. The nuclear lysate was sonicated for 8 minutes (30s on/30s off cycles) at 4°C to obtain a chromatin fragment size of 500 bp. Lysates were incubated at 4°C with protein G magnetic beads that had been pre-incubated with antibodies specific to IRF5, Pol II or IgG isotype control. Following an overnight incubation protein-DNA complexes were washed 6 times with ice-cold ChIP wash buffer and complexes eluted in 120 μ l ChIP elution buffer. Samples were reverse crosslinked overnight at 65°C, along with non-immunoprecipitated lysate for 'whole cell extract' input control, followed by DNA purification. ChIP-qPCR reactions were performed in duplicates using primers designed against IL1a, IL1b, IL12b and IL6 gene promoters with SyBR green master mix. Data are normalised against chromatin amount in lysates and expressed as percentage of input for each gene. Data shown as mean values $\pm$ SEM from n=3 individual mice. Comparison by one-way ANOVA *P<0.05, **<0.01 with multiple test corrections by Tukey.

suppressed in macrophages treated with defactinib. Thus, pharmacological inhibitors of PYK2 phenocopies PYK2 deficiency in its impact on IRF5-driven gene expression in macrophages stimulated with LPS, and supports the idea that PYK2 is involved in IRF5 activation.

4.2.6.7. PYK2 controls IRF5 nuclear translocation

Phosphorylation of IRF5 is known to release IRF5 autoinhibition to promote its subsequent nuclear translocation, resulting in an increase in IRF5 mediated transcriptional activity. I then examined whether PYK2 promotes IRF5 translocation by examining the subcellular localisation of IRF5 following PYK2 inhibition. RAW264.7 macrophages were pre-incubated with defactinib (1 μ M) for 1 hour, and stimulated with LPS for a further 2 hours. Cytosolic and nuclear extracts were isolated by sucrose buffer separation and immunoblotted for IRF5, alpha tubulin (cytosolic loading control) and histone H3 (nuclear loading control). As shown in **Figure 4.19**, there was a good separation between cytosolic and nuclear fractions with similar loading control levels within the cytosol and nucleus. In unstimulated cells, IRF5 resides in both the cytosol and nucleus with similar protein levels in both compartments. IRF5 contains a nuclear localisation signal (NLS) as well as a nuclear export signal (NES)

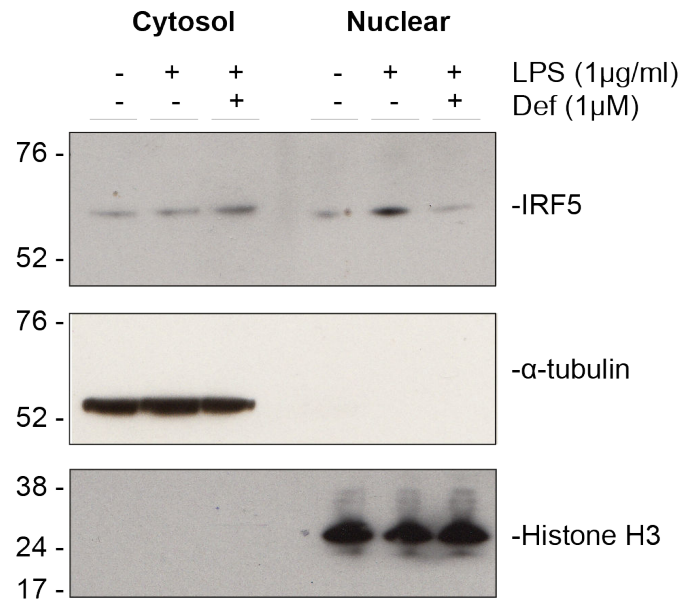


Figure 4.19. IRF5 translocation with inhibitor treatment 1×10^7 RAW 264.7 cells were pre-treated with 1 µM of defactinib (def) or DMSO control for 1 hour, followed by LPS stimulation (1 µg/ml) for a further 2 hours. Cells were lysed on ice with cytoplasmic lysis buffer with protease inhibitor. The lysate was layer on top of cold sucrose buffer and cleared by centrifugation where the supernatant was kept as the cytosolic extract. The nuclear pellet was lysed in cold RIPA lysis buffer and sonicated for 10 cycles (30-seconds on/30-seconds off). Lysates were cleared by centrifugation and the supernatant kept as the nuclear fraction. 20 µg and 60 µg of cytosolic and nuclear lysates, respectively were resolved on an SDS-PAGE gel. Proteins were transferred to PVDF membrane for immunoblotting with Histone H3 (nuclear loading control), alpha-tubulin (cytosolic loading control) and IRF5 antibodies. WB = Western blot

so it is likely that IRF5 shuttles in and out of the nucleus. Following LPS stimulation, IRF5 migrates from the cytosol and accumulates in the nucleus. However, upon defactinib treatment with LPS, IRF5 nuclear translocation is blocked and IRF5 resides predominantly in the cytosol. This observation highlights that PYK2 controls IRF5 activity by affecting its subcellular localisation.

To examine whether this block in IRF5 migration occurred at lower defactinib concentrations the inhibitor was further titrated. RAW264.7 macrophages were pre-stimulated with a dose response of defactinib at 0.3, 0.5 and 1.0 uM for 1 hour, and stimulated with LPS for a further 2 hours. As assessed by immunoblotting for IRF5, alpha-tubulin and histone H3 only 1.0 uM of defactinib reduced nuclear levels of IRF5, whereas

at lower defactinib concentrations IRF5 nuclear levels were similar to LPS stimulated only (Figure 4.20).

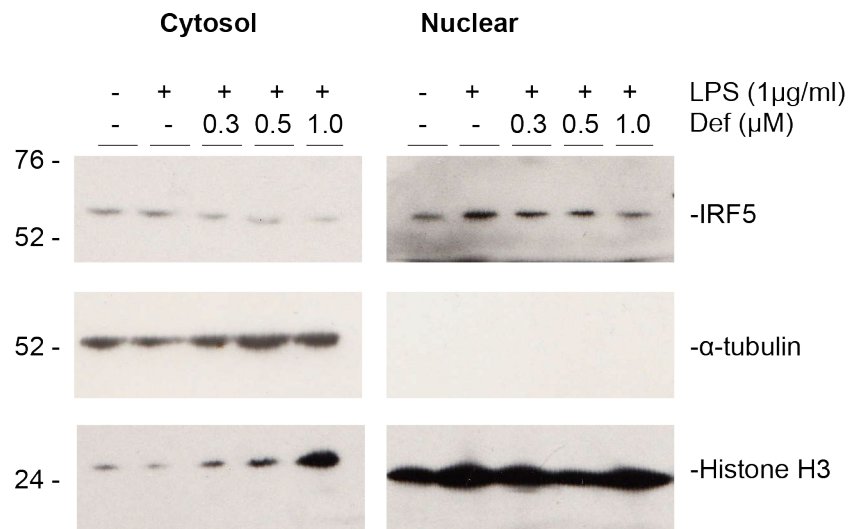


Figure 4.20. IRF5 translocation with inhibitor titration 1×10^7 RAW264.7 cells were pre-treated with 0.3/0.5/1 μM of defactinib (def) or DMSO control for 1 hour, followed by LPS stimulation (1 ug/ml) for a further 2 hours. Cells were lysed on ice with cytoplasmic lysis buffer with protease inhibitor. The lysate was layer on top of cold sucrose buffer and cleared by centrifugation where the supernatant was kept as the cytosolic extract. The nuclear pellet was lysed in cold RIPA lysis buffer and sonicated for 10 cycles (30-seconds on/30-seconds off). Lysates were cleared by centrifugation and the supernatant kept as the nuclear fraction. 20 μg and 60 μg of cytosolic and nuclear lysates, respectively were resolved on an SDS-PAGE gel. Proteins were transferred to PVDF membrane for immunoblotting with Histone H3 (nuclear loading control), alpha-tubulin (cytosolic loading control) and IRF5 antibodies. WB = Western blot

4.2.6.8. PYK2 activity in macrophages and tissues

PYK2 contains a tyrosine autophosphorylation site (Y402) that is essential for PYK2 activation and function (168). To assess PYK2 activation, autophosphorylation of PYK2 in RAW264.7 cells and in GM-BMDMs over a LPS stimulation time course was analysed. In line with a previous study (169), I also found LPS-induced PYK2 autophosphorylation on Y402 (Figure 4.21) with no changes in total PYK2 protein levels. RAW264.7 cells display basal activation of PYK2 activation that is induced with LPS stimulation, with the highest activation at 30 minutes. Along with previous data, I can now build a picture of LPS-induced

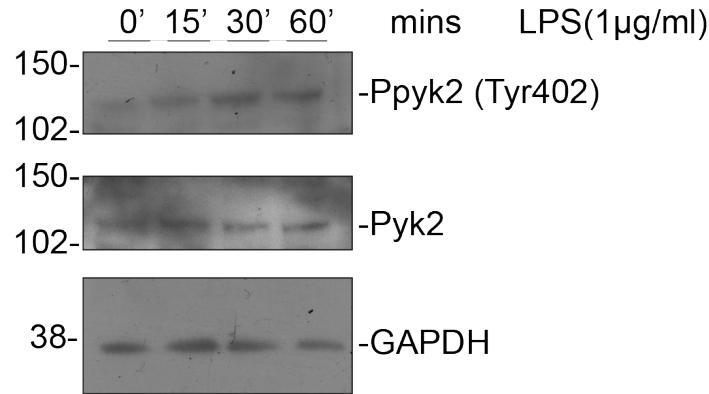


Figure 4.21. Phospho-PYK2 kinetics in RAW264.7 cells RAW264.7 cells were plated and incubated overnight. Media was replaced with serum-free media for 30 minutes. Cells were then stimulated with LPS (1 µg/ml) at indicated time points. Cells were lysed in whole cell lysis buffer (with added phosphatase and protease inhibitors) on ice for 30 minutes, then cleared by centrifugation to pellet cellular debris. 80 µg of protein lysate (as determined by the Qubit assay) was resolved by SDS-PAGE. Proteins were transferred to PVDF membrane for immunoblotting with P-PYK2 (Tyr402), PYK2 and GAPDH specific antibodies

IRF5 regulation in which PYK2 is initially autophosphorylated at 30 minutes, active PYK2 phosphorylates IRF5 at 60 minutes and IRF5 translocates to the nucleus and binds to target promoters at 2 hours.

LPS also stimulated PYK2 activation (at Y402) in GM-BMDMs in a time-dependent manner. Unstimulated GM-BMDMs display basal PYK2 autophosphorylation which is induced specifically 60 minutes post LPS stimulation and retained at 120 minutes with no changes in total PYK2 protein levels (**Figure 4.22**). Therefore, LPS stimulation for 60 minutes was selected as the optimum timepoint for PYK2 activation for subsequent experiments. The levels of IRF5 in GM-BMDMs was also analysed by western blotting. In agreement with the *Udalova* labs previous findings (47), IRF5 expression is elevated upon LPS stimulation particularly at 15-60 minutes. Unstimulated cells showed no levels of IRF5 protein, however this could be due to uneven protein loading.

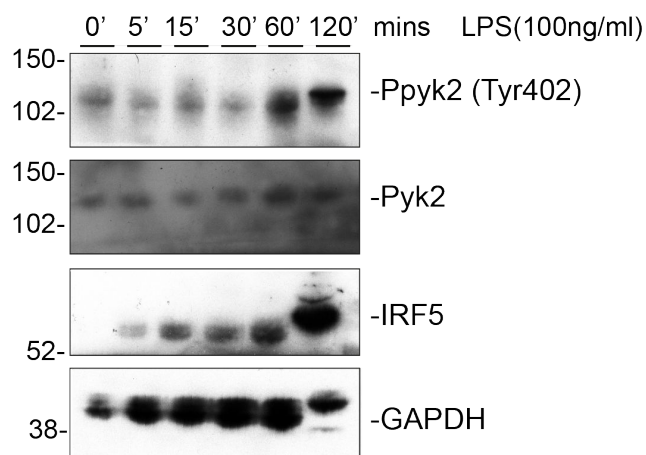


Figure 4.22. Phospho-Pyk2 kinetics in GM-BMDMs GM-BMDMs were plated and incubated overnight. Media was replaced with serum-free media for 30 minutes. Cells were then stimulated with LPS (100 ng/ml) at indicated time points. Cells were lysed in RIPA buffer (with added phosphatase protease inhibitor) on ice for 30 minutes, then cleared by centrifugation to pellet cellular debris. 100 µg of protein lysate (as determined by the Qubit assay) was resolved by SDS-PAGE. Proteins were transferred to PVDF membrane for immunoblotting with P-PYK2 (Tyr402), IRF5, PYK2 and GAPDH specific antibodies.

Both inhibitors (defactinib and PF-431396) have previously been shown to block PYK2 autophosphorylation in various cell lines and tissues including rat arterial smooth muscle tissue and myeloma cell lines (182, 183). I then tested the *in vitro* effects of PYK2 inhibitors on PYK2 activation in RAW264.7 cells. On the basis of the previous results, cells were stimulated with LPS for 30 minutes after 1 hour incubation with PYK2 inhibitor or carrier DMSO. As anticipated, LPS stimulation induced PYK2 autophosphorylation, whereas pre-treatment with either PYK2 inhibitors attenuated PYK2 activation to basal levels (**Figure 4.23**).

I then incubated WT and *Irf5*^{-/-} GM-BMDMs in the presence of 1 µM or 3 µM of defactinib or the carrier (DMSO) and examined the effect on LPS-induced PYK2 autophosphorylation.

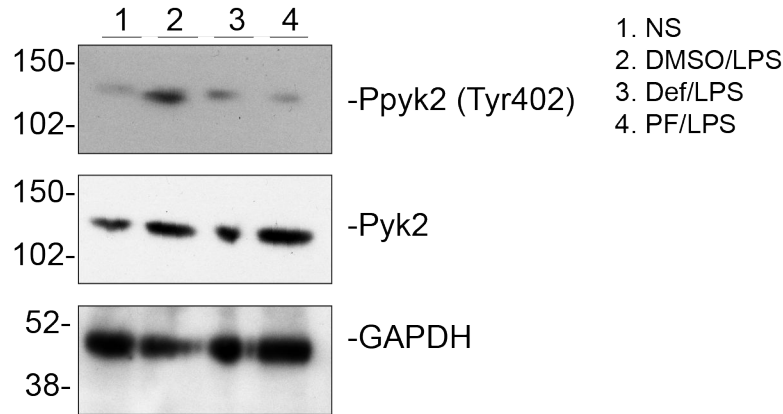


Figure 4.23. Phospo-PYK2 kinetics with inhibitor treatment RAW264.7 cells were plated and incubated overnight. Media was replaced with serum-free media for 30 minutes prior to pre-treatment with 1 μ M defactinib (def), 1 μ M PF-431396 (PF) or DMSO control for 1 hour followed by LPS (1 μ g/ml) stimulation for a further 30 minutes. Cells were lysed in whole cell lysis buffer (with added protease and phosphatase inhibitors) on ice for 30 minutes, then cleared by centrifugation to pellet cellular debris. 80 μ g of protein lysate (as determined by the Qubit assay) was resolved by SDS-PAGE. Proteins were transferred to PVDF membrane for immunoblotting with P-PYK2 (Tyr402), PYK2 and GAPDH specific antibodies. WB = Western blot

LPS induced PYK2 autophosphorylation at Tyr402 to similar levels in both WT and *Irf5*^{-/-} GM-BMDMs with no changes in total PYK2 levels (**Figure 4.24**). At 1 μ M and 3 μ M , defactinib reduced LPS-induced phospho-PYK2 protein levels in WT and KOs to basal levels, confirming that defactinib targets PYK2 in macrophages.

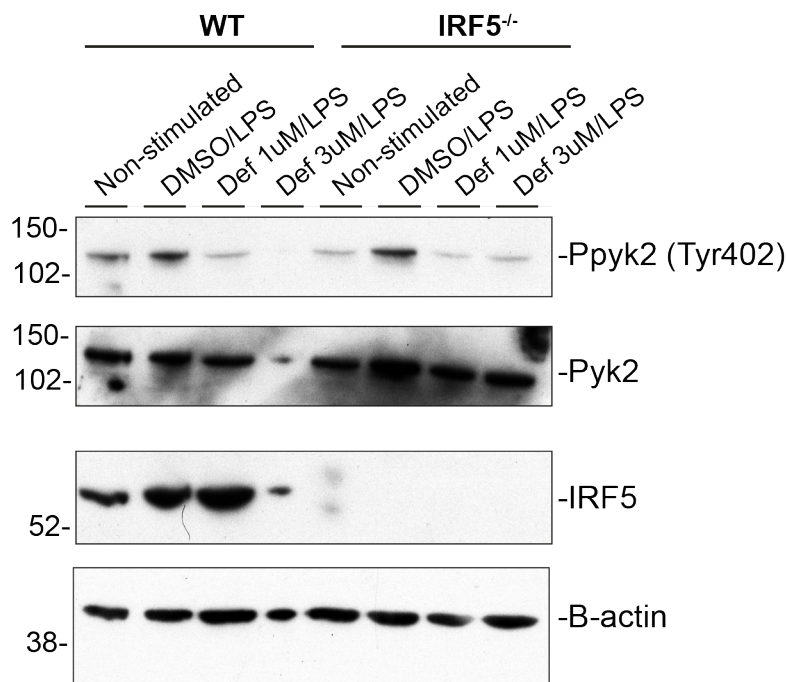


Figure 4.24. Phospho-PYK2 levels in defactinib treated GM-BMDMs GM-BMDMs cells were plated and incubated overnight. Cells were pre-treated with 1 μ M or 3 μ M defactinib (def), or DMSO control for 1 hour followed by LPS (100 ng/ml) stimulation for a further 60 minutes. Cells were lysed in RIPA cell lysis buffer (with added protease and phosphatase inhibitors) on ice for 30 minutes, then cleared by centrifugation to pellet cellular debris. 80 μ g of protein lysate (as determined by the Qubit assay) was resolved by SDS-PAGE. Proteins were transferred to PVDF membrane for immunoblotting with P-PYK2 (Tyr402), PYK2, IRF5 and B-actin specific antibodies. WB = Western blot

I next examined whether levels of phosphorylated-PYK2 (Y402) could be detected in mouse tissues. This is useful for future *in vivo* models to see if the PYK2 inhibitor is targeting PYK2 and whether it reduces pathology in murine colitis. Colon tissues were taken from *Citrobacter rodentium*-induced colitis mice (tissues prepared by Claire Pearson, Powrie lab) and protein levels of pPYK2 (Y402), PYK2, IRF5, and β -actin were assessed by immunoblotting (**Figure 4.25**). A sample from LPS-stimulated GM-BMDMs was included as a positive control. PYK2 autophosphorylation was undetectable in the steady state sample and colons taken from 3 out of 5 *Citrobacter rodentium*-induced colitis mice. Even though total levels of PYK2 and β -actin were similar between mice, pPYK2 was detectable in 2 out of 5 colon samples. Interestingly, the samples in which pPYK2 was detectable also seem to have increased levels of IRF5. It may be the case that PYK2 activation is linked to increased levels of IRF5.

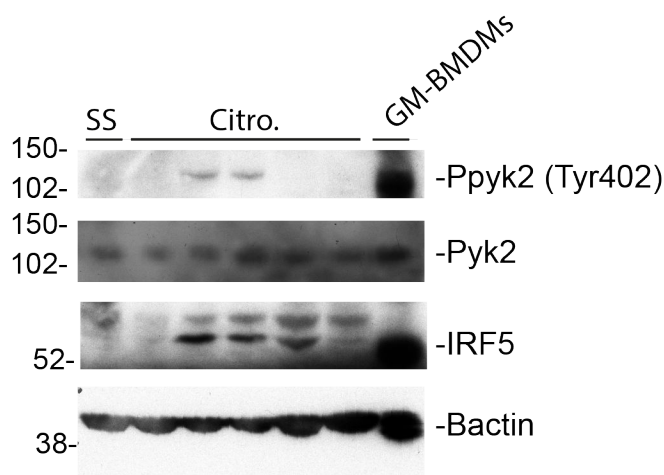


Figure 4.25. Phospho-PYK2 levels in colon tissues Colon tissues were taken from one steady state (SS) mouse and five *Citrobacter rodentium*-induced colitis mice. 50 mg tissues were lysed in RIPA buffer (with added protease and phosphatase inhibitors) on ice for 30 minutes, sonicated with protein extraction beads and cleared by centrifugation to pellet cellular debris. A LPS-stimulated GM-BMDM samples was included as a positive control. 80 μ g of protein lysate (as determined by the Qubit assay) was resolved by SDS-PAGE. Proteins were transferred to PVDF membrane for immunoblotting with P-PYK2 (Tyr402), PYK2, IRF5 and B-actin specific antibodies. A sample of LPS-stimulated GM-BMDMs was included as a positive control. WB = Western blot

I then looked at PYK2 autophosphorylation in colon tissue samples taken from vehicle treated and defactinib treated mice on day 7 of *Hh*-aIL10 induced colitis mice (tissues prepared by Alastair Corbin). In this model *Hh* infection concomitant with the administration of an anti-IL 10 Receptor (α IL10R) antibody triggers IL-23 dependent intestinal inflammation with a robust Th1/Th17 cell response, and carries many features of human IBD (184–186). On days 3 and 6 of *Hh*-aIL10 induced colitis (55), mice were orally gavaged with 25mg/kg defactinib, and colon tissues were taken for immunoblot analysis. As shown in **Figure 4.26**, no PYK2 autophosphorylation was detected in vehicle treated or defactinib treated mice. The absence of pPYK2 could be attributed to less total levels of PYK2 loaded as the bands for total PYK2 are faint. Another reason could be from the reuse of antibody reagents during the immunoblot as the pPYK2 band for the positive GM-BMDMs control is much fainter than previous blots. Since phosphorylation is a transient process it may be the case that phosphorylation is no longer sustained at the point the tissues were collected. This experiment requires further optimisation to ensure pPYK2 is detectable in this model and can be targeted by defactinib.

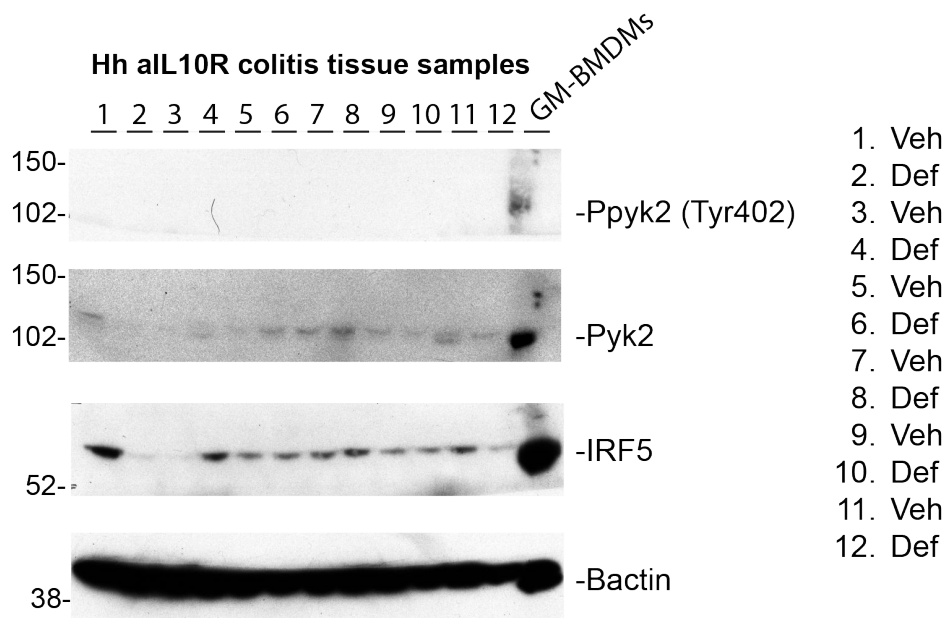


Figure 4.26. Phospo-PYK2 levels in defactinib treated mice during Hh-aIL10R colitis 50 mg tissue from 12 mice were lysed in RIPA buffer (with added protease and phosphatase inhibitors) on ice for 30 minutes, sonicated with protein extraction beads and cleared by centrifugation to pellet cellular debris. 80 μ g of protein lysate (as determined by the Qubit assay) was resolved by SDS-PAGE. Proteins were transferred to PVDF membrane for immunoblotting with P-PYK2 (Tyr402), PYK2, IRF5 and B-actin specific antibodies. A sample of LPS-stimulated GM-BMDMs was included as a positive control. WB = Western blot

The administration of defactinib was altered with mixed intravenous (i.v) and intraperitoneal (i.p) as the onset of drug action is more rapid than oral gavage (**Figure 4.27a**). In brief, on days 0, 1, and 2 mice were injected i.v. with defactinib/vehicle and injected i.p with defactinib/vehicle daily from days 3. The immunoblot in **Figure 4.27b**, shows that active PYK2 was detectable in all colon samples. In general, defactinib treatment reduced protein levels of phospho-PYK2 indicating that the inhibitor targets PYK2.

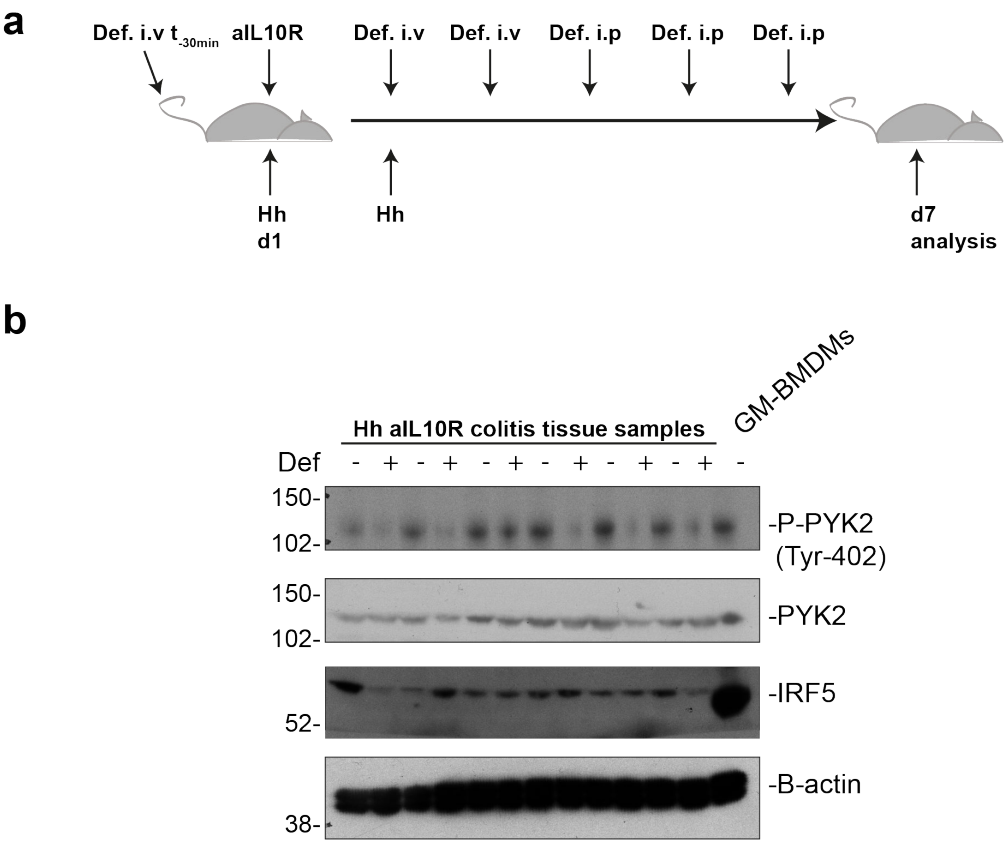


Figure 4.27. Defactinib in Hh/anti-IL10R model of murine colitis (a) Defactinib regime during the initiation phase of mouse Hh-anti-IL-10R colitis. i.v = intravenously, i.p = intraperitoneal **(b)** PYK2 autophosphorylation (Tyr-402) in colon tissue of vehicle or defactinib treated mice (6 mice each) assessed by western blot analysis. Lysates from LPS-stimulated GM-BMDMs included as a positive control).

4.2.6.9. Defactinib suppresses cytokine production in primary human cells and patient biopsies

The following work (**Section 4.2.6.9**) was carried out by Alastair Corbin so will be referred to in plural. Having demonstrated the pharmacological affect of defactinib in mouse macrophages, we set out to examine the effects of defactinib in the human setting. Peripheral blood monocytes from healthy human donors were differentiated *in vitro* into macrophages and treated with LPS in the presence or absence of defactinib. Increasing doses of defactinib at concentrations not affecting cell viability (**Supplementary Figure S5a-b**) reduced LPS-induced levels of IL-6 and TNF α (**Figure 4.28a**). Similar to our findings in mouse BMDMs, we saw robust inhibition of LPS-induced cytokine expression levels of

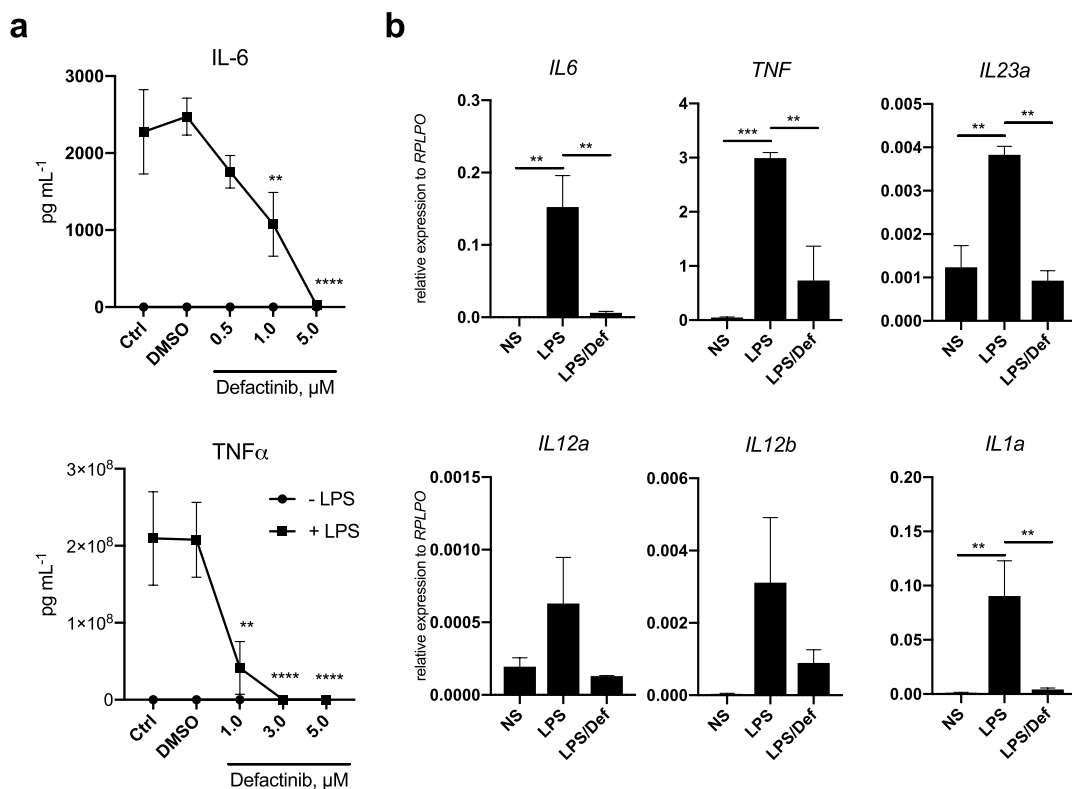


Figure 4.28. Decreased cytokine production in defactinib-treated primary human cells (a) IL6 and TNF α cytokine protein levels in human monocyte-derived macrophages pre-treated with DMSO vehicle or with various amounts of defactinib for 1 h, followed by LPS stimulation (100 ng/ml) for 24 h. (b) Cytokine mRNA expression levels in human monocyte-derived macrophages pre-treated with defactinib (def, 5 μ M) for 1 h followed by LPS stimulation (100 ng/ml) for 2 h. Transcript levels of IL6, Tnf, IL23a, IL12a, IL12b, IL1a, and RPLPO (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta$ Ct-method. Data are shown as means $\pm$ SEM for n=4 and analysed by one-way ANOVA where **P<0.01 ***P<0.001, ****P<0.0001.

Il6, *Tnf*, *Il23a*, *Il12a*, *Il12b*, and *Il1a* (**Figure 4.28**). Overall, this work highlights that defactinib controls proinflammatory gene expression.

The *PTK2B* gene encoding PYK2 in humans is located in one of the genomic loci conferring susceptibility to inflammatory bowel disease (151). As IBD is a complex disease involving many cell types apart from myeloid cells, we tested the impact of PYK2 inhibition in biopsies derived from the colonic mucosa of patients with active ulcerative colitis at concentrations not affecting cell viability in these samples (**Supplementary Figure 5c**). We found elevated cytokine production in colon biopsies obtained from the sites of active inflammation in comparison to those from adjacent non-inflamed sites (**Figure 4.29**). Incubation with increasing doses of defactinib significantly lowered IL-6 and IL-12p70, identified as part of a cassette of inflammatory molecules that mark anti-TNF α -resistant IBD (187), but not IL-1b levels. Therefore, pharmacological inhibition of PYK2 effectively dampens pro-inflammatory response in primary human cells and *ex vivo* cultured fragments of inflamed intestinal mucosal tissue.

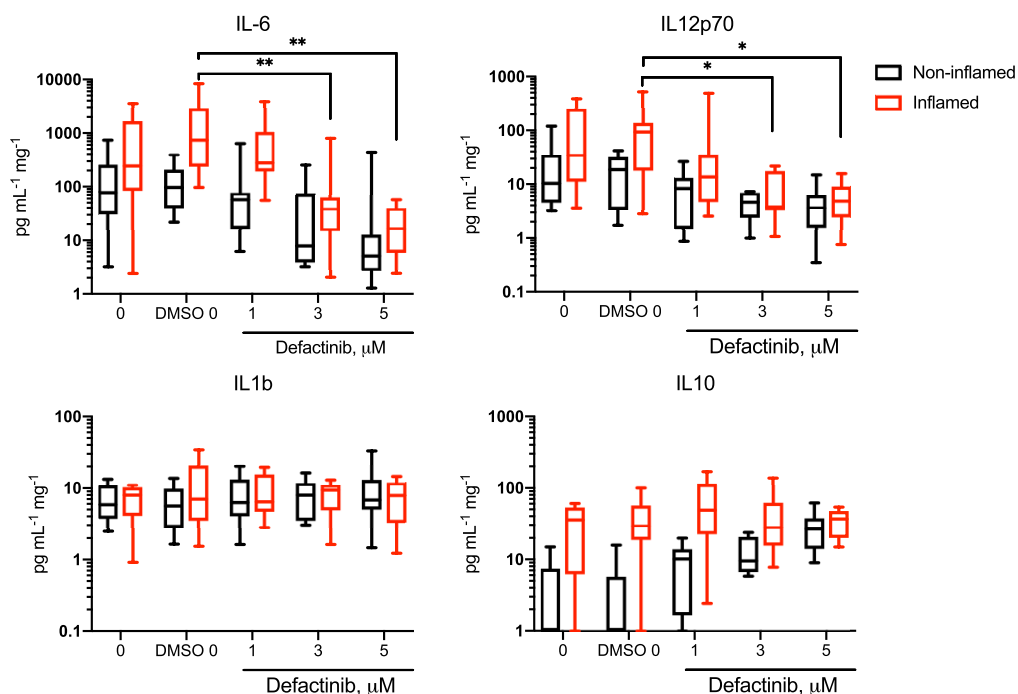


Figure 4.29. Decreased cytokine production in IBD biopsies Cytokine protein levels in biopsies from ulcerative colitis patients from non-inflamed (black) and inflamed tissues (red) treated with defactinib at indicated concentrations. Levels of IL-6, IL12p70, IL1b and IL10 were measured by ELISA or Cytometric Bead Array. Data are shown as means $\pm$ SEM for n=10 human donors and analysed by two-way ANOVA where *p<0.05 **p<0.01.

4.3. CONCLUSION

PYK2 is a non-receptor tyrosine kinase of the FAK family that has been implicated as a regulator of cell adhesion, migration and proliferation. The majority of studies have focused on the role of PYK2 in cancer (178). However, little work has been carried out to investigate its role in the context of inflammation. Following on from Chapter 3 of this thesis, in which PYK2 was identified as a lead IRF5 candidate kinase, I investigated the functional importance of PYK2. In this chapter, I have identified PYK2 as a pivotal regulator of IRF5-dependent pro-inflammatory responses. I have demonstrated that PYK2 physically interacts with IRF5 and triggers its phosphorylation, nuclear translocation and binding to dedicated gene promoters. PYK2-deficient cells are impaired in LPS-induced IRF5-driven transcriptional programmes. Moreover, pharmacological inhibition of PYK2 blocks IRF5 activity in *in vitro* assays, and suppresses LPS-induced pro-inflammatory responses in primary human monocyte-derived macrophages. RNAseq data indicates that defactinib acts in an IRF5-specific manner. In addition to blocking LPS-driven programmes, PYK2 inhibitors also block induction of cytokines stimulated with Dectin-1 agonist WGP or a mouse bacterial pathobiont *Hh*. Finally, treatment with a PYK2 inhibitor lowers IL-6 and IL-12 production in inflamed colonic biopsies from human patients with ulcerative colitis.

This data is supported by the existing literature: PYK2 was found to regulate the inflammatory response in a model of acute lung injury (ALI) via its effect on cell migration and chemotaxis (188). Moreover, use of a PYK2 peptide inhibitor had a protective impact on LPS-induced ALI with a reduction in both lung neutrophil infiltration and protein leakage in the bronchoalveolar lavage fluid. The role of PYK2 in other diseases such as asthma, vasculitis and osteoporosis has also been highlighted (154, 161, 162).

PYK2 kinase inhibition in LPS-stimulated macrophages blocks inflammatory cytokine production. Transcriptional profiling suggests that this block in mRNA expression by defactinib acts in an IRF5-specific manner where defactinib treated LPS-stimulated WT macrophages share a similar transcriptional profile to LPS-stimulated IRF5 KO macrophages. Interestingly, the inhibitor had no further effect on the transcriptional programmes in IRF5 KOs suggesting that PYK2 regulates IRF5-mediated transcripts. Further analyses of these genes are needed to decipher pathways, for instance, to separate chemotaxis and migration from inflammation.

The data suggests that PYK2 acts downstream of not only LPS/TLR4, but also WGP/dectin-1 pathway. PYK2 is known to mediate signal transduction in cells treated with TLR ligands (189), although it has never previously been linked to IRF5. MyD88 is likely to be the essential linking adaptor between TLRs and PYK2/IRF5 complex as previous studies have shown impairment of PYK2 activation in LPS-deficient cells (169). I also show that PYK2 KO cells are unable to induce IRF5 activation in response to WGP, which are recognised by the C-type lectin receptor Dectin-1 (190). This is an agreement with prior studies demonstrating an essential role of IRF5 in the Dectin-1 pathway (191). PYK2 was further shown to be downstream of the Dectin-1/Syk axis in zymosan-stimulated macrophages (192). It is probable that PYK2 signals downstream of SYK to control IRF5 activation as both IRF5 and PYK2 can act downstream of dectin-1 - SYK pathways. Taken together, these data indicate that PYK2 is a general signalling component of IRF5-activating innate sensing pathways.

I have identified several LPS induced IRF5 phosphorylation sites in WT and PYK2 deficient cells. One of which is the IKK β dependent site Ser-445 (mouse) which is agreement with other studies in which IKK β phosphorylates IRF5 at this site upon TLR activation (95, 96). Although serine phosphorylation has been shown to activate IRF5, little is known about the

consequence of tyrosine phosphorylation of IRF5. The only known reported tyrosine phosphorylation site Tyr-104 has been linked to negative regulation of IRF5 transcriptional activity (69). However, my functional studies show that Tyr-104 has no effect on IRF5 transactivation. IRF5 is phosphorylated on several tyrosine sites that I've identified by MS in which Tyr-171 (mouse) is targeted by PYK2 and is important for IRF5 activation. I have also identified sites Tyr-312 and Tyr-334 (mouse) as important for IRF5 transactivation, indicating involvement of other upstream tyrosine kinases. This warrants further exploration to identify the responsible kinases and identify the functional connection between different tyrosine sites as they all appear non-redundant for IRF5 activity. Ongoing studies with global phosphoproteome experiments will help answer if defactinib specifically targets IRF5 at a cell signalling level and help pinpoint the cellular pathways targeted by PYK2.

The molecular dissection of the PYK2/IRF5 axis carried out in cell-based assays has a direct translational potential. Indeed, here I show the role of PYK2 in dampening IBD-associated, IRF5-regulated inflammatory cytokine secretion in inflamed mucosal biopsies from patients with ulcerative colitis, which fits with data from genome-wide association studies. The human *PTK2B* gene is located in one of the IBD susceptibility loci (151), while SNPs in the *IRF5* gene have been associated with IBD risk (53). Thus, the two genetic risk factors can be linked together where PYK2 can likely be mapped to the IRF5 inflammatory pathway. Future studies should focus on the use of defactinib in *in vivo* colitis models. Somewhat contradictory to our expectations a recent study has shown that PYK2 deficient mice are phenotypically normal under homeostatic conditions but hyper-sensitive to DSS-induced colitis (153). However, phenotypes associated with PYK2 KO may be distinct from PYK2 inhibition as the KO may affect the development of immune cells, whereas inhibitors act in a transient manner. Furthermore, it seems possible that knocking out PYK2 may be affecting important scaffolding functions important for intestinal epithelium integrity, whereas inhibitors only block PYK2 kinase activity without effecting its scaffolding functions.

Defactinib, a PYK2 inhibitor tested in this study, is currently in clinical trials for treatment of ovarian cancer (181). Given that defactinib has passed all the necessary drug checkpoints, has a good toxicological profile and has demonstrated promising pre-clinical data on its efficacy in mice, primary cells and patient samples, defactinib and related PYK2 inhibitors are attractive molecules for repurposing to treat patients with inflammatory conditions such as ulcerative colitis, rheumatoid arthritis or lupus, where IRF5 plays an important role.

Several emerging studies have demonstrated the role of IRF5 in colitis, including *Hh*-aLLR and T-cell transfer models (54, 55). Interestingly, we have observed reduced recruitment of leukocytes with mixed i.v/i.p defactinib treatment during *Hh*-induced colitis (data not shown) in ongoing experiments in the Udalova lab. Coupled with the identity of PYK2 as a genetic risk factor for IBD, the discovery of PYK2 as an IRF5 kinase and the promising data with PYK2 inhibitors in biopsies from patients with UC this could lead to potential therapeutics. Overall, my findings reveal a potentially pharmacologically targetable mechanism underlying IRF5 activation in which PYK2 plays a pivotal role in coordinating IRF5 activity, and blocking PYK2 may represent an opportunity to prevent IRF5 activation in IRF5-driven inflammatory conditions.

5.0. MEKK3 KINASE VALIDATION

5.1. INTRODUCTION

Follow on from results Chapter 3.0, MEKK3 came up as one of the lead IRF5 candidate kinases. MEKK3 acts as a branching point downstream of important pro-inflammatory signalling pathways in the innate immune response including NF- κ B, JNK and p38 pathways and our initial data suggests a possible involvement in the IRF5 pathway. In this chapter, I sought to examine the role of MEKK3 in IRF5 activation.

Mitogen activate protein kinase kinase 3 (MEKK3, MAP3K3) is a serine/threonine kinase belonging to the MEKK/STE11 subgroup of the MAP3K family of kinases, and is ubiquitously expressed in several tissues. MEKK3 is reported to play a role in several cellular processes including proliferation, cell cycle progression, differentiation, migration, apoptosis and inflammatory response (193–196). *In vitro* MEKK3 mediates the activation of several MAPKs including c-Jun N-terminal kinases (JNKs), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), p38 and ERK5 (197–199). Genetic studies have shown that MEKK3 is necessary for embryonic cardiovascular development particularly in angiogenesis and heart development (200). MEKK3 knockout mice are embryonically lethal between embryonic days 10 and 11, but a conditional knock-out line has been generated (201).

Numerous studies have demonstrated a link between increased MAP3K3 expression and activation in cancers including non-small cell lung cancer, pancreatic cancer, hepatocellular carcinoma, renal clear cell carcinoma and oesophageal squamous cell carcinoma (202–205). Overexpression of MEKK3 leads to increased NF- κ B activation and confers resistance to apoptosis in cancer cells (206). Despite its role in cancer, to date no MEKK3-specific inhibitors have been developed. The FDA-approved kinase inhibitor Ponatinib designed to target BCR-ABL tyrosine kinase to treat acute myeloid leukemia (207) has

recently been shown to inhibit MEKK3 (208) but also displays inhibitory activity towards MEKK2 and other kinases (209, 210).

MEKK3 acts as an essential signal transducer required for activation of NF- κ B induced by TNF- α , IL-1 and LPS (194–196). During TNF-induced NF- κ B activation, MEKK3 functions downstream of TRAF2 and RIP, but upstream of IKK where it directly phosphorylates IKK *in vitro* (194). Moreover, MEKK3 functions downstream of the MyD88-IRAK-TRAF6 complex in IL-1R TLR4 signalling. Several studies have shown that TAK1 directly binds and inhibits MEKK3 to negatively regulate cytokine secretion (211, 212). Knockdown of MEKK3 expression in RAW264.7 macrophages impairs LPS induced IL-6 and GM-CSF production but not of TNF- α and IL1 β .

In the absence of any MEKK3-specific inhibitors, I test MEKK3 inhibitors developed by LAB282, Evotec. Often is the case that probes designed to target a specific kinase, also displays off-target effects to other kinases, as kinases all share similar structural frameworks in their active sites. For example SP600125 was originally developed to inhibit c-Jun-N-terminal kinases (JNKs) (213). But when profiled against the kinome, SP600125 inhibited several kinases to a similar or greater extent than JNKs (214). This promiscuity towards 'off-target' members of the kinome can be advantageous for repurposing or act as a starting point to improve by medicinal chemistry. For example, Imatinib (Gleevec) was originally designed for the treatment of chronic myelogenous leukaemia as an oral inhibitor of BCR-ABL (215). The off-target effects of Imatinib to receptor tyrosine kinases c-kit and platelet derived growth factor receptor α (PDGF-R α) has led to Imatinib's use for treatment of gastrointestinal stromal tumour (216). Similarly, crizotinib was initially developed as a MET inhibitor, but its off-target activity towards ALK led to its use as an ALK inhibitor in ALK- fusion positive lung cancer (217).

Current inhibitors can serve as chemical starting points for further improvement. As a proof-of-concept, probes against the lung cancer survival marker MELK (maternal embryonic leucine zipper kinase) have been identified as initial leads (218). Through the use of kinase assays, 16 probes including FDA approved inhibitors nintedanib and sunitinib displayed selectivity towards MELK. Co-crystallisation of lead probes with MELK identified essential amino acids within the ATP pocket that may be useful for the development of selective and potent MELK inhibitors. By screening existing probes combined with structural information it is now possible to develop inhibitors against a desired target. In this Chapter, the use of starting points for focused lead discovery efforts are used to develop and test MEKK3 inhibitors.

5.2. RESULTS

5.2.1. Reduced TNF-reporter activity in MEKK3 knockouts

MEKK3 was a positive hit in the initial validation assays (Chapter 3.0). In this section I further validate MEKK3 as a candidate IRF5 kinase using the MEKK3-deficient RAW264.7 cells. I first investigated whether MEKK3 has any effect on IRF5s transcriptional activation of the TNF promoter using the TNF-luciferase reporter assay. RAW264.7 cells were transfected with the TNF-reporter construct, HA-IRF5 or empty expression plasmids, and renilla luciferase as a control of transfection efficiency. After 24 hours in transfection media, cells were stimulated with LPS for 6 hours, and luciferase activity was measured using the dual-luciferase assay system. As shown in **Figure 5.1**, LPS stimulation of WT cells ectopically expressing IRF5 induced a ten-fold increase in TNF-luciferase activity. In contrast, cells deficient in MEKK3 failed to induce luciferase activity to the same levels and induced only a five-fold increase. There was no effect in cells expressing the empty vector

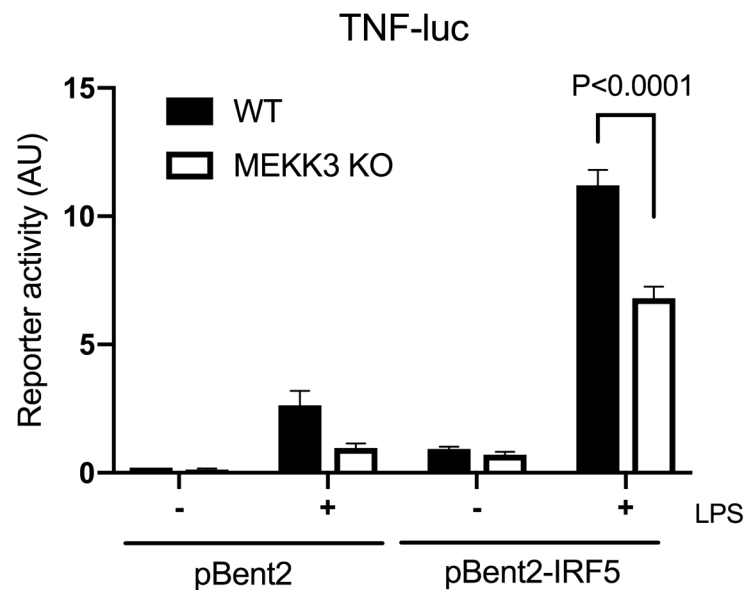


Figure 5.1. IRF5-driven TNF-reporter in MEKK3 KOs WT and MEKK3 KOs (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation) in a 96-well polylysine coated plate. Following an overnight incubation at 37°C, cells were stimulated with LPS (1 µg/ml) for 6 hours. Firefly and Renilla luciferase activity was measured on the FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of n=3 independent experiments, with each experiment performed in triplicates. Statistical analysis by unpaired Student's t-test.

control. This observation suggests that MEKK3 is involved in IRF5 driven *TNF*-transactivation.

5.2.2. MEKK3 deletion reduces pro-inflammatory cytokine gene expression

I then proceeded to explore whether IRF5-induced cytokines were similarly regulated in a MEKK3 dependent manner. To do this, I performed qPCR experiments in WT, IRF5 KO and MEKK3 KO RAW264.7 cells stimulated with LPS to trigger TLR4 signalling. Increases in *Il1a*, *Il1b*, *Il6* were observed in WT cells at early and later time points (**Figure 5.2**). The expression of pro-inflammatory cytokines *Il1a*, *Il1b*, and *Il6* were downregulated in IRF5 KOs and MEKK3 KOs in comparison to WT cells at 4 h LPS stimulation. Whereas, LPS-induced levels of the anti-inflammatory cytokine *Il10* was increased in both KO cell lines compared to WT at 2 and 4 h LPS stimulation. Interestingly, levels of *Il1a* were reduced further and levels of *Il10* were increased further in MEKK3 KOs compared to IRF5 KOs at 4 h. This suggests that MEKK3 targets other pathways involved in expression of these cytokines. Overall, these results highlight that MEKK3 may be necessary for LPS-induced

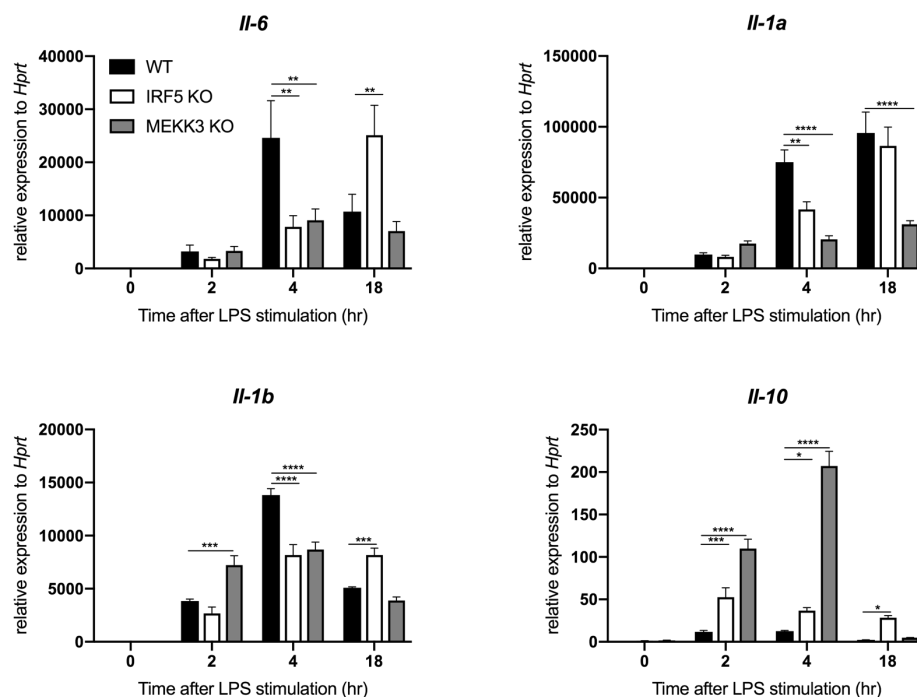


Figure 5.2. Cytokine profiles of LPS-stimulated RAW264.7 cells WT, IRF5 knockout, and MEKK3 knockout RAW264.7 cells (1×10^6) were stimulated with LPS (500 ng/ml) for 1, 2, 4, and 18 hours. RNA was extracted and 1 μ g (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il6*, *Il1a*, *Il1b*, and *Il10* were measured with real-time PCR and normalised to *Hprt* levels (housekeeping gene). Gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data shown as means $\pm$ SEM of three independent experiments conducted in duplicates. Statistical analysis was performed by 2-way ANOVA and Bonferroni's multiple comparison $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

expression of *Il1a*, *Il1b*, *Il6* and *Il10* in macrophage RAW264.7 cells where MEKK3 deficient cells phenocopies IRF5 deficient cells.

I then assessed the involvement of MEKK3 downstream of the Dectin-1 only agonist whole glucan particles (WGP) in WT, IRF5^{-/-} and MEKK3^{-/-} RAW264.7 cells over a time course. In response to WGP, WT cells produce high level of *Il10*, which was further increased in IRF5 and MEKK3 knockout cells at basal levels and with LPS stimulation (**Figure 5.3**). Levels of *Il6* and *Tnf* were similar in all cell lines at all timepoints. Overall, LPS stimulation seemed to have the most profound effect on cytokine induction, whereas WGP stimulation affects cytokine levels less robustly.

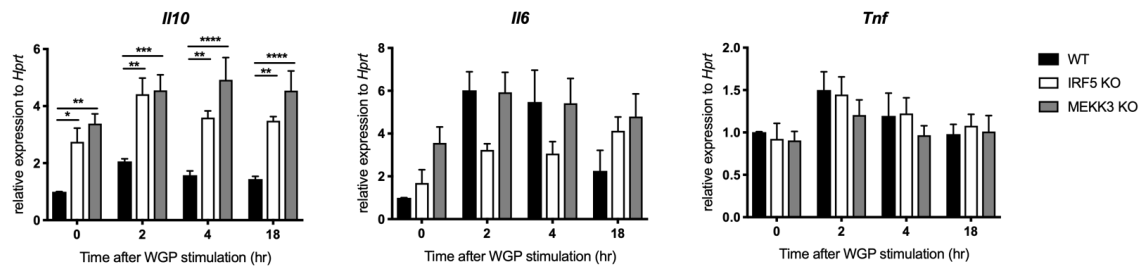


Figure 5.3. Cytokine profile of WGP-stimulated RAW264.7 cells WT, IRF5 knockout, and MEKK3 knockout RAW264.7 cells (1×10^6) were stimulated with WGP (100 μ g/ml) for 1, 2, 4, and 18 hours. RNA was extracted and 1 μ g (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of *Il10*, *Il6*, *Tnf* and *Hprt* (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data shown as means $\pm$ SEM of three independent experiments conducted in duplicates. Statistical analysis was performed by 2-way ANOVA and Bonferroni's multiple comparison $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$.

5.2.3. IRF5 kinetics in MEKK3 knockouts

Upon TLR4 activation with LPS, MEKK3 is activated on a specific serine site located within its activation loop and is essential for TLR-induced Il6 production (219). Serine phosphorylation on IRF5 is essential for its activation, so I next investigated whether MEKK3 had an effect on IRF5 serine phosphorylation. WT and MEKK3 KO RAW264.7 cells were stimulated with LPS and a phospho-serine antibody was used to detect the induction of IRF5 serine phosphorylation in cells immunoprecipitated with IRF5 antibody (**Figure 5.4**). LPS induced IRF5 serine phosphorylation in WT cells with similar levels of IRF5 immunoprecipitated between wells, confirming previous IRF5 kinetics carried out in Chapter 3.2. Serine phosphorylation was also induced with LPS in MEKK3 KOs to similar levels as WT. Western blot is probably too crude a method to detect any differences in phosphorylation on a single site, so it is possible that MEKK3 may target IRF5 on a site specific serine. Use of mass spectrometry would answer whether MEKK3 is capable of phosphorylating IRF5 and at which site(s). The contribution of other serine kinases in the absence of MEKK3 such as IKK β (95, 96) and other unknown kinases cannot be excluded as a possibility.

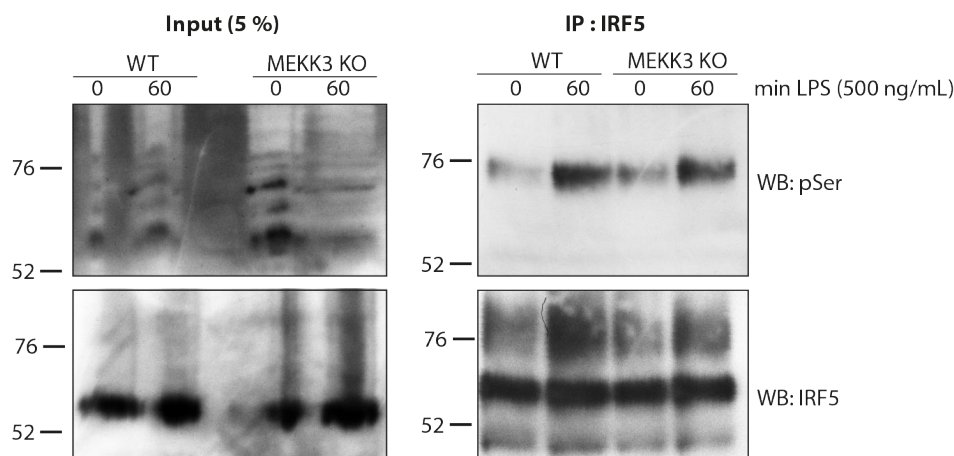


Figure 5.4. IRF5 immunoprecipitation in WT and MEKK3 KO cells IRF5 was immunoprecipitated from cell lysates (WT and MEKK3 CRISPR KO) after 60 min LPS treatment (500 ng/ml). Samples were resolved by SDS-PAGE and transferred to PVDF membrane for immunoblotting with IRF5 and phospho-serine antibodies. 5 % of non-immunoprecipitated lysate was kept for input control. WB = Western blot, IP = immunoprecipitation.

5.2.4. Testing of MEKK3 kinase inhibitors Batch I

Through our collaboration with *LAB282 (Evotec)*, a preliminary small panel identified 16 probes with >56% inhibition of MEKK3 at 1 μ M and half maximal inhibitory concentration (IC_{50}) in the nanomolar range (**Supplementary Figure S6**). Inhibitors were assessed for selectivity against a small panel of related MAP3K/IRF5 pathway targets to avoid confounding pharmacological interpretation. Of these 16 probes, 4 compounds were taken forward as leads on the basis of displaying less off-target effects to other kinases. The 4 lead compounds included Ruxolitinib (Probe EV'008) a JAK1/2 inhibitor, Neratinib (Probe EV'010) a dual HER2/EGFR inhibitor, PP121 (Probe EV'015) a dual tyrosine and phosphoinositide kinase inhibitor, and 5n (Probe EV'030) an ALK inhibitor. These 4 compounds were put forward for testing in cellular based assays (**Supplementary Figure S7**).

5.2.4.1. Cell viability of Batch I compounds

I tested the effect of the MEKK3-inhibitor compounds provided by *LAB282 (Evotec)* on cell viability to establish the concentration range (0.1 μ M-10 μ M) well tolerated by RAW264.7 cells. After 1 hr pre-treatment with the inhibitors or DMSO carrier, RAW264.7 cells were stimulated with LPS for a further 2h, 6h or 17h. Cell viability was assessed by the CTG viability assay and the IC_{50} drug dose which is required to achieve 50% percent cell viability was determined based on the curve of the slope (**Figure 5.5**). No effect was observed with the control DMSO vehicle compound at all time points and concentrations. Compounds EV'030 (5N) and EV'015 (PP121) displayed lower IC_{50} cell viability values with longer inhibitor incubations. Cells incubated with compound EV'010 (Neratinib) were viable at 3 h and 7 h treatment, but the compound affected cell viability at longer 18 h incubations with an IC_{50} cell viability value of 6 μ M. Cells treated with compound EV'008 (Ruxolitinib) displayed greater than 80 % cell viability at all incubation times.

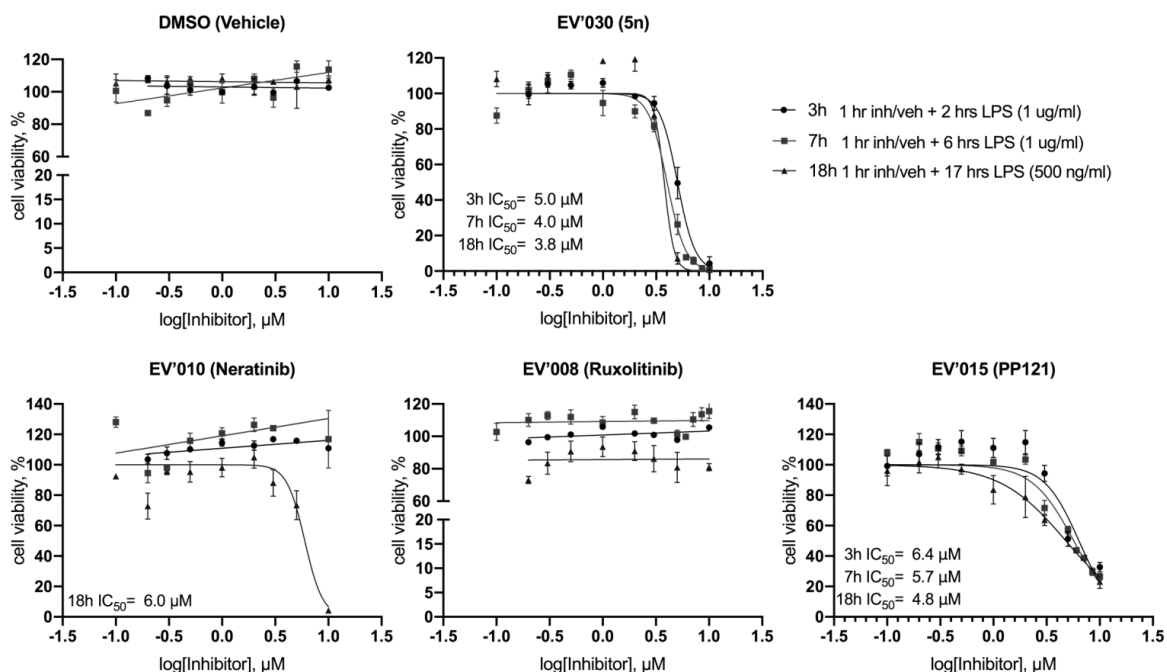


Figure 5.5. Cell viability of RAW264.7 cells titrated with Batch I compounds RAW264.7 cells (3×10^5) plated in triplicates were pre-treated with inhibitor Probes EV'030, EV'010, EV'008, EV'015 or DMSO vehicle at indicated concentrations ($\log_{10}$ transformed) for 1 hour, followed by LPS stimulation ($1 \mu\text{g/ml}$) for a further 2 h, 6 h, or 17 h. Cell viability was assessed with the CTG viability assay by normalising luminescence signals from treated cells to untreated cells and expressed as a %. Cell viability IC_{50} values for each probe are indicated on the graphs and calculated from the curve of the slope.

5.2.4.2. *TNF-reporter*

I next examined whether the probes had any effect on IRF5-dependent gene expression. To this end, a IRF5-driven TNF luciferase reporter assay was carried out in RAW264.7 cells pre-treated with inhibitor or DMSO carrier control and stimulated with LPS. Based on the previous viability data, cells were treated with compounds EV'008 and EV'010 at dose concentrations of $\leq 5 \mu\text{M}$, and compounds EV'030 and EV'015 at dose concentrations of $\leq 2 \mu\text{M}$. Co-expression of IRF5 and LPS stimulation strongly induced *Tnf*-luciferase reporter activity in the DMSO control (**Figure 5.6**). Ectopic expression of IRF5 and LPS stimulation induced TNF-reporter activity to similar levels to DMSO control in cells treated with probes EV'010, EV'030 and EV'015 even at higher compound dose concentrations. In contrast, LPS-stimulated IRF5 expressing cells treated with increasing doses of probe EV'008 display a marked increase in TNF luciferase activity. I hypothesised that this effect was due to other kinases and signalling pathways, as probe EV'008 displayed inhibition to several

kinases including IRAK1, JAK2, MEKK2 and TAK1 in the small kinase panel (Supplementary Figure S6), and likely to display specificity to other kinases in the kinome.

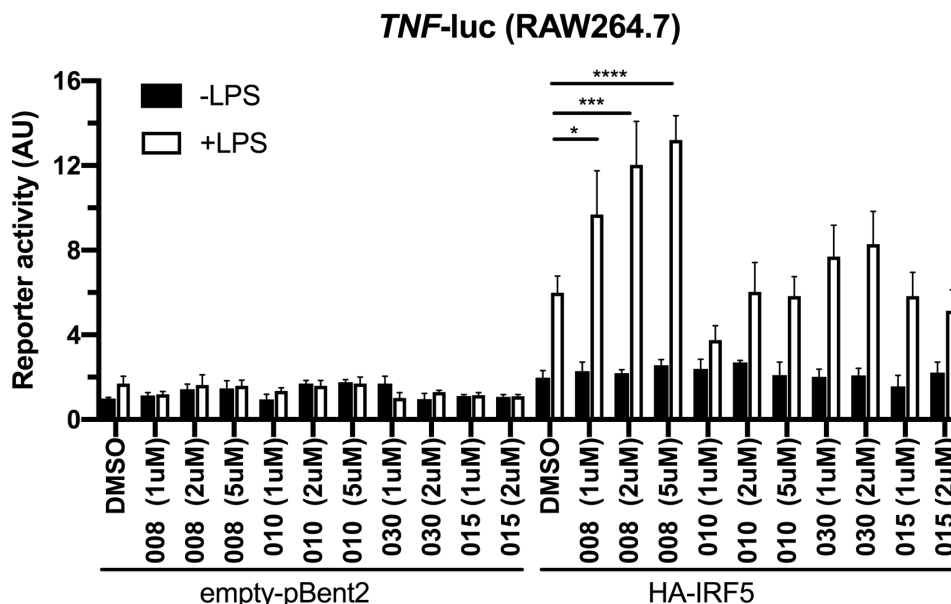


Figure 5.6. TNF-reporter with Batch I compounds WT RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pretreated with 1/2/5 μ M EV'008 probe (Ruxolitinib), EV'010 (Neratinib), EV'030 (5N), EV'015 (PP121) or DMSO vehicle control for 1 hour, followed by LPS (1 μ g/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of $n=3$ independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

To elucidate whether this effect was a MEKK3-driven process or due to other kinases I carried out the reporter assay in WT and MEKK3 deficient RAW264.7 cells. As observed previously increasing doses of EV'008 significantly increased TNF-reporter activity compared to DMSO only in LPS-stimulated IRF5 expressing cells (**Figure 5.7**). A similar effect was found in MEKK3 KO cells suggesting that the compound affected the activity of other kinases or other endogenous transcription factors involved in regulation of the *Tnf* gene.

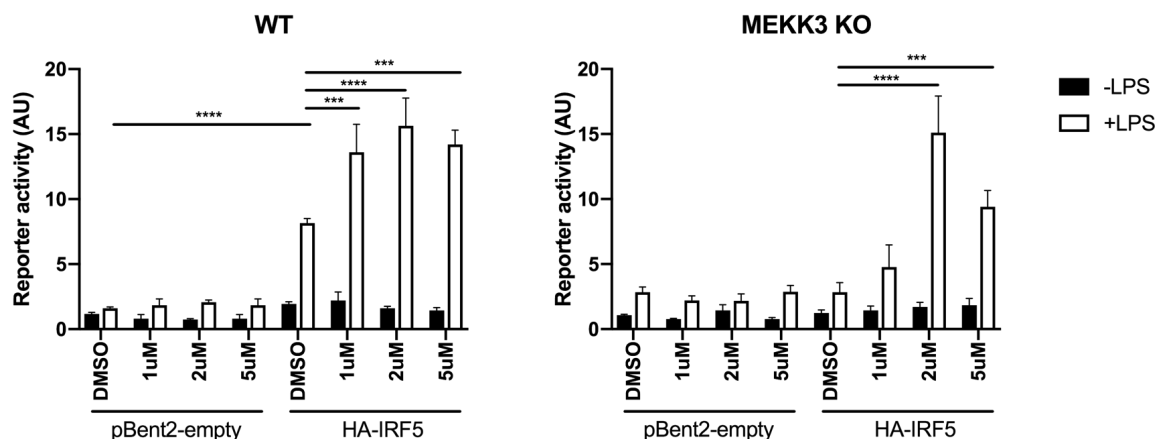


Figure 5.7. TNF-reporter with EV'008 inhibitor WT and MEKK3 KO RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pretreated with 1/2/5 μM EV'008 probe (Ruxolitinib) or DMSO vehicle control for 1 hour, followed by LPS (1 μg/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of $n=3$ independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test *** $P \leq 0.001$, **** $P \leq 0.0001$.

5.2.5. Testing of follow up compounds Batch II

Analogues of EV'010 (Neratinib) were designed by Evotec including EV'037, EV'487, EV'039, EV'041, EV'902. Both EV'037 and EV'487 display similar potency to Neratinib with similar MEKK3 IC_{50} values. By deleting the Michael acceptor of Neratinib, both compounds EV'037 and EV'487 display better selectivity, with reduced inhibition towards MEKK2 (**Figure 5.8**). A aniline to phenol swap of Neratinib to generate probe EV'039 improved selectivity in the

EOAI	3411488 (Probe 010)	3999037	3411487	3999039	3983902
Structure					
CHUK (IKK alpha) - Adapta - Km app	-6	6	1	-9	0
IKKB (IKK beta) - ZLYTE - Km app	-9	6	0	4	6
IRAK1 - Adapta - Km app	86	41	50	1	4
IRAK4 - ZLYTE - Km app	10	17	17	18	-8
JAK2 - ZLYTE - Km app	2	2	-4	-1	2
MAP3K11 (MLK3)	30	44	47	12	6
MAP3K2 (MEKK2)	81	77	61	13	-1
MAP3K5 (ASK1)	2	-26	-14	-3	-15
MAP3K7/MAP3K7IP1 (TAK1-TAB1)	15	37	22	3	15
MAP3K8 (COT) - ZLYTE - 100	35	67	65	4	11
MAPK7 (ERK5) - ZLYTE - Km app	-2	17	16	9	-2
PTK2B (FAK2) - ZLYTE - Km app	n/a	33	33	-9	5
RIPK2	10	8	8	4	13
STK4 (MST1) - ZLYTE - Km app	99	94	96	76	6
TBK1 - ZLYTE - Km app	n/a	15	13	5	4
MEKK3 IC_{50} (nM)	25	23	31	197	143

kinase involved in IRF5 phosphorylation

selectivity data @ 1 μM

< 40% inhibition
 40-80% inhibition
 > 80% inhibition

Figure 5.8 Screening panel for Probe EV'010 follow-up compounds (Batch II) Potency and selectivity data at 1 μ M on analogs of Probe EV'010 (Neratinib) including EV'037, EV'487, EV'039 and EV'902. Red dashed boxes indicate points at which chemical structures were modified. Reported IRF5 kinases in blue, < 40% inhibition (green), 40-80% inhibition (yellow), >80% inhibition (red). Data and table generated by Evotec (LAB282).

mini kinase panel albeit with lower potency towards MEKK3. The pyridine replacement of Neratinib to generate analog EV'902 appears even more selective with better potency than EV'039 in the mini panel.

5.2.5.1. Cell viability of Batch II compounds

To assess the toxicity of probe EV'010 follow-up compounds, CTG viability assays were performed in RAW264.7 cells 3 h and 7 h after treatment with 0.1-10 μ M of inhibitor. These assays revealed no significant apparent toxicity to RAW264.7 cells at all concentrations tested when pre-treated 1 hour with inhibitor and stimulated with LPS for a further 2 or 6 h

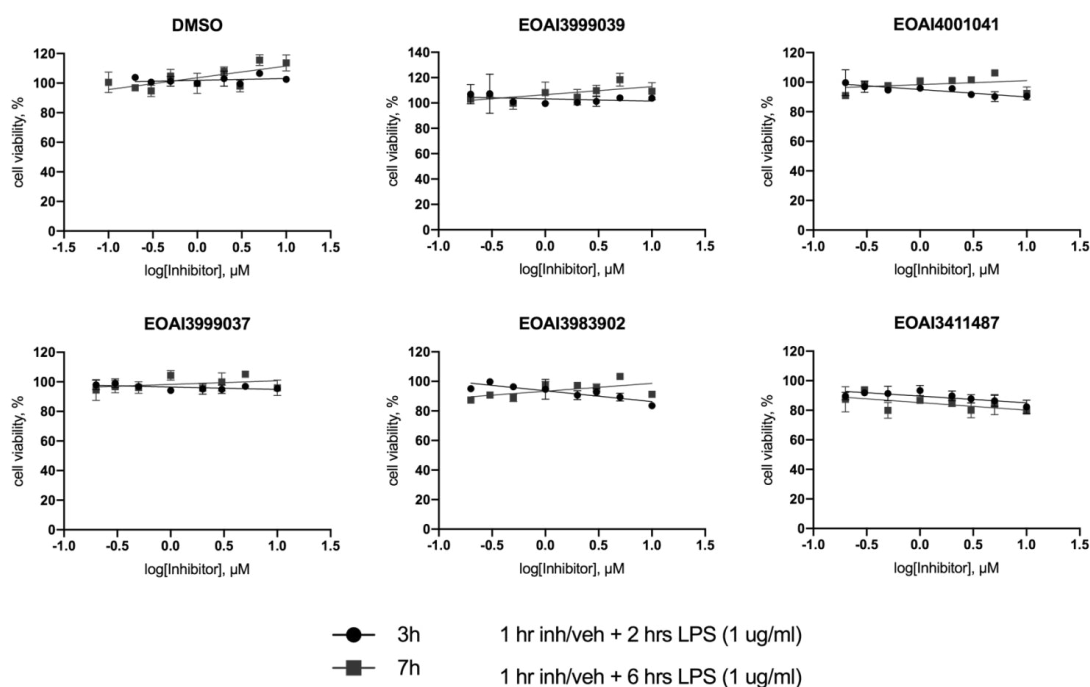


Figure 5.9. Cell viability of RAW264.7 cells titrated with EV'010 follow-up compounds RAW264.7 cells (3×10^5) plated in triplicates were pre-treated with inhibitor Probes EV'039, EV'041, EV'037, EV'902, EV'487 or DMSO vehicle at indicated concentrations ($\log_{10}$ transformed) for 1 hour, followed by LPS stimulation (1 μ g/ml) for a further 2 h or 6 h. Cell viability was assessed with the CTG viability assay by normalising luminescence signals from treated cells to untreated cells and expressed as a %. Cell viability IC_{50} values for each probe are indicated on the graphs and calculated from the curve of the slope.

(**Figure 5.9**). No effect was observed with DMSO control vehicle at both time points. As an initial starting point 2 and 5 μM of inhibitor was selected for further experiments.

5.2.5.2. *TNF-reporter*

The IRF5-driven *Tnf* reporter gene assay was carried out in RAW264.7 cells treated with probe EV'010 analogs to see the effect of the inhibitors on IRF5 transactivation. Transfected cells were pre-incubated with EV'010 follow-up compounds at 2 and 5 μM , followed by LPS stimulation. As shown in **Figure 5.10**, LPS stimulated IRF5 expressing cells treated with compounds EV'039 and EV'037 displayed reduced TNF-reporter activity at both concentrations tested compared to DMSO control. Whereas the remaining probes (EV'041, EV'902 and EV'487) had a similar effect on reporter activity to DMSO vehicle control. This

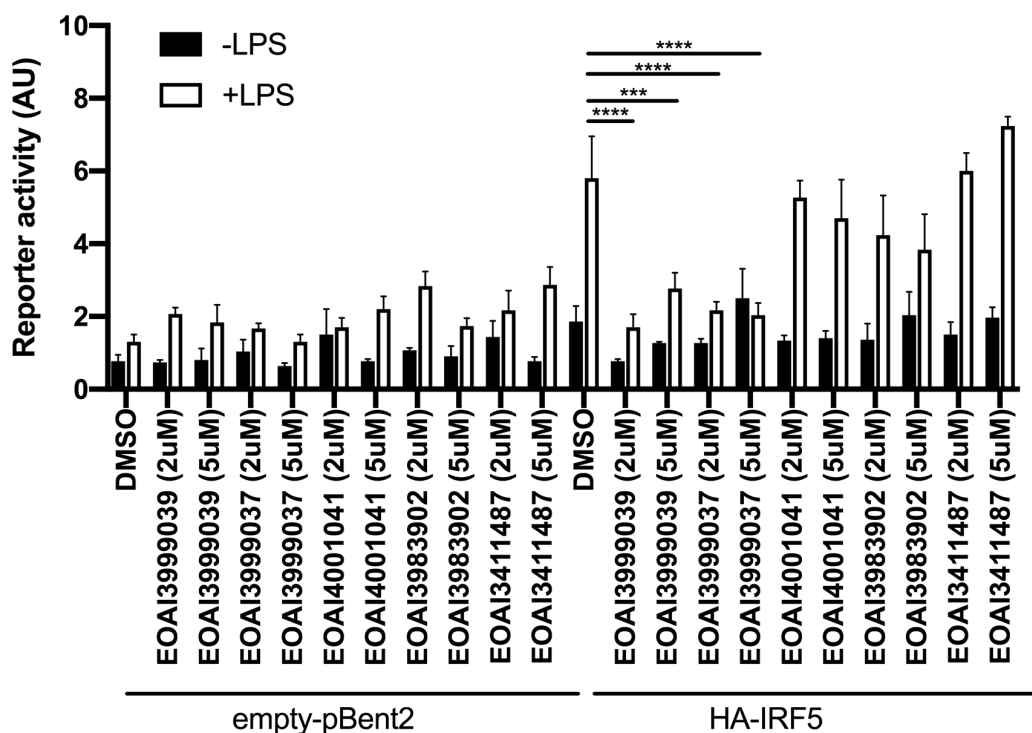


Figure 5.10. TNF-reporter with EV'010 follow-up compounds WT RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C , cells were pretreated with 2/5 μM EV'039, EV'037, EV'0341, EV'902, EV'039, EV'487 or DMSO vehicle control for 1 hour, followed by LPS (1 $\mu\text{g}/\text{ml}$) stimulation for a further 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay using a FLUOstar Omega microplate reader to measure luminescence levels. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of $n=3$ independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test where *** $p \leq 0.001$, **** $p \leq 0.0001$.

data suggests that compounds EV'039 and EV'037 may target MEKK3 to control IRF5-driven TNF activation.

Compounds EV'039 and EV'037 were taken forward as lead compounds and were titrated to lower doses to test whether the effect on reporter activity occurred at lower concentrations. Transfected cells were pre-incubated with the probes at 0.5, 1 and 5 μ M or DMSO carrier and stimulated with LPS. Ectopic expression of IRF5 and LPS stimulation induced TNF-luciferase reporter activity (**Figure 5.11**). Pre-treatment of cells with compound EV'037 reduced TNF-reporter activity at all concentrations tested, whereas compound EV'039 affected reporter activity only at concentrations above 1.0 μ M.

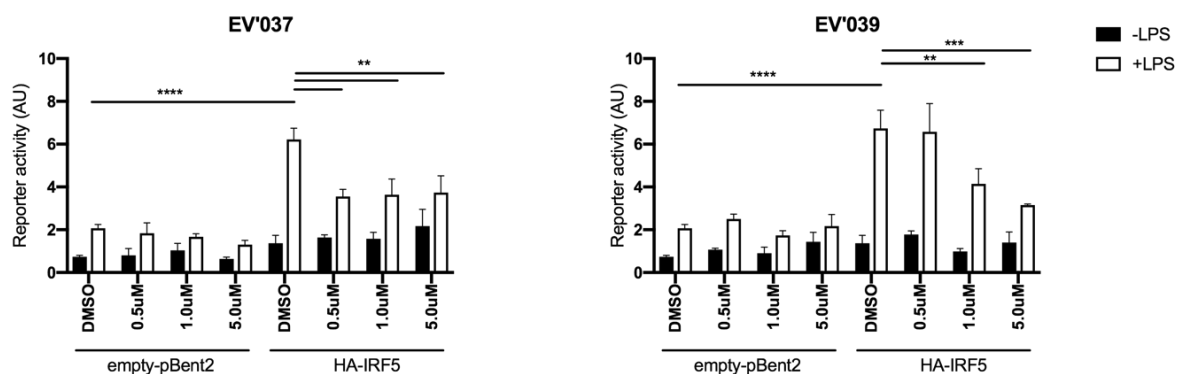


Figure 5.11. Titration of compounds EV'037 and EV'039 WT RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pretreated with 0.5/1/5 μ M EV'039, EV'037, or DMSO vehicle control for 1 hour, followed by LPS (1 μ g/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader to measure luminescence levels. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of $n=3$ independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test where ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

Given the promising results of compounds EV'037 and EV'039, I assessed its selectivity by testing in MEKK3 and IRF5 deficient cells. Confirming previous results, both probes reduced TNF-reporter activity in WT LPS-stimulated IRF5 expressing cells (**Figure 5.12**). In MEKK3 KO cells, LPS stimulation with ectopic IRF5 expression increased reporter activity, which was reduced in cells incubated with compound EV'037 but not compound EV'039. This suggests that probe EV'037 is not specific to MEKK3 and displays off-target

effects, whereas EV'039 displays specificity to MEKK3. This correlates well with the screening panel data (**Figure 5.8**) where EV'037 displayed more kinase off-target effects than EV'039. In IRF5 deficient cells in which IRF5 is put back in the system by ectopic expression, EV'037 had no further effect on LPS-induced reporter activity compared to DMSO control, whereas EV'039 reduced reporter activity. This suggests that the effects of EV'039 are possibly delivered by IRF5 but requires further investigation. Overall, these results show that EV'039 displays the desired profile with inhibition of LPS/IRF5 induced TNF activity with limited effect on MEKK3 KO cells and similar effects in IRF5 KO cells in which IRF5 is ectopically expressed.

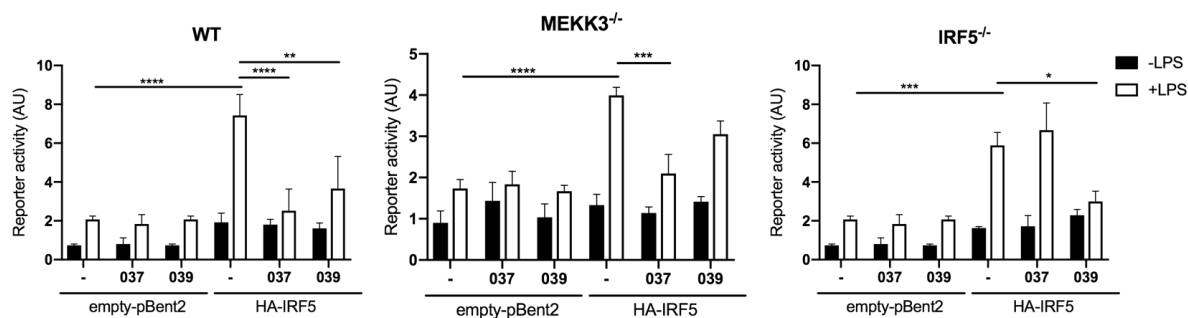


Figure 5.12. TNF reporter in knockout lines WT, MEKK3^{-/-}, IRF5^{-/-} RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pretreated with 1 μ M EV'039, EV'037, or DMSO vehicle control for 1 hour, followed by LPS (1 μ g/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader to measure luminescence levels. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of n=3 independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

5.2.5.3. Gene expression profile in macrophages

The compounds were tested in RAW264.7 cells to see their effect on cytokine gene expression. Cells were pre-treated with inhibitor or DMSO for 1 hour, followed by LPS stimulation for a further 2 hours. Compound EV'039 was selected as the lead probe and EV'010 as the compound EV'039 was derived from as a comparison. Stimulation with LPS/DMSO increased cytokine expression of *Il1a*, *Il1b*, *Il6*, and *Tnf* which was reduced with treatment with compound EV'010 at low and high concentrations (**Figure 5.13**). However,

compound EV'039 which affected TNF-reporter activity had no further effect on gene expression at both concentrations tested. This negative result may be due to compound EV'039 being less potent to MEKK3 compared to EV'010 with an eight-fold higher IC_{50} value towards MEKK3 (**Figure 5.8**). Although probe EV'039 appears more selective, the kinase profile panel was screened against a subset of pathway relevant kinases, and a full kinome profile is needed to determine its specificity.

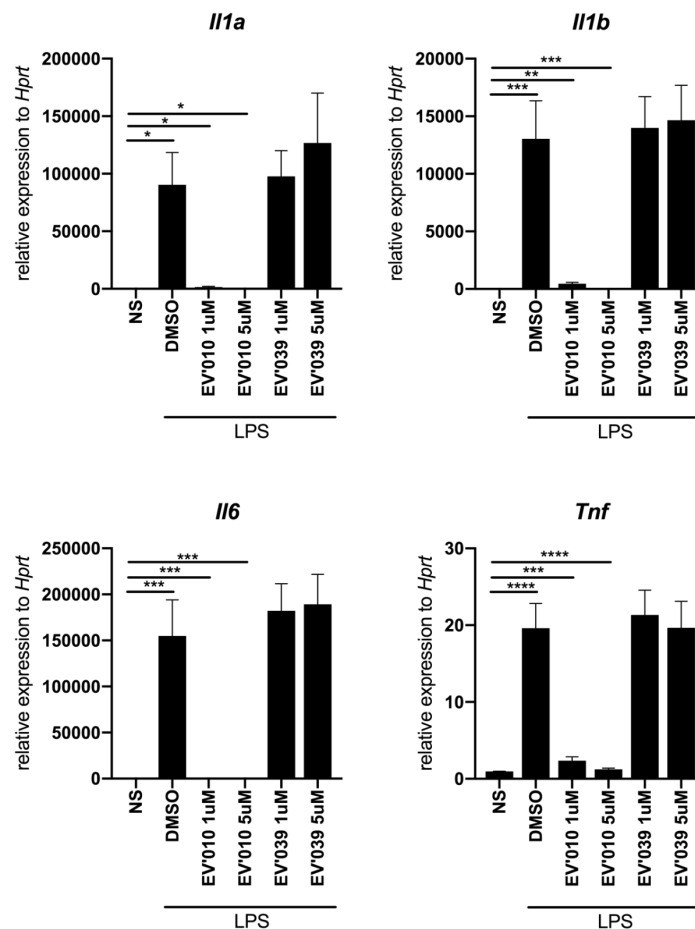


Figure 5.13. Gene expression of RAW264.7 cells with compound Cytokine level profiles of RAW264.7 cells pre-treated with DMSO, EV'010, EV'039 at 1 or 5 μ M for 1 hour, followed by LPS (1 μ g/ml) stimulation for 2 hours. RNA was extracted and 150 ng (as determined by Nanodrop quantification) reverse transcribed to cDNA. Transcript levels of IL1a, IL1b, IL6, Tnf, and Hprt (housekeeping gene) were measured with real-time PCR and gene expression was analysed using the change in threshold $\Delta\Delta C_t$ -method. Data are shown as means $\pm$ SEM for $n=3$ individual experiments and analysed by one-way ANOVA * $P<0.05$, ** <0.01 *** <0.001 , **** $P<0.0001$.

5.2.6. Testing of follow up compounds Batch III

The last set of 15 compounds developed by *Evotec* were tested for cell evaluation. These included analogs of batch II compounds (EV'039, EV'487, EV'902) with improved MEKK3 IC_{50} values and solubility (**Supplementary Figure S8**).

5.2.6.1. Cell viability of Batch III compounds

All 15 compounds synthesised by *Evotec* were tested in RAW264.7 cells for their viability. Cells were pre-treated with DMSO vehicle control or the compound at a range of concentrations (0.3-10 μ M) for 1 h, followed by LPS stimulation for a further 6 h. No effect on cell viability was observed with incubation with the DMSO carrier, EV'355, EV'272, EV'328, EV'326, EV'475, EV'433, EV'476, EV'432, EV'692 and EV'771 (**Figure 5.14**). However, at higher concentrations probes EV'691, EV'355, EV'693, EV'771, EV'694 were toxic to the cells with cell viability IC_{50} values indicated on the graphs. Based on these results an initial probe concentration of 2 μ M was selected for further experiments.

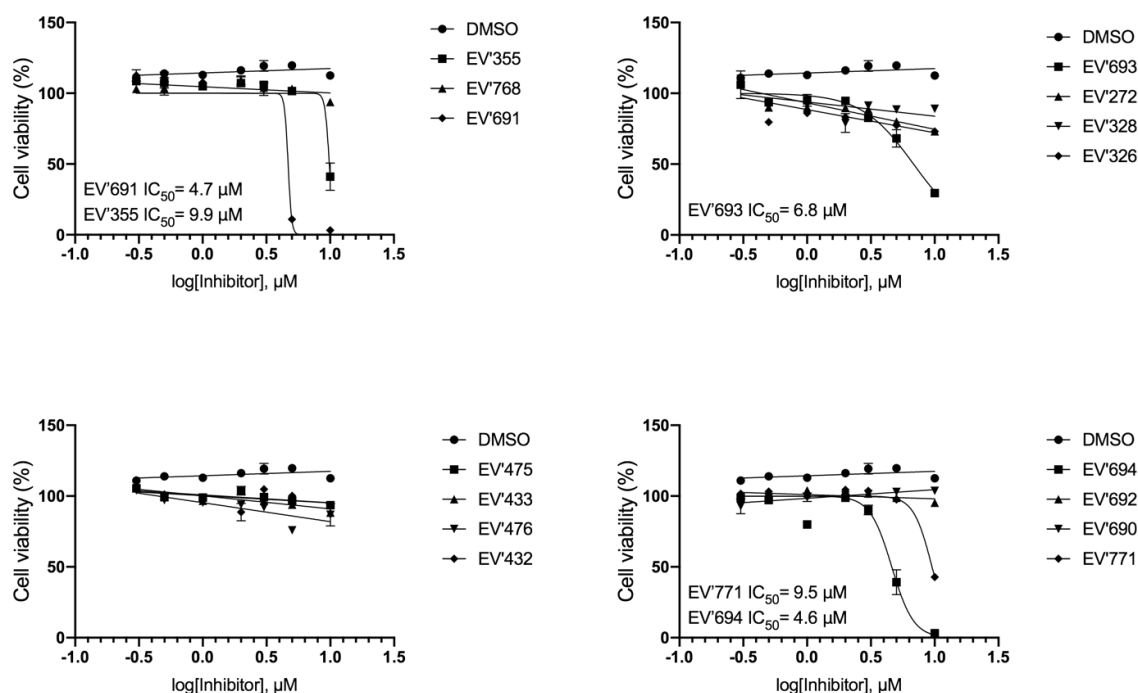


Figure 5.14. Cell viability of RAW264.7 cells titrated with LAB282 follow-up compounds RAW264.7 cells (3×10^5) plated in triplicates were pre-treated with inhibitor probes EV'355, EV'768, EV'691, EV'693, EV'272, EV'328, EV'326, EV'475, EV'433, EV'476, EV'432, EV'694, EV'692, EV'690, EV'771 or DMSO vehicle at indicated concentrations ($\log_{10}$ transformed) for 1 hour, followed by LPS stimulation (1 μ g/ml) for a further 6 hours. Cell viability was assessed with the CTG viability assay by normalising luminescence signals from treated cells to untreated cells and expressed as a %. Cell viability IC_{50} values for each probe are indicated on the graphs and calculated from the curve of the slope.

5.2.6.2. *TNF-reporter in WT cells*

All 15 compounds were tested in RAW264.7 cells along with compound EV'039 for comparison. Cells were pre-treated with DMSO vehicle carrier or inhibitor for 1 hour at 2 μ M followed by LPS stimulation for a further 6 hours. Ectopic expression of IRF5 increased LPS induced TNF-reporter activity seven-fold (**Figure 5.15**). As anticipated and confirming previous findings, pre-incubation with inhibitors EV'039 reduced reporter activity. EV'039 follow-up compounds EV'475, EV'433 also reduced reporter activity to the same levels. However, EV'694 a EV'039 analog had no further effect on LPS-IRF5 driven reporter activity. Compounds EV'432 and EV'272 (probes derived from EV'487) also reduced reporter activity. The remaining probes increased reporter activity to the same levels as DMSO only treated cells, so were excluding for further experiments.

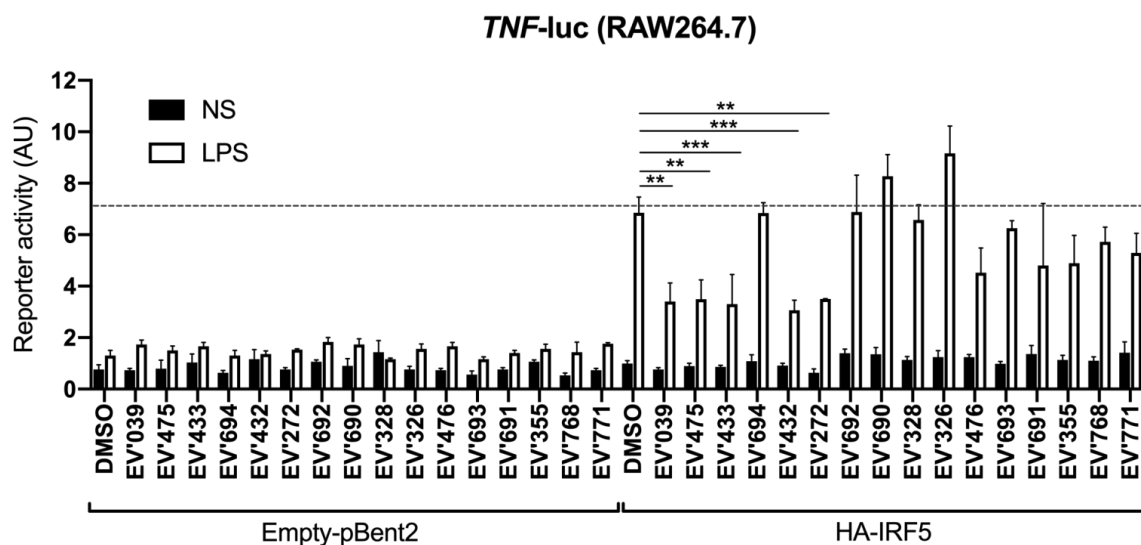


Figure 5.15. TNF-reporter of LAB282 batch III compounds WT RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pre-treated with 2 μ M of EVOTEC probes or DMSO vehicle control for 1 hour, followed by LPS (1 μ g/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader to measure luminescence levels. Firefly luciferase levels were normalised to Renilla luciferase levels Error bars show SEM of n=3 independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test where ** $p \leq 0.01$, *** $p \leq 0.001$.

5.2.6.3. *TNF-reporter in knockouts*

The most promising batch III compounds (EV'432, EV'272, EV'475, EV'433) were taken forward for retesting in WT, IRF5 KOs and MEKK3 KOs. I also included the compounds in which the probes were derived from as comparisons - EV'039 for EV'475 and EV'433, EV'487 for EV'432 and EV'272. Compounds EV'475 and EV'433 display improved MEKK3 IC₅₀ values than EV'039 but with similar kinase selectivity (**Supplementary Figure S8**). Compound EV'272 displays better kinase selectivity but less potent to MEKK3 compared to its derived compound EV'487, where EV'432 displays similar MEKK3 potency and kinase selectivity to EV'487 (**Supplementary Figure S8**).

In agreement with previous data all the compounds excluding EV'487 reduced reporter activity in LPS stimulated WT cells overexpressing IRF5 (**Figure 5.16**). Likewise all the compounds excluding EV'487 reduced reporter activity in IRF5 KO cells in which IRF5 is put back into the system suggesting the effects of the inhibitor are delivered via IRF5. LPS stimulation and ectopic expression of IRF5 induced TNF-reporter activity in MEKK3 KOs but to a lesser extent than WTs. Pre-incubation with the probes had no further effect in MEKK3 KOs suggesting that the inhibitors display specificity to MEKK3. Overall, after rounds of chemical modification of the lead compound Neratinib, the data generated here has resulted in the identification and synthesis of derivatives with biochemical potency towards MEKK3, improved selectivity against a small panel of pathway relevant kinases and the desired effect in cell based assays.

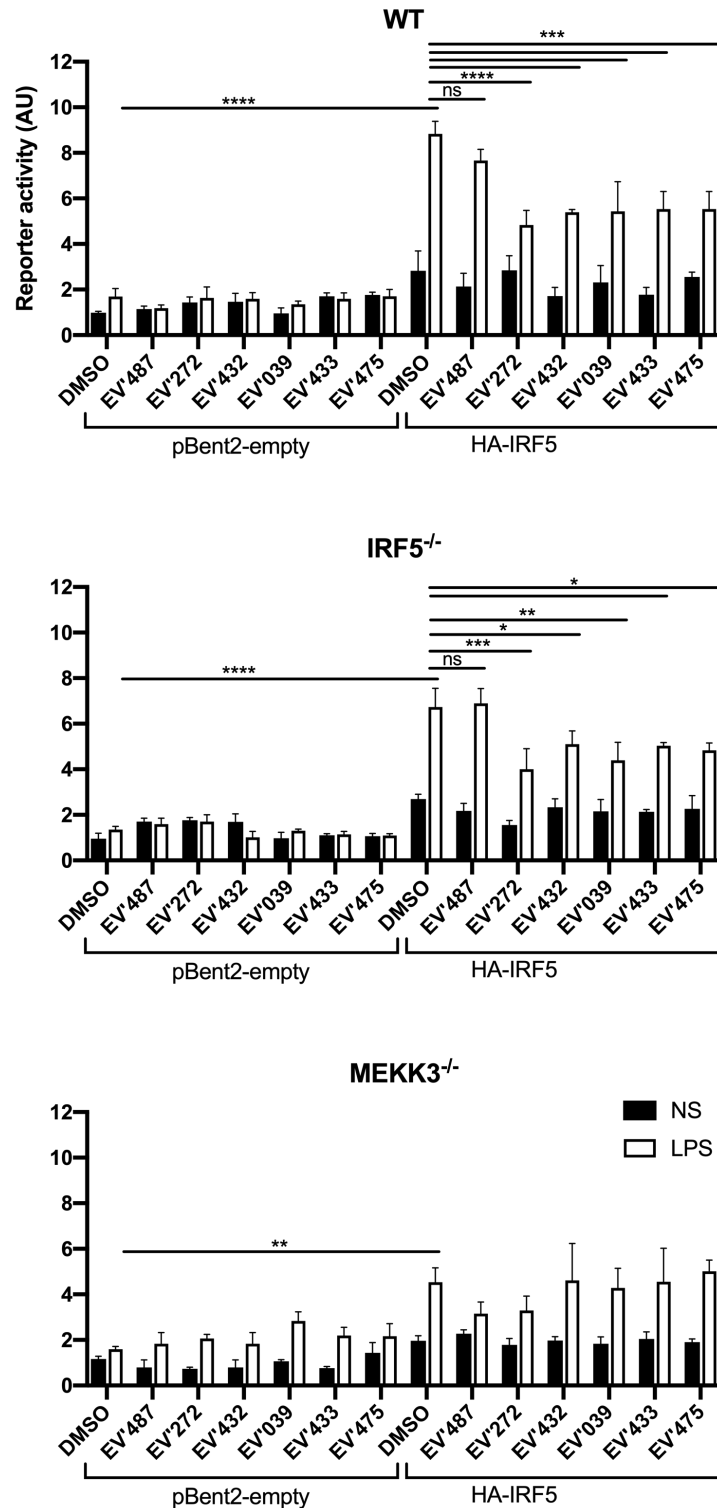
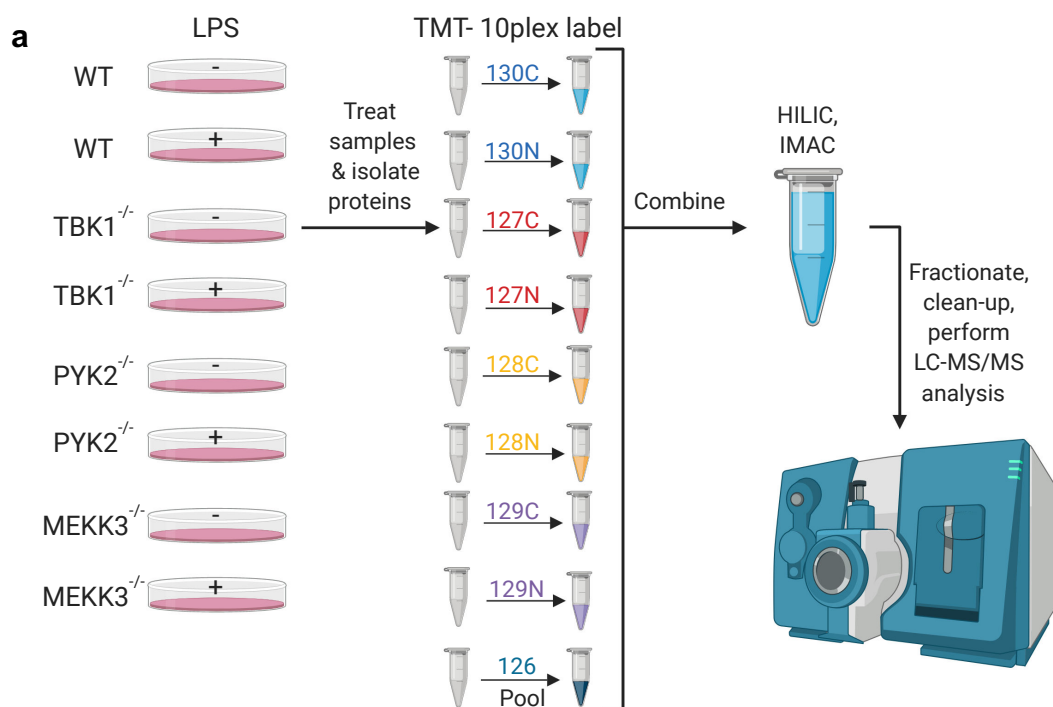


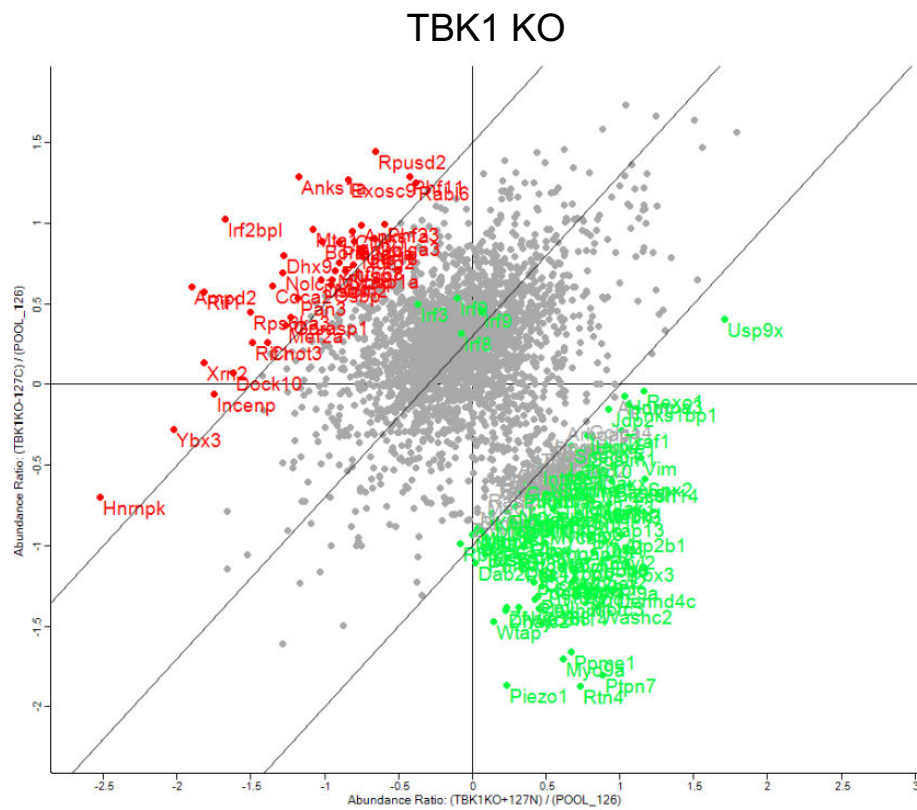
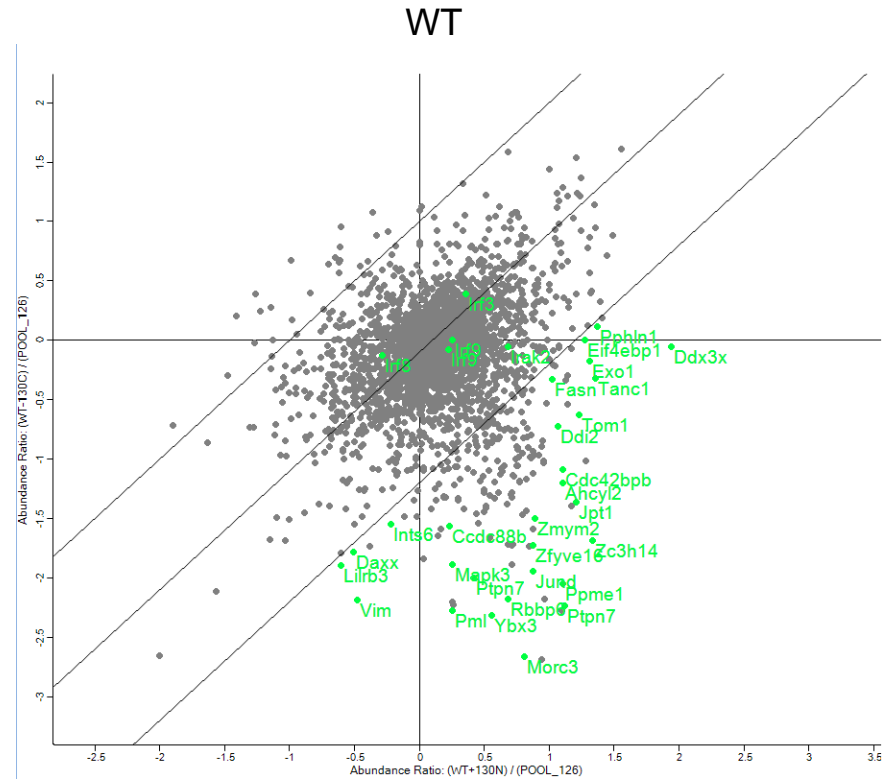
Figure 5.16. TNF reporter in knockout lines WT, MEKK3 KO, and IRF5 KO RAW264.7 cells (5×10^5) were transfected with TNF-luciferase, HA-IRF5/pBent2 empty vector, and with a Renilla luciferase expression plasmid (for normalisation). Following an overnight incubation at 37°C, cells were pretreated with 2 μ M EV'487, EV'272, EV'432, EV'039, EV'433, EV'475 or DMSO vehicle control for 1 hour, followed by LPS (1 μ g/ml) stimulation for 6 hours. Firefly and Renilla luciferase activity was measured by Dual Glo luciferase assay on the FLUOstar Omega microplate reader to measure luminescence levels. Firefly luciferase levels were normalised to Renilla luciferase levels. Error bars show SEM of $n=3$ independent experiments, each performed in triplicates. Statistical analysis by unpaired Student's t-test where * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$.

5.2.7. Global phosphoproteome of kinase deficient cell lines

I next sought to compare the phosphoproteome of unstimulated and LPS-stimulated samples in WT and kinase knockout lines (MEKK3, PYK2, TBK1) to glean information into the potential substrates and signaling pathways modulated by each kinase. I performed a global phosphoproteome using Tandam mass tagging (TMT) isobaric labelling which enables the simultaneous measurement of up to 10 samples labelled on peptide level, followed by phosphopeptide enrichment coupled to tandem mass spectrometry. Each sample was labelled by a different tag and a pooled sample was also tagged to facilitate equal sample treatment and better quantification (**Figure 5.17a**). Following tryptic digestion, phosphopeptide enrichment, and quantitative mass spectrometry, I evaluated the effect of each cell line and stimulus in each cell population. A total of ~5,000 phosphosites were identified. The preliminary data shows the phosphopeptide abundance for each cell line plus/minus LPS relative to the pooled sample (**Figure 5.17b**).



b



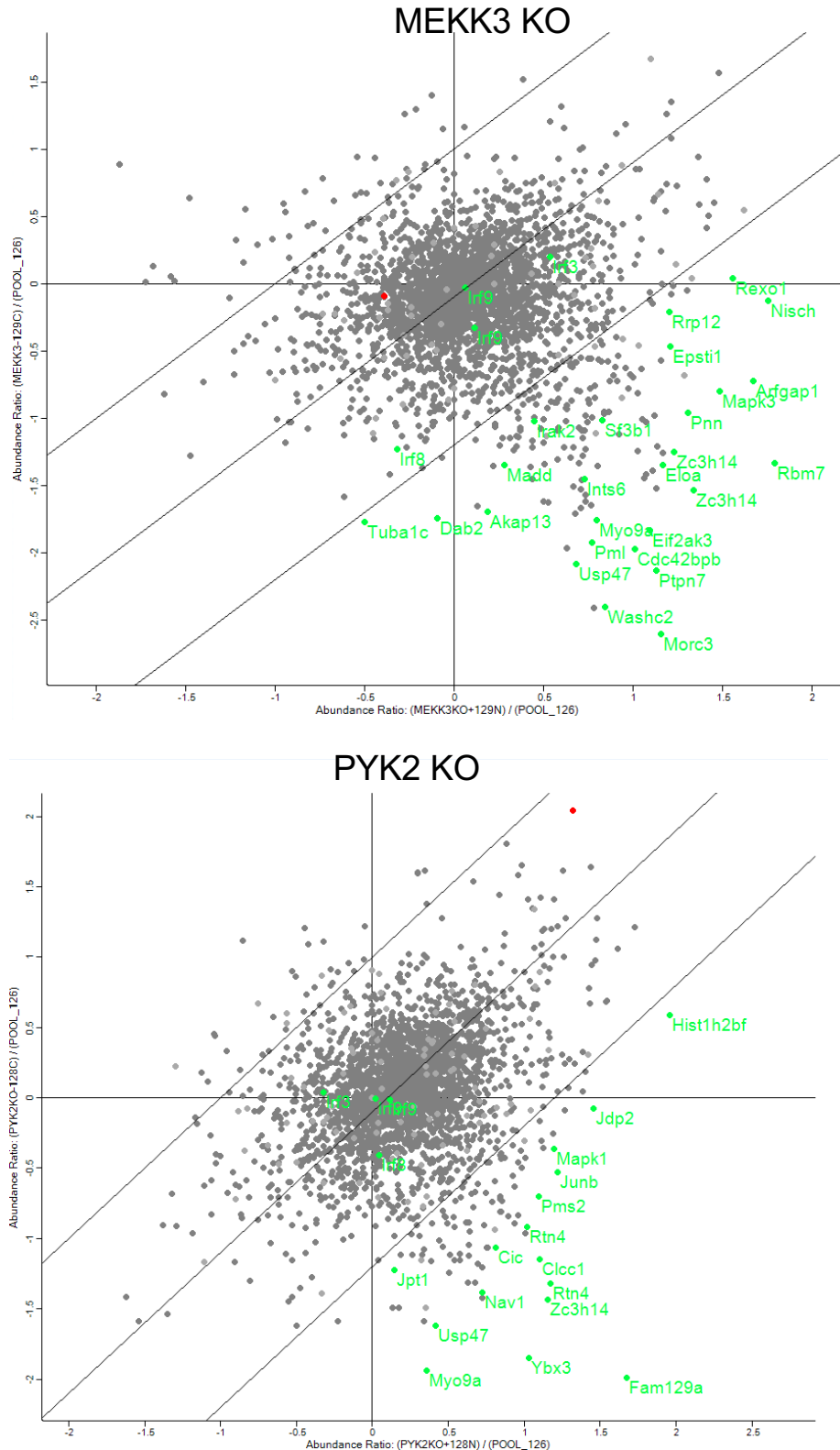


Figure 5.17. Global phosphoproteome of WT and kinase deficient cells lines (a) Experimental setup and design. WT, TBK1 KO, PYK2 KO and MEKK3 KO cells were stimulated with LPS (500 ng/ml) for 1 h or left untreated. Cell lysates for each condition, and a pooled sample were TMT labelled with different isotopes, fractionated and enzymatically digested into peptides. Phosphopeptides were enriched on TiO₂ beads and analysed by LC-MS/MS. **(b)** Scatter plots of peak reporter ion intensities for phosphopeptides derived from WT and kinase KO lines and were subjected to LPS stimulation (x axis) or no-stimulation (y axis). Data normalised to the pooled sample. Plots were generated on Perseus software.

I found that that the LPS targets mitogen-activated protein kinase 3 (MAPK3), fatty acid synthase (FASN), and DEAD-box helicase (DDX3X) behaved as predicted (220–222) (**Figure 5.17b, Table 5.1**). FASN has been reported to be essential for the synthesis of cholesterol during LPS activation of macrophages (220), and the upregulation of FASN validates this. The global phosphoproteome showing LPS-induced DDX3X phosphorylation in all cells excluding TBK1 KO confirms the finding that DDX3X is phosphorylated by TBK1 (221) (**Figure 5.17b, Table 5.1**). However, the lack of induction of IRF3, a known TBK1 substrate in WT cells highlights that the global phosphoproteome is not capturing all the phosphorylation events. Other reported TBK1 substrates such as the tyrosine phosphatase PTPN12, should be increased in TBK1 KO (223), however my results does not reflect this. Likewise, paxillin a known PYK2 substrate (170) is induced with LPS but not further affected in PYK2 deficient cells.

Protein Name	Gene Name	Fold Change			
		WT	MEKK3 KO	PYK2 KO	TBK1 KO
DEAD-Box Helicase 3 X-Linked	DDX3X	3.98	2.62	2.81	0.83
Fatty acid synthase	FASN	2.56	2.74	2.68	2.92
Heterogeneous nuclear ribonucleoprotein F	HNRNPF	5.43	1.55	3.13	3.74
Interferon regulatory factor 3	IRF3	0.98	0.65	0.78	0.55
Interferon regulatory factor 8	IRF8	0.89	0.88	1.37	0.76
Interferon regulatory factor 9	IRF9	1.23	1.48	1.02	0.64
Inhibitor Of Nuclear Factor Kappa B Kinase Subunit Beta	IKBKB	1.10	1.10	1.10	1.15
Mitogen-Activated Protein Kinase 3	MAPK3	4.39	1.82	2.43	3.08
Paxillin	PXN	2.84	2.44	2.21	2.13
Protein Tyrosine Phosphatase Non-Receptor Type 12	PTPN12	1.34	1.41	1.05	0.94
Ubiquitin Specific Peptidase 47	USP47	3.90	1.40	4.10	2.21

Table 5.1. Ratios of phosphopeptide intensities for selected proteins LPS stimulated/non-treated normalised to pool control for WT, MEKK3, TBK1 and PYK2 KO for proteins mentioned in main text.

The reduced phosphorylation of MAPK3/ERK1 specifically in MEKK3 KO is consistent with reports of MEKK3 targeting MAPK3 (222) (**Figure 5.17b, Table 5.1**). In RAW264.7 cells, LPS induced phosphorylation of MAPK3 at later timepoints (60 and 120 min post-LPS stimulation) (222), which is consistent with my findings. The downregulation of phosphosites of reported LPS-induced proteins including heterogeneous nuclear

ribonucleoprotein F (HPRNTP) and Ubiquitin Specific Peptidase 47 (USP47) (224, 225), specifically in MEKK3 KO cells suggests a role for MEKK3. These targets have not been reported to be linked to MEKK3 and could be possible MEKK3 substrates worth exploring. The absence of p38 and JNK in the global phosphoproteome which should be reduced in MEKK3 deficient cells (222), again highlights that there are issues with phosphoproteome coverage.

The lack of LPS induction of expected proteins such as IRFs (IRF3,8 and 9) and IKBKB in cells, coupled with the absence of known LPS induced proteins including transcription factors, kinases and adaptor proteins (e.g. IRF5, p65, TANK, STING and NEMO) highlights that there are several issues with the experimental set up that need to be addressed. The labelling method was initially chosen as the cell populations and conditions can be mixed and treated as a single sample during processing steps and eliminates variables that can induce quantification error. However, the TMT-labeling method is limited by the amount of material that can be labelled and may affect the phosphoproteomic coverage. Label-free methods are known to provide a deeper coverage of the proteome than label-based approaches but quantification is not as efficient, particularly for low-abundance proteins (226). Moreover, phosphopeptides may be lost during sample preparation where the abundance of unlabelled peptides may mask labelled peptides of lower abundance. Ongoing experiments using label-free methods with phosphopeptide enrichment optimised by the *Kessler lab*, and increasing the starting cell material should improve the coverage.

Another issue is that 1 hour LPS stimulation may not be an appropriate time point for harvesting whole cell extracts for phosphoproteomic analysis. The 1 hour timepoint I used may be too late where phosphorylation reaches basal levels. An earlier time point such as 30 minutes may capture more LPS-induced phosphorylation events. Alongside this, verification of known LPS-TLR4 targets by western blot would help confirm that the ideal timepoint is used. It is possible that signaling events not captured by MS are identified by immunoblotting. Of course, the lack of detection in my phosphoproteomic analyses may

also reflect a simple loss of phosphorylation, possibly through phosphatase activity. Overall, although the global phosphoproteome confirmed substrates of MEKK3 such as MAPK3, adjustments to the experimental set up will help improve the phosphoproteomic depth to help identify potential substrates of MEKK3.

5.3. CONCLUSION

Based on the validation rounds in Chapter 3.0, I have prioritised MEKK3 as an IRF5 candidate kinase. MEKK3 represents a novel kinase for drug targeting IRF5 activity in inflammatory disorders. It is strongly expressed in macrophages, plays a role in pro-inflammatory cytokine production in macrophages, and does not appear to be currently in pipeline for advanced stage drug development.

CRISPR/Cas9 deletion of MEKK3 demonstrated its essential role in LPS-induced *Il1a*, *Il1b*, *Tnf* and *Il10* expression in RAW264.7 macrophages. The effect of MEKK3 on *Il6* levels is consistent with another study showing MEKK3 is important for LPS-induced *Il6* production (222). Contradictory to a previous study in which levels of *Il1b* were not affected in MEKK3 deficient RAW264.7 cells (222), I found that MEKK3 deficiency reduced levels of *Il1b*. This may be due to use of siRNA mediated knockdown where MEKK3 is still expressed at low levels and may be inducing *Il1b* levels, whereas I generated a MEKK3 knockout line.

At the onset of this study, inhibitors of MEKK3 have not been available. Therefore, one of my aims was to develop and test MEKK3 inhibitor compounds. In collaboration with LAB282, current kinase inhibitors that display some off-target inhibition to MEKK3 was used as a basis to make more potent and specific to MEKK3 through chemical techniques. Initially, 16 clinically validated kinase inhibitors were reported to have off-target effects to MEKK3 with MEKK3 potency $IC_{50} < 200$ nM. From this, 4 compounds were taken forward for proof-of-concept cellular evaluation as they displayed inhibitory activity towards MEKK3 and were selective against a small panel of related MAP3K/IRF5 pathway targets. Rounds of chemical modifications of the probes by LAB282 resulted in the development of compounds which were more potent and selective towards MEKK3. By testing all these probes in the IRF5-driven TNF promoter driven firefly luciferase reporter assay I have narrowed down on Probes EV'432, EV'272, EV'475, EV'433 which are all derived from

Neratinib (Probe 010) as the lead MEKK3 inhibitors. All 4 compounds exhibited effects on the TNF-reporter activity in an IRF5 and MEKK3 specific manner. Importantly, these compounds display IC_{50} s for MEKK3 in the nanomolar range, and displayed improved selectivity on a small panel of pathway relevant kinases.

I used a global phosphoproteome approach to identify substrates or signalling intermediates regulated by MEKK3. Although, the identity of MAPK3, a known MEKK3 substrate (222) is encouraging, it is clear that there are several issues with the set up used. No significant changes in phosphorylation were observed upon LPS stimulation for the IRFs (IRF3, IRF8, IRF9) and IRF5 was absent. This could be due to phosphoproteomic depth, loss of phosphopeptides during sample preparation and the time point chosen may not have captured phosphorylation events in the signalling pathway. For example MyD88-dependent pathways are known to have an early response to TLR4 stimulation (227). It is likely that the signalling events leading to IRF activation are lost by 1 hour and have reached basal levels. Ongoing experiments include using an earlier timepoint, increasing starting cell material, and using a label-free method should improve the results.

In summary, I have identified the first set of MEKK3 specific inhibitors that will form the basis for testing for future experiments. Future work should test the lead compounds against the kinome to confirm that the probes engage with MEKK3 and to highlight potential off-target kinases inhibited by the compounds. The compounds should also be tested for their effects in inhibiting LPS induced inflammatory gene expression in macrophages by focusing on a panel of IRF5-induced genes. Moreover, global phosphoproteomic analysis in LPS stimulated WT and MEKK3 KO RAW264.7 cells treated with the lead inhibitors will identify MEKK3 dependent substrates, allow comparison of inhibitor action to MEKK3 deficiency, and identify potential off target effects.

6.0. GENERAL DISCUSSION

The results of my thesis have provided insights into the IRF5 signalling pathway. In particular, my work has focused on the kinases responsible for IRF5 activation. Previous studies in the *Udalova* lab have demonstrated that IRF5 is a multifunctional regulator of immune responses with a critical role in controlling the pro-inflammatory response of macrophages, and modulates innate and adaptive immune responses (24, 45, 60). In line with this, IRF5 is a genetic risk factor for many inflammatory and autoimmune diseases such as SLE, IBD and RA (53, 142, 228). Cell signalling triggered in response to pathogenic products such as LPS leads to the activation of protein kinases, which are believed to phosphorylate at the C-terminal serine rich region of IRF5 (37, 86, 109). Phosphorylation is essential for IRF5 activation, which results in IRF5 dimerisation, nuclear translocation and binding to its dedicated gene promoters (86, 127). The purpose of this study was to identify novel IRF5 kinases as potential targets for specific control of IRF5-driven inflammatory conditions.

Protein kinases are the fundamental drivers of intracellular cell signalling. Dysregulated kinase activity is implicated in many human diseases, with more than 400 human diseases associated either directly or indirectly to protein kinases (229). The success of kinase inhibitors in cancer-related conditions has spurred interest in targeting kinases in signal transduction pathways related to inflammation and immunity. For example, the SYK inhibitor fostamatinib has recently been FDA approved for the treatment of adult patients with chronic immune thrombocytopenia (ITP) (230). Another example is the JAK kinase inhibitor tofacitinib which has proved effective in treating moderate-severe ulcerative colitis (UC) in a phase 2 trial (231). Since signalling pathways are dysregulated in many inflammatory and autoimmune conditions, targeting these pathways using kinase inhibitors is one such approach for the treatment of such diseases. The current study has identified kinases PYK2 and MEKK3 as such targets that may be involved in IRF5 signalling.

In Chapter 3.0, a systemic kinase inhibitor screen identified several candidate IRF5 kinases including reported IRF5 kinases IKK β , IKK ϵ , TBK1, RIP2 and TAK1 (44, 92, 95, 96) and previously unpublished novel candidates. Importantly, I acknowledge that the kinase inhibitor library screen targets half the kinome and, therefore, IRF5 kinases may be overlooked. Despite this the effectiveness of this screening approach is supported by the identify of published IRF5 kinases. Among them IKK β , has already been described to phosphorylate a specific serine site on IRF5 which is involved in dimeric interactions (95, 96). Since IKK β is involved in the activation of several targets such as I κ B in the NF- κ B pathway its inhibition is unlikely to result in the specific inhibition of IRF5 function. Likewise, TAK1 plays an essential role in several signalling pathways including the MAPK cascade and IKKs (118) and so was ruled out as a target for inhibition. Other kinases from the IKK family, including TBK1 and IKK ϵ are proposed to phosphorylate and activate IRF5 in response to TLR7 stimulation (31, 109). However, phosphorylation of IRF5 by either kinases failed to induce nuclear translocation of IRF5 (31), and there is no direct evidence that IRF5 phosphorylation by these kinases occurs *in vivo*. Mycobacteria can activate IRF5 in a TLR-independent manner via the intracellular NOD2 receptor pathway (113) which also involves RIP2 kinase. A RIP2 phosphorylation site, serine 436 (IRF5 isoform v3/v4), was identified at the C-terminus of IRF5 (92) but the physiological role of this modification remains unexplored.

Via rounds of validation with overexpression and endogenous systems kinases PYK2 and MEKK3 were identified as lead candidates. Both kinases are expressed predominantly in myeloid cells, bind to IRF5, are capable of phosphorylating IRF5 in kinase assays, and had an effect on LPS/IRF5-driven TNF-luciferase reporter activation. These kinases are novel for the IRF5 signalling pathway and are apparently not currently in pipelines for drug development. Their selective inhibition may turn out to be more efficacious than current standard of care for inflammatory conditions, more cell-specific and produce limited side

effects. For example in IBD, traditional therapies such as glucocorticosteroids are hampered by low remission rates and adverse side effects. Targeting IRF5 could be an a more efficacious and alternative treatment to anti-TNF therapy. Although anti-TNF therapy reduces inflammation in many patients, approximately 30 percent of patients do not respond to the treatment and in some cases patients acquire resistance over time or are susceptible to infections. Targeting upstream kinases to block IRF5 may be advantageous as basal levels of TNF activity are retained and not completely blocked, leading to less adverse effects to general cell function. Moreover, simultaneous inhibition of several IRF5-dependent pro-inflammatory cytokines would, at least in theory, allow for more extensive resolution of the inflammatory response.

In this Chapter, I also show that IRF5 is phosphorylated in response to LPS on serine residues. Phosphorylation of carboxyl serine residues are essential for IRF5 function; phosphorylation of S436 (human isoform v3/v4) contributes to the stabilisation of the activated dimer, whereas phosphorylation of S425, S427 and S430 (human isoform v3/v4) are essential for release of the C-terminal autoinhibitory conformation (89, 94). This study confirms that these serine sites are essential for IRF5 activation and provide a rationale for identifying the kinases involved in targeting these sites. Another interesting finding was IRF5 is also phosphorylated on tyrosine sites, as they have not been extensively studied. Phosphorylation on site Tyr-104 (human/mouse) by BCR-ABL kinase in chronic myeloid leukaemia cells has been linked to negative regulation of IRF5 transcriptional activity (69). However, I later show in Chapter 4.0, that phosphorylation of this site has no further effect on IRF5-driven TNF reporter activation. Although the non-receptor tyrosine kinase LYN is capable of phosphorylating IRF5 on tyrosine sites, its kinase activity is dispensable in inhibiting the PTMs of IRF5 (38). Further investigation into the role of IRF5 tyrosine phosphorylation will undoubtedly contribute to our understanding of how IRF5 is regulated.

Chapter 4.0 focused on validation of PYK2 as a candidate kinase with the use of PYK2-deficient cells and PYK2 specific inhibitors. Here, I demonstrate that PYK2 deficiency has an effect on IRF5 activation, IRF5 recruitment to the genomic loci, and transcription of IRF5 target inflammatory genes. Furthermore, I have mapped the PYK2 dependent phosphosite within IRF5 (Tyr-171, mouse) using a mass spectrometry assisted phosphoproteomic approach and demonstrated its importance in IRF5 activation by *in vitro* mutagenesis. This is the first study showing that IRF5 tyrosine phosphorylation is important for IRF5 activation. However, the exact role of IRF5 tyrosine phosphorylation remains unclear. The biochemical and functional significance associated with the PYK2-dependent IRF5 tyrosine site as well other tyrosine sites identified in MS may be worth investigating in future studies as other upstream kinases may be involved. It is possible that the phosphorylation event alters IRF5 conformation to alter its activity, it may block its auto-inhibitory state, or it may create a recruitment site for other interacting proteins.

In this Chapter, I explore the use of defactinib a PYK2 inhibitor which currently in phase clinical trials for ovarian cancer (181). This study demonstrates that defactinib blocks IRF5 nuclear translocation, IRF5 recruitment to genomic loci, and IRF5 transcription and inflammatory gene expression in mouse and human macrophages. The fact that defactinib only targets a specific subset of genes downstream of LPS activation in an IRF5-dependent manner, highlights the promising possibility of specifically targeting IRF5-induced pro-inflammatory function. A global phosphoproteomic screening for PYK2 substrates in LPS stimulated WT and PYK2 deficient cells with defactinib would illuminate the major cellular functions of this kinase and provide further information on the specificity of the inhibitor. It is important to note that defactinib is a dual inhibitor of both PYK2 and FAK proteins. Further studies will be required to distinguish the effects of PYK2 inhibition from FAK inhibition during inflammation. Potent and selective inhibitors of FAK with 50-250- fold selectivity for FAK over other protein kinases are available and would clarify this issue. Despite the dual

nature of defactinib, the lack of non-specific targeting outside the FAK/PYK2 family is of clear and immediate therapeutic potential.

Finally, I show that defactinib suppresses cytokine production in biopsies taken from the inflamed gut mucosal tissue of patients with UC. Defactinib is currently in clinical trial for treatment of patients with ovarian cancer. Given its good toxicological profile and promising pre-clinical data on its efficacy in primary cells and patient cells, defactinib is an attractive compound for repurposing to treat patients with inflammatory conditions such as UC, where both the *PTK2B* and *IRF5* gene loci have been associated with increased genetic risk (53, 151). There are currently several papers demonstrating that IRF5 plays a key role in modulating intestinal inflammation in murine colitis models (54, 55). The *Udalova* lab have shown that deficiency in IRF5 ameliorates colitis severity, assessed by reduction in pathology, inflammatory cellular infiltrate, production of inflammatory cytokines and Th1/Th17 response (55). Pre-clinical studies in the *Udalova* lab have begun to assess the effects of defactinib pre-treatment and daily IV/IP administration of defactinib at the early onset (day 7) of mouse models of *Hh*-induced colitis. As expected, *Hh*-anti IL10R infected mice developed inflammation in the colon after a week. Initial data suggests defactinib treatment improved intestinal immunopathology with a reduction in immune cell infiltrate and expression of IRF5-regulated cytokines and chemokines in the colon of defactinib-treated *Hh* IL10R infected mice.

However, this was a prevention protocol with inhibitor pre-treatment raising the question of whether defactinib ameliorates disease after colitis initiation. Defactinib administration after colitis disease onset instead of pre-treatment before induction of disease to see whether defactinib prevents all manifestations of colitis should be investigated in future studies. Therapeutic inhibition after disease onset is more translationally relevant such as that expected in a newly diagnosed patient with UC and this would provide further assessment of the therapeutic properties of defactinib i.e. whether its administration during the early

stages of colitis is sufficient to halt disease progression. The model should also be conducted in PYK2 deficient mice to assess whether there is an endogenous PYK2 mediated suppression of colitis in the *Hh* model. Lack of an effect from administration of defactinib in PYK2 KO mice will validate the specificity of treatment. Furthermore, using PYK2 deficient mice for assessing the activation of IRF5 and other immune relevant pathway activation in disease would be informative.

Translating novel findings from pre-clinical assessment of defactinib into future clinical benefits for patients would be the ultimate goal. Defactinib represents an appealing treatment in patients with UC who experience treatment failure or intolerance to conventional treatment of a biologic drug, thus future clinical studies in the efficacy of defactinib should be investigated in both induction and maintenance treatment. Further work into pre-clinical evaluation of defactinib in UC should be carried out such as stratification of defactinib responsive and non-responsive patient subgroups by IRF5 expression. For example, endoscopic and histological scores could be correlated with cytokine and IRF5 expression, whilst patient metadata (age, sex, disease duration, and therapies) can be used to identify patient subgroups that are more responsive to defactinib treatment. This would help determine whether sub-populations of patients with UC are more prone to intervention with defactinib.

To date, genome-wide association studies have identified over 200 genomic loci linked to IBD, yet it remains unclear how these genes interact to influence the pathology (232). The use of single cell genomics has made it possible to determine which cells and pathway disease-associated genes are acting during disease. A recent pioneer single-cell sequencing study reveals underlying building blocks of UC by analysing intestinal tissue from patients with UC and healthy individuals to generate a high-resolution map of this complex disease (233). For example, patient samples had higher proportions of inflammatory fibroblasts, inflammatory monocytes, microfold-like cells and T cells that co-

express CD8 and IL-17. Inflammatory associated fibroblasts and monocytes are able to bypass TNF pathways and evade anti-TNF drugs via oncostatin M (OSM) signalling. This study has provided a framework for linking GWAS risk loci with specific cell types and functional pathways and helped to nominate causal genes across GWAS loci, amongst them IRF5 and PYK2, where IRF5 is enriched in monocytes, macrophages, dendritic cells and follicular cells in inflamed mucosa of patients with UC. By improving our understanding of the cell-types and genes that contribute to inflammatory conditions, specific therapies can be developed with more limited unexpected off-target effects.

It is clear that tissue specific, and cell-specific analysis of PYK2 is required for the understanding of its function in IBD. Therapeutic strategies to target sufficient quantities of defactinib to the site of inflammation with preferential uptake in macrophages to achieve maximal local drug concentration while minimising systemic side effects is desirable. Cell type specificity to accurately deliver drugs to macrophages may be achieved through the use of nanoparticles (234). The use of nanoparticles as drug carriers offers several advantages including their ability to penetrate physiological barriers, ability to evade phagocytosis, display rapid mixing kinetics, accept high loading concentrations, confer little or no toxicity and resist degradation. For example, colon homing nanoparticles to deliver TNF- α siRNA to M1 macrophages in a model of IBD has been reported (235). Thus, macrophage-targeted pharmacological intervention could be explored as a viable option for targeted delivery of defactinib.

This work has mapped a clear mechanistic interaction between two established IBD risk genes which will allow future investigations to map the specific effects of the IRF5 and PYK2 risk variants on their respective genes and downstream effects. The mechanism by which PYK2 and IRF5 polymorphisms lead to increased disease risk is not well established. The risk variant for PYK2 impacts gene expression in monocytes and macrophages, but the direction of this effect varies by condition. For example, the risk variant increases

expression in naïve and interferon-gamma stimulated monocytes, but decreases it in LPS-stimulated monocytes (236). The IRF5 risk variant has consistent effects across monocytes and macrophages conditions, but also impacts gene expression and splicing across a wide range of other immune cells and tissues (237). Mapping the effects of these two risk variants on PYK2 related IRF5 phosphorylation and downstream signalling across different cell types and stimulation conditions will allow us to understand which of these effects are casually related to disease, and possibly to tailor treatment to the correct cell context. The pro-inflammatory function of PYK2 may be confounded to myeloid cells where it forms the axis with IRF5 and may not exist in other cell types e.g. epithelial cells (153). In addition, these two IBD risk variants also have very different risk profiles for other diseases. The PYK2 IBD risk variant also decreases the risk of Alzheimer's disease (238), whereas the IRF5 polymorphism has no detectable effect on Alzheimer's. By contrast, the IRF5 IBD risk variant increases the risk of SLE (239), whereas the PYK2 variant does not. Understanding how the mechanistic effects of these two risk variants differ will shed light on the shared pathways across these different diseases, and how these shared pathways can produce divergent disease phenotypes.

My findings reveal a novel regulatory circuit in macrophages that involves TLR4/PYK2/IRF5. Based on my results and previously published data, I propose the following model for the regulation of IRF5 in macrophages (**Figure 6.1**). Upon TLR4 stimulation by LPS or Dectin-1 stimulation by glucans, PYK2 is activated by autophosphorylation at Tyr-402 (169, 192). PYK2 phosphorylates IRF5 on site Tyr-171 (mouse) leading to translocation of IRF5 to the nucleus, binding to gene promoters and subsequent transcription of pro-inflammatory cytokines. Upon Dectin-1 stimulation by glucan, the recruitment of PYK2 to IRF5 is likely to be Syk-dependent (192). Further investigations need to be carried out to prove this hypothesis and clarify the mechanisms involved.

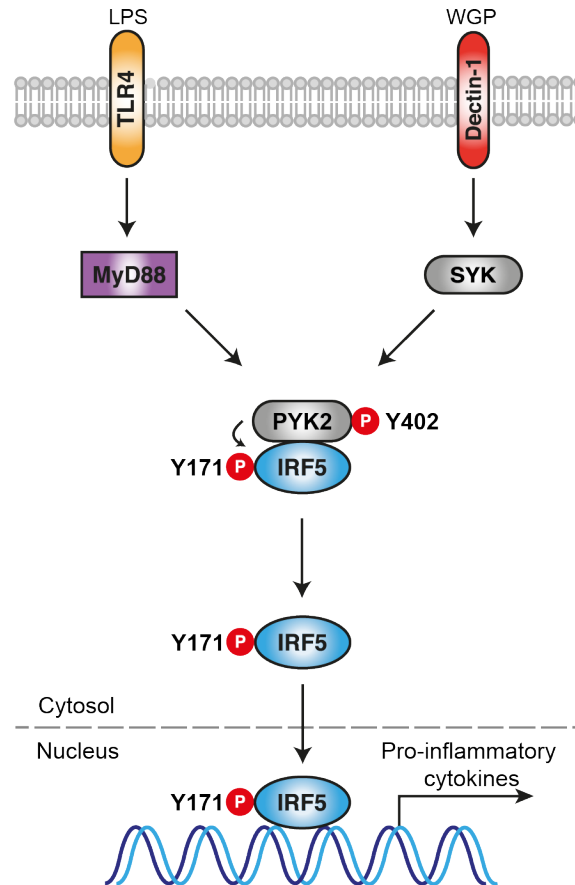


Figure 6.1. Proposed model of IRF5 activation by PYK2 in macrophages LPS stimulation of TLR4 leads to PYK2 autophosphorylation on site Y402. Activated PYK2 phosphorylates IRF5 at site Y171 (mouse). IRF5 translocates to the nucleus and activates target genes. Dectin-1 stimulation by whole glucan particles also leads to IRF5 mediated transcription and is likely to be Syk-dependent.

In Chapter 4.0, MEKK3 is further validated as a candidate IRF5 kinase. MEKK3 knockouts show reduced TNF-reporter activity, and phenocopies IRF5 knockouts in terms of cytokine gene expression. Based on previous published data, I propose the following model of IRF5 activation: LPS/TLR4 signalling triggers the MyD88-dependent pathway through the MyD88-IRAK-TRAF6 complex, which leads to activation of MEKK3 (195, 219). MEKK3 may directly phosphorylate IRF5 or act via an intermediate substrate such as IKK β (194) to activate IRF5 (**Figure 6.2**). Further studies should explore whether MEKK3 is capable of phosphorylating IRF5 and whether MEKK3 effects IRF5 recruitment to targeted promoters. Although several targets have been described as potential substrates of MEKK3 such as IKKs and MEK5 (194, 240), the proteins phosphorylated by MEKK3 in macrophages remain to be identified, and no systematic screen for cellular MEKK3 substrates have been

reported. Identification of the key substrates of MEKK3 is essential, as this data would highlight the major cellular functions of this kinase.

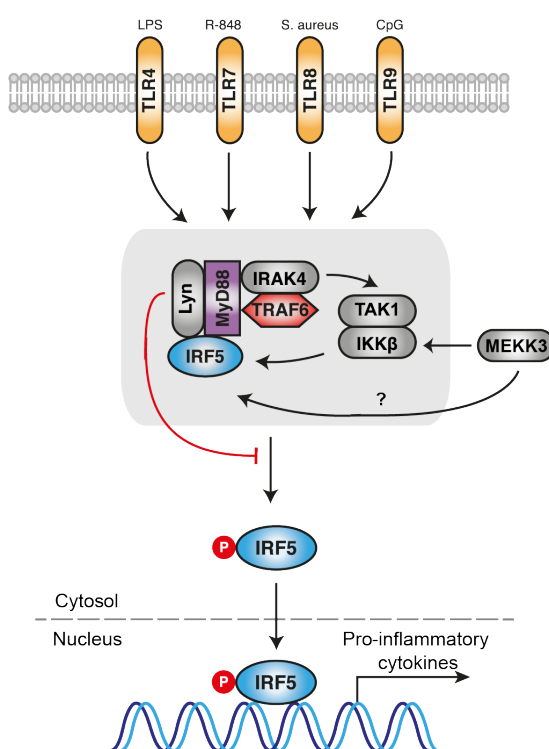


Figure 6.2. Proposed model of IRF5 activation by MEKK3 in macrophages TLR stimulation leads to formation of the MyD88-IRAK4-TRAF6 complex to activate MEKK3. MEKK3 may directly activate IRF5 or via an intermediate kinase such as IKKβ.

To date, no MEKK3 specific inhibitors exist so in this Chapter MEKK3-specific inhibitors were developed by *Lab282*, *Evotec* and tested in cell based assays. By performing a small primary screen of existing kinase inhibitors, *Evotec* identified 4 compounds which showed inhibition to MEKK3 and less off-target effects to other kinases. Although these initial compounds are not selective to MEKK3, the structures of these compounds give insights into chemical structures that potentially inhibit MEKK3 and used as initial leads to design highly selective inhibitors of MEKK3. Compound EV'010 (Neratinib) a HER2/EGFR inhibitor, had been identified as one of the leads going forward with the lowest MEKK3 IC₅₀ value. Thus, optimisation of the compound was carried out. At the onset of this study, one of the challenges with developing MEKK3-specific inhibitors is that MEKK2 and MEKK3 are structurally similar with residue similarity across the kinase domain as a whole. Through

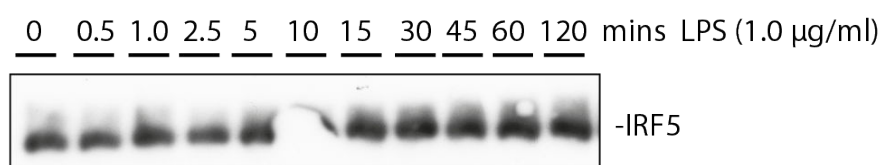
chemistry efforts, *Evotec* have improved compound EV'010 and developed probes that are less selective to other kinases including MEKK2, while maintaining selectivity to MEKK3. Through testing of these compounds in cell based assays I narrowed down on 4 promising probes (EV'475, EV'433, EV'432, EV'272) derived from compound EV'010. These 4 probes act in an IRF5 and MEKK3-specific manner in the IRF5/TNF-reporter assay, and are reasonably potent and selective in the mini kinase panel.

Although several probes have now been identified with the desired cellular read outs, a full characterisation of the cellular mechanism of action and direct link between MEKK3 biochemical inhibition and cellular effects still need to be established. Further studies should investigate the inhibition of inflammatory gene expression in macrophages, and a full kinase profile of the lead inhibitors will give an idea of the off-target effects of the inhibitors. Moreover, phosphoproteomic analysis will provide an overview of the substrates and signalling pathways targeted by these inhibitors. Overall, this chapter describes a pragmatic, flexible approach to drug discovery, via optimising existing inhibitors through rational drug design to yield compounds that inhibit desired targets. These lead compounds are the most interesting inhibitor derivatives and are worthy of further investigation.

To summarise the data presented here has made novel findings in identifying IRF5 kinases for therapeutic intervention. PYK2 targets IRF5 to control IRF5-mediated inflammatory responses. PYK2 is a druggable target, and through its targeted inhibition with defactinib, IRF5 driven cytokine responses can be significantly diminished. The identity of MEKK3 as another promising IRF5 candidate kinase target will pave the way for investigations into testing MEKK3-specific probes as a potential way to modulate the IRF5 pathway.

7.0. APPENDIX

7.1. SUPPLEMENTARY FIGURES



Supplementary Figure S1. LPS-induced IRF5 phosphorylation RAW264.7 cells were stimulated with LPS (1.0 μ g/ml) at indicated times points (minutes) and subjected to Phos-tag immunoblot analysis using antibodies against IRF5.

WT

Protein List									
Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	#Peptides	#Unique	PTM	Avg. Mass	Description
1	6	trIQ3U169 Q3U169_MOUSE	387.41	91	159	159	Y	56005	Interferon regulatory factor 5 OS=Mus musculus GN=Irf5 PE=1 SV=1
1	7	P56477 IRF5_MOUSE	387.41	91	159	159	Y	56005	Interferon regulatory factor 5 OS=Mus musculus GN=Irf5 PE=1 SV=1
2	1	P38647 GRP75_MOUSE	360.41	75	87	84	Y	73461	Stress-70 protein, mitochondrial OS=Mus musculus GN=Hspa9 PE=1 SV=3
5	2	P20029 GRP78_MOUSE	332.71	62	60	58	Y	72422	78 kDa glucose-regulated protein OS=Mus musculus GN=Hspa5 PE=1 SV=3
7	9	P63017 HSP7C_MOUSE	299.58	62	52	34	Y	70871	Heat shock cognate 71 kDa protein OS=Mus musculus GN=Hspa8 PE=1 SV=1
11	15	P52480 KPYM_MOUSE	285.59	58	40	36	Y	57845	Pyruvate kinase PKM OS=Mus musculus GN=Pkm PE=1 SV=4
3	12	Q922U2 K2C5_MOUSE	279.47	44	65	23	Y	61767	Keratin, type II cytoskeletal 5 OS=Mus musculus GN=Krt5 PE=1 SV=1
3	13	trIQ32P04 Q32P04_MOUSE	279.47	44	65	23	Y	61767	Keratin 5 OS=Mus musculus GN=Krt5 PE=1 SV=2
4	14	P50446 K2C6A_MOUSE	278.42	42	60	5	Y	59335	Keratin, type II cytoskeletal 6A OS=Mus musculus GN=Krt6a PE=1 SV=3
10	3	trIQ5FWJ3 Q5FWJ3_MOUSE	270.37	64	46	43	Y	53688	Vimentin OS=Mus musculus GN=Vim PE=1 SV=1

PYK2 KOs

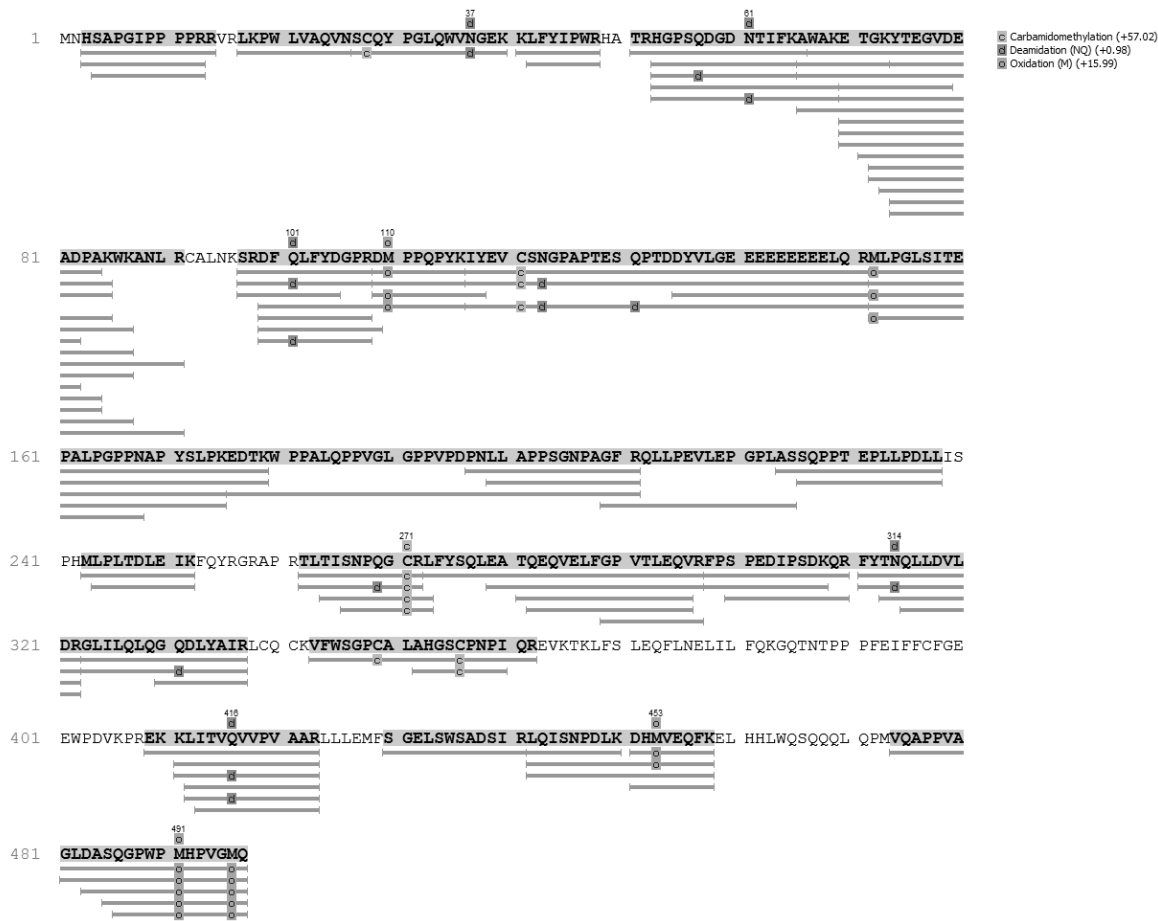
Protein List									
Protein Group	Protein ID	Accession	-10lgP	Coverage (%)	#Peptides	#Unique	PTM	Avg. Mass	Description
1	3	trIQ5FWJ3 Q5FWJ3_MOUSE	411.43	99	299	280	Y	53688	Vimentin OS=Mus musculus GN=Vim PE=1 SV=1
1	4	P20152 VIME_MOUSE	411.43	99	299	280	Y	53688	Vimentin OS=Mus musculus GN=Vim PE=1 SV=3
3	1	P38647 GRP75_MOUSE	348.41	75	74	71	Y	73461	Stress-70 protein, mitochondrial OS=Mus musculus GN=Hspa9 PE=1 SV=3
5	2	P20029 GRP78_MOUSE	333.93	59	52	50	Y	72422	78 kDa glucose-regulated protein OS=Mus musculus GN=Hspa5 PE=1 SV=3
2	6	trIQ3U169 Q3U169_MOUSE	317.22	81	77	77	Y	56005	Interferon regulatory factor 5 OS=Mus musculus GN=Irf5 PE=1 SV=1
2	7	P56477 IRF5_MOUSE	317.22	81	77	77	Y	56005	Interferon regulatory factor 5 OS=Mus musculus GN=Irf5 PE=1 SV=1
7	9	P63017 HSP7C_MOUSE	296.54	52	44	28	Y	70871	Heat shock cognate 71 kDa protein OS=Mus musculus GN=Hspa8 PE=1 SV=1
4	12	Q922U2 K2C5_MOUSE	272.60	44	63	20	Y	61767	Keratin, type II cytoskeletal 5 OS=Mus musculus GN=Krt5 PE=1 SV=1
4	13	trIQ32P04 Q32P04_MOUSE	272.60	44	63	20	Y	61767	Keratin 5 OS=Mus musculus GN=Krt5 PE=1 SV=2
8	14	P50446 K2C6A_MOUSE	269.30	34	50	2	Y	59335	Keratin, type II cytoskeletal 6A OS=Mus musculus GN=Krt6a PE=1 SV=3

Supplementary Figure S2. Mass spectrometry protein hit list Mascot database top 10 protein hits from IRF5 immunoprecipitation in LPS-stimulated WT (top table) and PYK KOs (bottom table). List sorted by descending protein score.

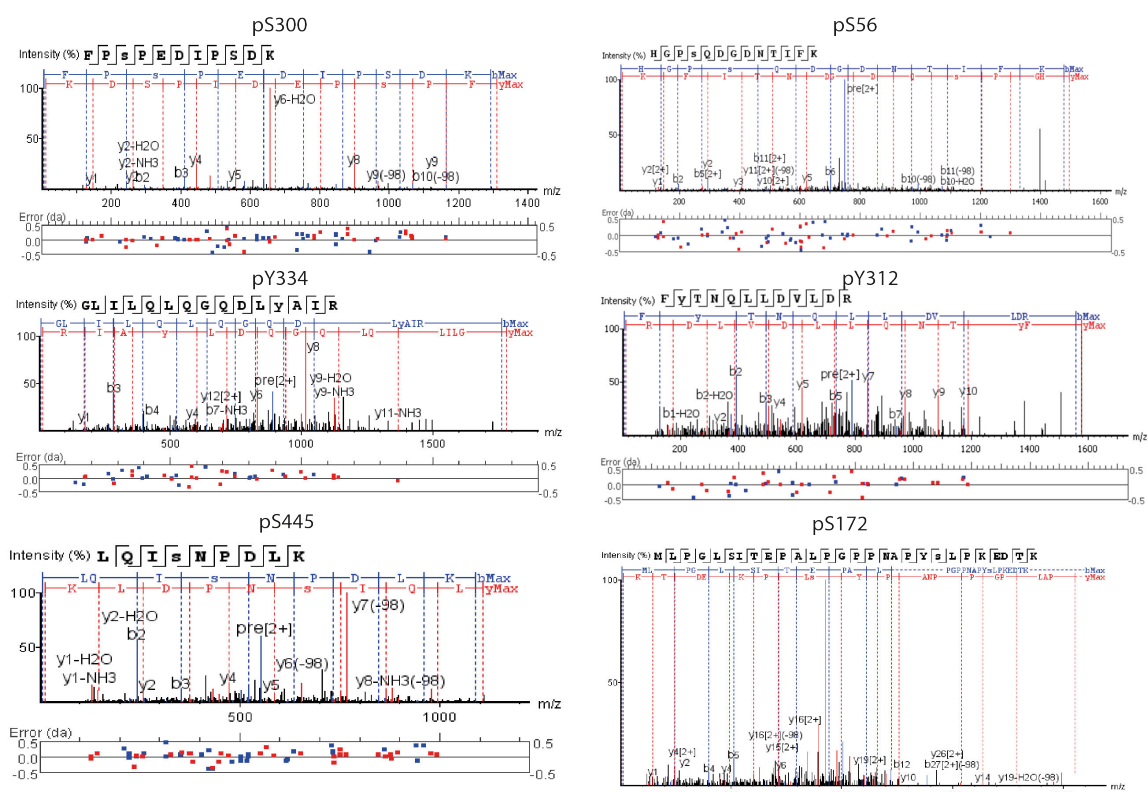
WT



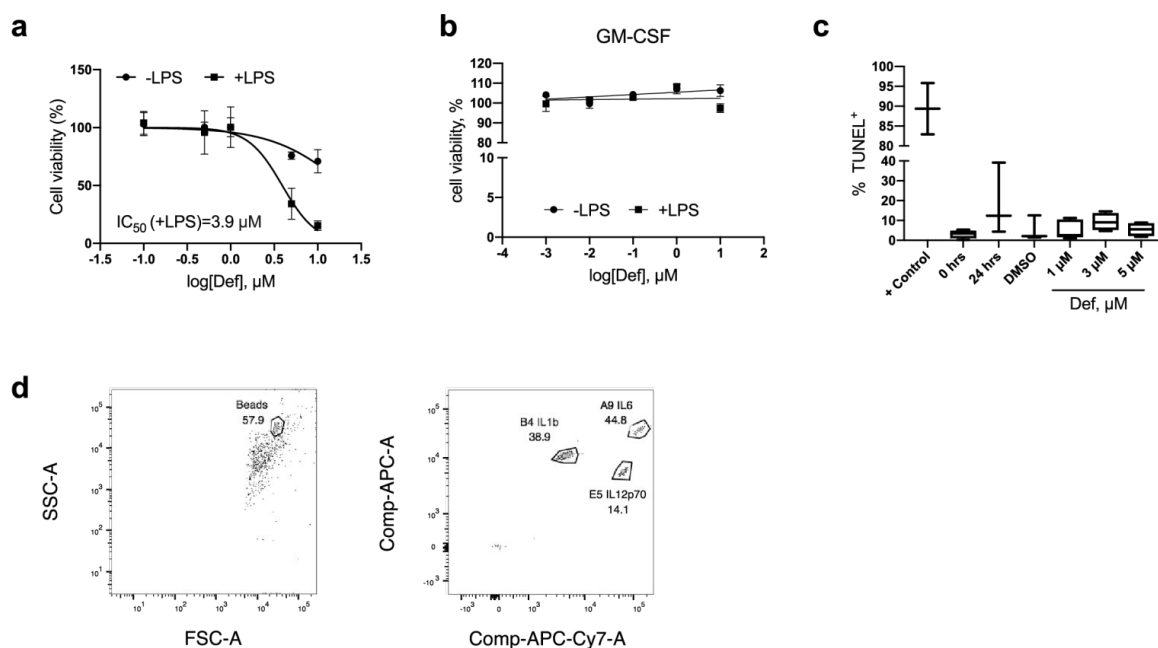
PYK2 KOs



Supplementary Figure S3. PEAKS SPIDER search analysis of peptides and post-translational modifications Mouse IRF5 from WT (top panel) and PYK2 KOs (lower panel) in which the peptides identified by LC-MS/MS are underlined below amino acid sequence. Each line corresponds to a unique MS/MS spectrum. Post-translational modifications including cysteine carbamidomethylation (c), deamidation (d) and oxidation (o) are indicated.



Supplementary Figure S4. MS/MS spectrum of the IRF5 derived tryptic peptides Spectra of remaining IRF5 phosphorylation sites (corresponding to Figure 4.7). Phosphorylation sites at position S300, S56, Y334, Y312, S445, S172 (mouse). Fragmentation ions of the b- and y- series are indicated in blue and red, respectively.



Supplementary Figure S5. Impact of defactinib treatment on viability of primary human cells and colon biopsies Cell viability was measured in human monocyte-derived macrophages after 24 hrs **(a)** and 3 hrs **(b)** of treatment with defactinib by CTG assay. **(c)** Cell viability by TUNEL assay was measured in colon biopsies after 24 hr treatment with defactinib **(d)** Flow cytometry gating strategy to detect IL1b, IL6 and IL12p70 levels by Cytometry Bead Array.

	Probe 1	Probe 2	Probe 5	Probe 6	Probe 7	Probe 8	Probe 9	Probe 10	Probe 11	Probe 13	Probe 15	Probe 18	Probe 19	Probe 30	Probe 31	Probe 38
MEKK3 IC50 nM	87	80	10.1	19.9	37.1	319	51.4	24.9	52.4	40.5	69	129	76.3	310	2080	431
%inh @ 1 μ M																
MEKK3	74	96	104	103	97	75	103	98	94	96	95	82	91	88	56	70
IKK alpha	48	9	58	66	3	-8	-5	-4	-9	47	4	76	-17	12	-12	100
IKK beta	65	-4	14	36	-3	15	1	-9	-1	16	0	23	-6	-1	-4	69
IRAK1	100	99	99	97	100	87	58	86	100	100	32	98	79	-1	13	100
IRAK4	55	9	54	46	96	10	14	10	24	101	13	32	52	7	6	29
JAK2	95	99	96	102	98	102	89	2	91	71	73	85	59	91	99	80
MAP3K8/COT	17	63	75	35	99	-6	19	35	6	85	9	17	39	15	2	32
MAPK7/ERK5	-3	-4	0	2	-2	-3	1	-2	0	49	3	2	8	6	1	3
STK4/MST1	4	62	101	78	99	15	91	99	80	101	92	56	94	101	92	94
MAP3K11/MLK3	98	77	99	98	44	17	40	30	19	98	17	77	46	58	26	94
MAP3K2/MEKK2	80	88	96	100	90	79	92	81	94	97	94	87	92	89	78	83
MAP3K5/ASK1	5	6	95	74	63	1	-2	2	4	28	2	25	-6	18	0	66
MAP3K7/TAK1	83	69	110	105	108	41	108	15	56	102	45	98	89	71	29	104
RIPK2	61	99	39	87	95	6	74	10	77	76	99	47	41	4	6	84

< 100 nM
 100 nM < < 1000 nM
 > 1000 nM

< 40% inhibition @ 1 μ M
 > 80% inhibition @ 1 μ M

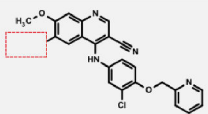
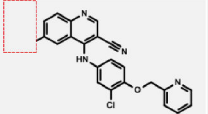
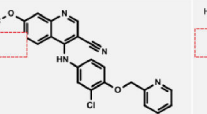
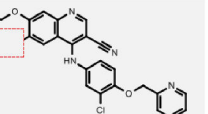
Supplementary Figure S6. Screening panel for kinase inhibitors Potency and selectivity data at 1 μ M of probes against a small panel of kinases. Probes EV'008 (Ruxolitinib), EV'010 (Neratinib), E'015 (PP121) and EV'030 (5n) were taken forward for further testing. Data and table generated by Evotec (LAB282).

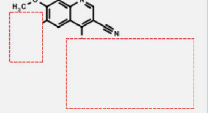
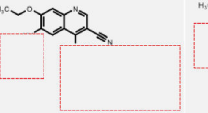
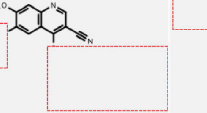
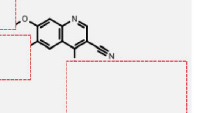
	Probe 8	Probe 10	Probe 15	Probe 30
Known names	Ruxolitinib	Neratinib	PP121	5n
MEKK3 IC50 nM	319	24.9	69	310
%inh @ 1 uM				
IKK alpha				
IKK beta				
IRAK1				
IRAK4				
JAK2				
MAP3K8/COT				
MAPK7/ERK5				
STK4/MST1				
MAP3K11/MLK3				
MAP3K2/MEKK2				
MAP3K5/ASK1				
MAP3K7/TAK1				
RIPK2				

Not-Selective

Selective

Supplementary Figure S7. Screening panel for Batch I compounds Potency and selectivity data at 1 μ M on Probes EV'008 (Ruxolitinib), EV'010 (Neratinib), E'015 (PP121), EV'030 (5n) against a small panel of kinases. Not selective in green, selective in pink. Data and table generated by Evotec (LAB282).

EOAI	3411487	4003272	4004326	4005432
Lab book No	EV-XID001-048-003	EV-GLB001-177-001	EV-GLB001-181-001	EV-GLB001-190-001
Structure				
MEKK3 IC ₅₀ (nM)	31	151	106	32
IRAK1 - Adapta - Km app	50	26	25	31
MAP3K11 (MLK3)	47	10	18	59
MAP3K2 (MEKK2)	61	13	31	82
MAP3K8 (COT) - ZLYTE - 100	65	29	6	72
STK4 (MST1) - ZLYTE - Km app	96	32	71	101
aq Solubility UV (uM)	poor calibration curve linearity	106.3	191.0	647.3

EOAI	3999039	4005475	4005433	4006694
Lab book No	EV-LOJ001-079-002	EV-XID001-080-003	EV-GLB001-191-001	EV-GLB001-204-001
Structure				
MEKK3 IC ₅₀ (nM)	197	60	79	134
IRAK1 - Adapta - Km app	1	23	12	21
MAP3K11 (MLK3)	12	-2	0	32
MAP3K2 (MEKK2)	13	0	10	55
MAP3K8 (COT) - ZLYTE - 100	4	-18	12	17
STK4 (MST1) - ZLYTE - Km app	76	74	67	93
aq Solubility UV (uM)	poor calibration curve linearity	429.2	poor calibration curve linearity	715.8

Supplementary Figure S8. Screening panel for Batch III compounds Potency and selectivity data at 1 μ M on Probes derived from EV'487 and EV'039 against a small panel of kinases. Dashed red boxes indicate regions where chemical modifications were carried out. Not selective in green, selective in pink. Data and table generated by Evotec (LAB282).

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