FUNCTIONAL CHARACTERISATION OF A NOVEL TIR-DOMAIN CONTAINING PROTEIN IN IMMUNE SIGNALLING

by

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A thesis presented to

The National University of Ireland

In fulfilment of the thesis requirement

For the degree of Master.

Biology Department

National University of Ireland Maynooth

October 2012

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Table of Contents

Acknowledgements

I wish to thank my supervisor, Prof. Paul Moynagh, for his assistance and guidance throughout the project. I would also like to thank the members of the Molecular Immunology lab, particularly Mark and Paola for their direction and encouragement. And also for their (Along with Emily Hams and Padraig Fallon) *in vivo* data which I have used in Figures 3.40 and 3.41! Thanks to Tang, Figs and Gargan for providing the lab with so much entertainment. A special thanks to Ronan Bergin, Marc Healy and Ruaidhri Jackson for chalking it down over the last number of years. Finally, I am particularly grateful for the moral (and financial!) support given to me by my Mum and the rest of my family.

Abstract

Dysregulation of innate immune signalling pathways has been implicated in a host of chronic inflammatory disorders including Multiple Sclerosis, Crohn's Disease and Rheumatoid Arthritis. As a result, it is critical that there are tight regulatory mechanisms in place to rigidly control such signalling pathways. The IL-17 family of cytokines, which comprises six members in mammals, are potent mediators of inflammation. Produced primarily by $CD4^+$ T helper 17 (Th17) cells, they signal through IL-17 receptor family complexes and protect the host against bacterial infection. Each member of the IL-17R family is composed of an extracellular Fibronectin III-like (FnIII) domain, a single transmembrane domain and an intracellular similar expression to FGF genes (SEF)/IL-17R (SEFIR) domain, which is crucial for IL-17 signalling and is homologous to the TIR domains of Toll-Like Receptors (TLRs).

Here, we describe a role for IL-17RD, also known as Sef, the remaining orphan receptor of the family, as a functional regulator of innate immune signalling. Small interfering RNAs (siRNAs) and short hairpin RNAs (shRNAs) were used to suppress endogenous expression of IL-17RD leading to enhanced activation of NF-κB and NF-κB responsive genes by TLR ligands such as Lipopolysaccharide (LPS) and poyinosinic:polycytidylic acid (Poly I:C). We demonstrate that IL-17RD can differentially regulate the various pathways employed by IL-17A. Neutrophil recruitment, in response to *in vivo* administration of IL-17A, is abolished in IL-17RD-deficient mice, correlating with reduced IL-17A-induced activation of p38 MAPK and expression of the neutrophil chemokine MIP-2. In contrast, IL-17RD deficiency results in enhanced IL-17A-induced activation of NF-κB and IL-6 and KC expression. IL-17RD disrupts the interaction of Act1 and TRAF6 causing differential regulation of NF-κB and p38 MAPK signalling pathways.

Abbreviations

Act1	NF-κB activator 1
Amp	Ampicillin
AP-1	Activating protein-1
APC	Antigen presenting cell
APS	Ammonium persulfate
ASC	Apoptosis-associated speck-like protein containing a CARD
ATF-2	Activating transcription factor-2
bp	Base Pair
BSA	Bovine serum albumin
BMDM	Bone marrow derived macrophages
cAMP	Cyclic adenosine 3', 5'-monophosphate
CARD	Caspase recruiting domain
CBP	CREB-binding protein
CD	Cluster of Differentiation
cDNA	complementary DNA
CpG DNA	2'-deoxyribo cytidine-phosphate-guanosine DNA
ChIP	Chromatin immunoprecipitation
CIP	Calf Intestinal Phosphatase
cm	centimetre
CMV	Cytomegalovirus
CNS	Central nervous system
CREB	cAMP-responsive element binding protein
CT	Control
DBD	DNA binding domain
DC	Dendritic cell
DD	Death domain
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleid triphosphates

dsRNA	Double-stranded RNA
DTT	Dithiothreitol
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ERK	Extracellular signal regulated kinase
EV	Empty vector
FADD	Fas-associated death domain
FBS	Foetal Bovine Serum
GNBP	β-glucan recognition protein
GFP	Green fluorescent protein
GR	Glucocorticoid receptor
GTP	Guanosine triphosphate
h	Hour(s)
HEK	Human Embryonic Kidney
HPRT	Hypoxanthinephosphoribosyltransferase
HSP	Heat shock protein
IAD	IRF association domain
IAP	Inhibitor of apoptosis protein
IB	Immunoblot
ICAM	Intercellular adhesion molecule 1
ID	Intermediate domain
IFN	Interferon
Ig	Immunoglobulin
IκB	Inhibitor of κB
IKK	IκB kinase
IKKi	Inducible IKK
IL	Interleukin
IL-1R	IL-1 Receptor
IL-1RAcP	IL-1R accessory protein
IL-17R	IL-17 Receptor
Imd	Immune deficiency
IP	Immunoprecipitation
IPS-1	IFNβ promoter stimulator 1

IR	Infrared
IRAK	IL-1R associated kinase
IRF	Interferon regulatory factor
ISRE	IFN-stimulated response element
JNK	c-Jun N-terminal kinase
K	Lysine
Kb	Kilobase
kD	KiloDalton
LB	Luria Bertoni
LBP	LPS-binding protein
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipoteichoic acid
Mal	MyD88-adaptor like
MAPK	Mitogen acticated protein kinase
MAVS	Mitochondria anti-viral signalling protein
MBL	Mannose-binding lectin
MCMV	Murine cytomegavirus
MD-1/2	Myeloid differentiation protein-1/2
MDA5	Melanoma differentiation-associated gene 5
mDC	Myeloid dendritic cell
MEKK1	Mitogen activated protein kinase kinase kinase 1
MHC	Major histocompatibility
min	minute
ml	milli litre
mM	milli molar
MMLV RT	Moloney murine leukemia virus reverse transcriptase
MMTV	Mouse mammary tumour virus
MMR	Macrophage mannose receptor
mRNA	Messenger RNA
MS	Mass Spectrometry
MyD88	Myeloid differentiation factor 88
NAP1	NF-κB-activating kinase (NAK)-associated protein1
NC	Non-specific competitor

NES	Nuclear export signal
NF-κB	Nuclear Factor-κB
ng	nano grammes
NIK	NF-κB interacting kinase
NK	Natural killer
NLR	Nod-like receptor
NLS	Nuclear localisation sequence
nM	nanomolar
NTP	Nucleotide triphosphate
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
Pam-Cys	Pam3CysSerLys4
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
pCAF	p300/CBP associated factor
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PGN	Peptidoglycan
PGRP	Peptidoglycan recognition protein
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PMSF	Phenylmethylsulfonyl Fluoride
Poly I:C	Polyinosinic:polycytidylic acid
PRD	Positive regulatory domain
PRR	Pattern recognition receptor
RA	Rheumatoid arthritis
RANTES	Regulated upon activation, normal T cell expressed and secreted
RH	Rel homology
RIG-I	Retinoic acid-inducible gene I
RIP	Receptor interacting protein
RISC	RNA-induced silencing protein
RNA	Ribonucleic acid

ROS	Reactive oxygen species
RP105	Radioprotective 105
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory synctical virus
RT	Room temperature
RT-PCR	Reverse Transcriptase PCR
s	second(s)
SARM	Sterile α and HEAT-Armadillo motifs
SC	Specific competitor
SDS	Sodium dodecyl sulphate
SEFIR	Sef / IL-17R domain
SH	domain *Src* homology domain
shRNA	short hairpin RNA
SIGGIR	Single immunoglobulin IL-1 related protein
siRNA	Small interfering RNA
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
SR	Super repressor
SRC	Steroid receptor-co-activator
SRF	Serum response factor
ssRNA	Single stranded RNA
ST2	Suppressor of tumourogenicity 2
STAT	Signal transducer and activator of transcription
sTLR	soluble TLR
TAB	TAK1-binding protein
TAE	Tris-acetate-EDTA
TAK	TGF-β-activated protein kinase 1
TANK	TRAF-family-member associated NF-κB activator
Taq	*Thermophilus aquaticus*
TBE	Tris-Borate-EDTA
TBK1	TANK-binding protein 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline containing Tween 20

TE	Tris-EDTA
TEMED	Tetramethylethylene-diamine
TGF-β	Transforming growth factor-β
$T_H1/2$	Helper type 1/2 T cell
TIR	Toll/IL-1R
TLR	Toll-like receptor
TNF	Tumour Necrosis Factor
TRAF	TNF receptor associated factor
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor inducing IFN-β
Ubc	Ubiquitin conjugating enzyme
Uev1A	Ubiquitin-conjugating enzyme E2 variant isoform
μg	micro grammes
μl	micro litre
μM	micro molar
UV	Ultraviolet
VCAM	Vascular cellular adhesion molecule 1
WCL	Whole cell lysate
WNV	West Nile Virus
WT	Wild Type

Publications

Mellett, M., Atzei, P., **Horgan, A**., Hams, E., Floss, T., Wurst, W., Fallon, P., and Moynagh, P.N. (2012) Orphan receptor IL-17RD tunes IL-17A signalling and is required for neutrophilia. *Nature Communications*

Chapter 1

General Introduction

1.1 The mammalian immune system

The immune system is the body's defence against invasion and colonisation by foreign material. Mammals possess a complex immune system consisting of two branches which function interdependently to stave off infection. These arms comprise of the innate and adaptive immune systems. The latter, which initially arose in gnathostomates or jawed vertebrates, encompasses specialised cells and processes which help relieve disease. The adaptive immune system is associated with immunological memory and specific tailored responses to particular pathogens. Its functions are performed by a specialised type of white blood cell called lymphocytes. T cells and B cells are the major types of lymphocytes. These cells have the ability to distinguish a wide selection of pathogens through their cell surface receptors. Each of these cells has a unique antigen receptor generated by somatic gene rearrangement which occurs during lymphocyte development. This process leads to a vast range or repertoire of receptors specific to each antigen (Janeway *et al*. 2005).

B cells and T cells are derived from hematopoietic stem cells. T cells are so called due to their development in the thymus while the B cell is named after the Bursa of Fabricius, an organ in birds in which their B cells mature. B cells are intimately involved in the humoral immune response, whereas T cells play a central role in cell-mediated immune responses. These lymphocytes circulate the lymphatic system with no effector function and act merely as surveillance agents. However, once their specific antigen is recognised they have the ability to proliferate and differentiate into effector cells. In addition to this recognition, in order for differentiation to occur, co-stimulatory molecules of Antigen Presenting Cells (APCs) from the innate immune system must be present (Medzhitov and Janeway. 1997).

B cell activation leads to plasma cell differentiation. These terminally differentiated cells produce antibodies. Antibodies are secreted proteins which recognise their specific foreign antigen. There are 3 main effector mechanisms by which antibodies can contribute to immunity. Firstly, in a process known as neutralisation, antibodies prevent the invading pathogen from entering the cell to subsequently cause damage by binding to the cell surface antigen. Phagocytosis by innate immune cells is triggered by opsonisation or the coating of the pathogens by antibody. Antibodies also stimulate activation of the complement system, a set of plasma proteins which function together to attack the bacteria which can result in either phagocytosis promotion or direct membrane pore creation resulting in pathogen destruction.

T cells are distinguished from other lymphocytes by the presence of a cell surface T cell receptor. There exist several subsets of T cell, each with distinct functions. Cytotoxic or killer T cells ($CD8^+$) do their work by releasing lymphotoxins, which cause cell lysis. Helper T cells secrete chemicals called lymphokines that stimulate cytotoxic T cells and B cells to grow and divide, attract neutrophils, and enhance the ability of macrophages to engulf and destroy microbes. Regulatory T cells are crucial for the maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells. As effective and specific as the adaptive immune system is, unfortunately, it can take anywhere from 4 to 7 days post infection for it to respond because of the duration of generation of T cell and B cell receptors, clonal selection and expansion. However, the older and more universal innate immune system is significantly more instinctive resulting in a rapid response which can halt the spread of infection during this time (Janeway *et al*. 2005)

The innate immune system has two main responsibilities, it acts as the first line of defence against invading pathogens and it determines the type of response to be mounted against the pathogens. It consists of the cells and mechanisms which protect the host from infection in a non-specific manner and thus is different from the adaptive system in that it does not confer any long lasting or protective immunity to the host (Janeway *et al*. 2005). It incorporates the action of white blood cells, antimicrobial proteins and the inflammatory response. Physical barriers are vital mechanisms of the innate response functioning as they do to keep pathogen burden low (Janeway 2001). The skin and mucous membranes are the initial anatomical obstacles. Secretions from these membranes are additional defense mechanisms. Chemical secretions such as sweat and tears can trap microorganisms, altering the environmental pH, which results in an unfavourable environment for the microbes. Desquamation of skin epithelium aids the removal of infectious agents that have become attached to external surfaces. In the gastrointestinal tract, microbial removal is achieved by the rhythmic movement due to peristalsis. Here, gut flora may also play a role by secreting toxins or merely competing with the would-be-colonisers for nutrients (Janeway 2001).

Acute inflammation is initiated by cells already present in the compromised tissues, mainly resident macrophages, dendritic cells, Kupffer cells and mastocytes. Chemical factors produced during the inflammatory response sensitize pain receptors, cause vasodilation of

the blood vessels at the scene, and attract other immune cells (Janeway 2001). Neutrophils are one of first cells to arrive at the scene of inflammation where they help phagocytose and remove the pathogenic invader (Kovach and Standiford 2012). They also trigger the recruitment of other parts of the immune system. Monocytes, macrophage precursor cells, are summoned to the site and subsequently differentiate into other macrophages which can release antimicrobial peptides and Nitric Oxide (NO). Meanwhile, other types of cells such as Mast Cells release histamine and proinflammatory cytokines such as TNF while NK cells kill virus infected cells (Liebson 2004). The later stages of the acute response result in the clotting of blood vessels which limits the spread of infection and promotes the healing of any damaged tissue following the removal of pathogens (Janeway 2001)

1.2 Pattern Recognition Receptors

An integral component of the innate immune system is the existence of germ line encoded pattern recognition receptors (PRRs). These PRRs have the ability to recognise a wide array of pathogen associated molecular patterns (PAMPs). PAMPs are highly conserved microbial motifs (Janeway and Medzhitov 2002). These motifs are not found on host cells and thus allow the PRRs to distinguish between self and infectious agents. A requirement of these motifs is that that they are highly conserved and necessary for microbial viability so that they are not lost from the genetic makeup of microorganisms given the high mutation rate during replication. Loss of the PAMP would render the microorganism undetectable. PAMPs may also be consistently present within classes of microbes thus enabling a limited number of PRRs to detect a vast panoply of microbial infections (Medzhitov 2001). Flagellin is a protein PAMP present in flagellated bacteria while another PAMP, Lipopolysaccharide (LPS), is a major component of gram negative bacterial cell walls (Newton and Dixit 2012). Double stranded RNA (dsRNA) is utilised by most viruses throughout their lifespan, but crucially not mammals, and is recognised by PRRs (Newton and Dixit 2012). Furthermore, damage associated molecular patterns (DAMPs) which are associated with cellular components released due to injury are also detected by PRRs. For example, ATP and adenosine are released in high concentrations after necrotic cell death and thus may function as DAMPs (Goh and Midwood 2012). PAMPs are found on all bacteria, viruses, fungi and protozoa. PRRs can be divided into three categories; secreted proteins, transmembrane receptors and intracellular receptors (Medzhitov and Janeway. 1997).

1.2.1 Secreted PRRs

Secreted PRRs are those which do not remain associated with the cell that produces them and are free to circulate through the blood (Medzhitov and Janeway. 1997). These types of PRRs include complement receptors, collectins, ficolins and pentraxins which are all secreted proteins (Roeder *et al* 2004). The mannose binding lectin (MBL) is one such receptor which is present as a free protein in blood plasma. MBL is a vital PRR of the innate immune response which can bind to a particular orientation of sugar residues on bacteria thus initiating the lectin pathway of complement activation (Takahashi 2011).

1.2.2 Transmembrane PRRs

The next set of receptors are the membrane bound PRRs. These include the C type Lectin Receptors (CLRs) which are a large family of carbohydrate recognition domain (CRD) containing proteins. One such CLR, Dectin-1, is responsible for the recognition of β-glucan, a polysaccharide, which is a major component of the fungal cell wall (Janeway and Medzhitov. 2002). One of the most important families of transmembrane PRRs to have emerged within the last 20 years has been the Toll-like receptors (TLRs). While they recognise a wide variety of PAMPs, their expression is very much cell-type specific in that they are primarily expressed on cells of the innate immune response including macrophages and Dendritic Cells (DCs) (Iwasaki and Medzhitov 2010). TLRs recognise PAMPs present on extracellular or endosomal surface pathogens. TLRs are categorised into several groups based on the PAMPs they recognise. TLR 1, 2, 4 and 6 recognize lipids. TLR4 recognises LPS, a major component of the bacterial cell wall, which is responsible for septic shock (Chow *et al*. 1999). TLR 2 can heterodimerise with TLR1, 6 and non- TLRs such as CD36 to detect a range of PAMPs such as peptidoglycan, lipopeptides and fungal zymosan. TLR1/2 and TLR 2/6 can discriminate between triacyl- and diacyl-lipopeptide (Farhat *et al*. 2008). TLR 5 and TLR 11 recognise protein ligands. TLR5, expressed highly in intestinal lamina propria cells, detects bacterial flagellin (Hayashi *et al*. 2001). The endosomal TLRs 3, 7, 8 and 9 detect microbial nucleic acids (Alexopoulou *et al*. 2001; Diebold *et al*. 2004; Bauer *et al*. 2001). Post PAMP recognition, TLRs then initiate downstream signaling cascades which result in activation of transcription factors such as nuclear factor (NF)-κB and the interferon regulatory factor (IRF) family of transcription factors (Iwaski and Medzhitov. 2004). This leads to the induction of inflammatory cytokine genes such as TNF,

IL-6, IL-1β and IL-12, anti-viral interferons (IFNs) and co stimulatory molecules on DCs which are critical for modulation of the adaptive immune response (Medzhitov *et al*. 1997).

1.2.3 Cytoplasmic PRRs

There are two main families of cytoplasmic PRRs which have been extensively characterised. The NOD-like receptor (NLR) is a large family of intracellular sensor of stress and microbial degradation products (Saleh 2011). The NLRs consist of 3 domains. The C terminus has, in a similar fashion to TLRs, a LRR which mediates ligand binding. Additionally, there is a central nucleotide-binding oligomerisation domain (NOD) and an N terminal effector domain which are required for propagation of the downstream signal (Saleh *et al* 2011). The NODs contain caspase recruiting (CARD) domains; NALPs have pyrin domains while NAIP has baculoviral repeat (BIR) domains (Ting *et al*. 2006). While some NLR signal transduction results in the activation of NF-κB, others form a multiprotein complex called the inflammasome (Martinon *et al* 2002). Inflammasomes ultimately lead to the production of key cytokines such as IL-1β and IL-18 by the cleavage of pro-IL-1β and pro-IL-18 by caspase-1. This pro-IL-1β is generally produced by TLR signalling highlighting a cooperative role between two different sets of PRRs (Creagh and O'Neill, 2006). Another family of cytoplasmic PRRs are the retinoic acid-inducible gene I (RIG-I)-like receptors (RLR), such as RIG-I and melanoma differentiation factor 5 (MDA5). RLR members are expressed in most cell types and detect viral RNA through their helicase domain and signal through their caspase recruitment domains. Both RIG-I and Mda5 recruit a CARD containing protein IFN-β promoter stimulator 1 (IPS-1) or mitochondrial anti-viral signalling (MAVS) via CARD-CARD interactions (Kawai *et al*. 2005; Meylan *et al*. 2005; Seth *et al*. 2005; Xu *et al*. 2005) MAVS displays two CARD domains that are important for initiating signalling downstream and for mitochondrial localisation. Signalling via IPS-1 leads to activation of IRF3 and NF-κB and consequently interferon production (Saleh 2011).

1.3 Toll-like Receptors

Toll, a gene first discovered in *Drosophila melanogaster,* is required for dorsoventral axis formation in fruit fly larvae and was subsequently discovered to have a role in antifungal immunity in *Drosophila* adults (Lemaitre *et al*. 1996). It was noted that the Toll pathway

shared similarity with the NF-κB pathway in mammals and this led to the discovery of the human Toll receptor, now known as TLR4 (Medzhitov *et al*. 1997). Since this initial breakthrough, 13 murine and 11 human TLRs have been identified each with a different specificity for the various PAMPs. TLRs have emerged as an important family of Type I transmembrane PRRs and are characterised by extracellular leucine-rich repeats that are responsible for PAMP-binding and a highly conserved intracellular Toll/IL-1R (TIR) domain that is shared with the IL-1 receptor family (Medzhitov *et al*. 1997; Rock *et al*. 1998). Interestingly, research has shown that the IL-1R family uses many of the same signalling intermediates as TLRs although TLRs do not possess the extracellular Immunoglobulin (Ig)-like domains which control ligand recognition in the IL-1R family (Martin and Wesche, 2002). Upon ligand binding, TLRs can either homo- or heterodimerise with other TLRs in order to be able to attract downstream TIR domain containing adaptor proteins responsible for signal propagation. Myeloid Differentiation factor 88 (MyD88) is a TIR domain containing adaptor protein which transduces the signal from all TLRs except TLR3. It also functions as a key mediator for the IL-1 and IL-18 receptors (Adachi *et al*. 1998; Janssens and Beyaert 2002). The recruitment of MyD88 to the TLR results in the downstream activation of the transcription factors NF-κB and AP-1, which regulate the expression of pro-inflammatory gene expression. TLR2 and TLR 4 require a bridging adaptor, MyD88 adaptor like (Mal) to link MyD88 to the receptor. TIR domain- containing adaptor inducing IFN- β (TRIF) is utilised by TLR3 and TLR4 leading to subsequent activation of NF-κB and IRFs (Jiang *et al*. 2004). TLR4 needs an additional adaptor protein TRIF-related adaptor molecule (TRAM) to act as a bridge between the receptor and TRIF (Oshiumi *et al*. 2003).

1.3.1 TLR1, 2, 6 and 10

TLR2 has the greatest repertoire of ligands. It is involved in recognition of lipoarabinomannan from mycobacteria (Underhill *et al*. 1999) glycoinositolphospholipids from protozoa (Campos *et al*. 2001) and glycolipids from spirochetes (Opitz *et al*. 2001). It was shown that TLR1 and TLR6 can form heterodimers with TLR2 when TLR2 dominant negative forms of both blocked TLR2 signalling (Ozinsky *et al*. 2000; Hajjar *et al*. 2001). TLR2 and TLR6 heterodimers are also crucial for recognising yeast zymosan, a cell wall component of *Saccharomyces cerevisae*. A C-type lectin; Dectin-1 can collaborate with TLR2 and TLR6 to mediate independent but cooperative signalling in response to zymosan.

Dectin-1 triggers release of reactive oxygen species (ROS) and promotes phagocytosis, while the TLR2-TLR6 heterodimer triggers NF-κB signalling leading to the production of inflammatory cytokines, such as IL-12 whose production is enhanced by Dectin-1 (Gantner *et al*. 2003).

1.3.2 TLR3

TLR3 is expressed on the endosomes of immune cells such as macrophages, NK cells and mDCs and recognises viral nucleic acids (Kumar *et al*. 2009). This is a strategic location for viral detection as these TLRs can detect nucleic acids released by bacteria and viruses that are internalised for delivery to the endosome. TLR3 is essential for recognising viral dsRNA which is the genetic information of some viruses. dsRNA is also produced by most other viruses at some point in their replication process. It can also recognise the stable synthetic dsRNA mimic polyriboinosinic : polyribocytidylic acid (Poly I:C) (Tabeta *et al*. 2004). TLR3 has been shown to have a protective role against viral infection highlighted by increased susceptibility to cytomegalovirus in TLR3 knockout mice (Tabeta *et al*. 2004). In contrast, it has been shown to increase disease pathogenesis in response to West Nile Virus (WNV) (Wang *et al*. 2004).

1.3.4 TLR4

Situated in the outer membrane of Gram negative bacteria, the endotoxin LPS consists of a Lipid A part and a core oligosaccharide with polysaccharide side chains. The most conserved region of the lipoglycan, Lipid A, is the region of LPS detected by TLR4. TLR4 is not sufficient in itself for LPS recognition. LPS is initially bound in serum by LPS-binding protein (LBP), which delivers the ligand to a membrane protein, CD14. CD14 exists as both a soluble form and a GPI-linked outer membrane protein and serves as a receptor for LPS (Aderem and Ulevitch. 2000). CD14 associates with TLR4 and an extracellular accessory protein MD2. Each member of this triumvirate is indispensible for ligand recognition and subsequent signalling (Shimazu *et al*. 1999). In addition to LPS, TLR4 has also been implicated in the recognition of a diverse range of other PAMPs. Interestingly, TLR4 has been implicated in recognition of viral-derived proteins. It was shown to have the ability to

recognise the fusion protein (F protein) of respiratory syntical virus (RSV) (Kurt-Jones *et al*. 2000) and the envelope protein from mouse mammary tumor virus (MMTV) (Rassa *et al*. 2002). Additionally, TLR4 can prompt an immune response to endogenous molecules. Heat-shock protein 60 (Hsp60) activates TLR4, serving as a danger signal for the innate immune system (Ohashi *et al*. 2000). Fragments of hyaluronic acid, the extra domain A (EDA) of fibronectin and the anti-microbial β-defensin 2 released during inflammation all activate TLR4 (Termeer *et al*. 2002; Okamura *et al*. 2001; Biragyn *et al*. 2002).

1.3.5 TLR 5 and TLR11

The primary ligand for TLR5 is flagellin, a protein found in the flagella of gram negative bacteria (Hayashi *et al* 2001). TLR5 is found expressed on a wide variety of immune cells including monocytes, dendritic cells, T lymphocytes, natural killer cells and also epithelial cells. It has been demonstrated that TLR5 and TLR9 have an effect on each other's signalling. TLR9, primarily found on expressed on plasmacytoid dendritic cells and B lymphocytes favours a T_H1 response promoting cell mediated immunity (Davis *et al* 1998). However, TLR5 initiates a T_H2-like response which favours humoral responses (Marshall *et al*. 2004). It was thus shown that flagellin can inhibit TLR9 mediated cell activation and cytokine production. Murine TLR11 shares a similar structure to TLR5. It is found highly expressed in the kidney and the bladder. TLR11 knockout mice have been shown to be susceptible to uropathogenic bacterial infection (Zhang *et al*. 2004). There is dose dependent NF-κB activation by murine TLR11 in response to profilin, an actin binding protein from the protozoan *Toxoplasma gondii* (Yarovinsky *et al*. 2005).

1.3.6 TLR7, TLR8 and TLR9

TLR 7 and 8 are involved in viral detection. In TLR7 deficient mice, there is abrogated cytokine and Type I interferon production in response to synthetic imidazoquinoline-like molecules including imiquimod and resiquimod in addition to guanosine analogs such as loxoribine (Hemmi *et al*. 2002). Human and murine TLR8 share significant similarity with TLR7 however only the human homolog responds to resiquimod

(Jurk *et al*. 2002). Uridine and guanosine are major components of viral ssRNA but they are also found in host nucleic acids. The localisation of TLR7 in endosomal compartments where host nucleic acids are usually absent allows for a specific response to viral RNA (Diebold *et al* 2006). However, host nucleic acid may still possess stimulatory activity. The inefficient removal of debris from apoptotic cells can result in host nucleic acids activating TLRs resulting in autoimmunity (Leadbetter *et al*. 2002).

Unmethylated 2'-deoxyribocytidine-phosphate-guanosine (CpG) DNA is detected by TLR9 on pDC and B cells in humans. This mechanism is employed to recognise intracellular pathogenic DNA of viral or bacterial origin. CpG sites are regions of DNA where there is a high frequency of cytosine nucleotides occurring next to guanosine nucleotides and are usually unmethylated turning the gene off. While unmethylated CpG DNA is relatively rare in mammals (~1%), it is more inherent in bacterial and viral DNA. Consequently, CpG has some vital characteristics of a PAMP. It is a property of microorganism cells but not of host cells (Medzhitov 2001). It is distinctive to a large number of otherwise unrelated microorganisms. Additionally, CpG is highly conserved being essential for the viability of the microbe and any mutants lacking the PAMP are unable to survive. In addition to CpG, TLR9 detects hemezoin from blood feeding parasites such as the Plasmodium spp., the causative agent of malaria (Coban *et al*. 2005). It is a hydrophobic heme polymer produced from the digestion of host haemoglobin by the parasites. Serving as a TLR9 agonist, it induces the production of inflammatory cytokines in macrophages and DCs.

It has been demonstrated that human TLR8 dimerises with both TLR7 and TLR9. Additionally, TLR8 ligands have been observed to function as TLR7 and TLR9 antagonists (Wang *et al* 2006). Furthermore, TLR9 interacts with TLR7, antagonising its signalling. This interplay exposes a hitherto unknown level of complexity governing TLR signalling networks.

1.4 TLR signalling

TLR signalling pathways stem from the cytoplasmic TIR domain. There are at least four TIR adaptors responsible for transducing the ligand recognition signal. These include MyD88, Mal, TRIF and TRAM. These proteins associate with the TLR through homotypic TIR-TIR interactions. MyD88 is viewed as a universal adaptor as it is shared by all TLRs with the notable exception of TLR3. Consequently, resulting TLR signalling cascades are defined by their employment of MyD88 (Takeda and Akira. 2004).

1.4.1 MyD88-dependent signalling

MyD88 is comprised of an N-terminal death domain and an intracellular C-terminal TIR domain. Upon ligand binding, a conformational change is induced which allows for MyD88 to recruit the IL-1 receptor-associated kinases (IRAKs) to TLRs via the interaction of the death domain of both molecules (Wesche *et al*. 1997; Burns *et al*. 1998; Medzhitov *et al*. 1998). The members of the IRAK serine/threonine kinase family include: IRAK1, IRAK2, IRAKM, and IRAK4. However, only IRAK1 and IRAK4 are active kinases (Li *et al*. 2002). IRAKs are activated by phosphorylation. IRAK1 phosphorylation leads to dissociation of the IRAKs from the receptor MyD88 complex and subsequent association with TNF receptor-associated factor 6 (TRAF6) (Qian *et al*. 2001). TRAF6 is a ubiquitin E3 ligase. It functions along with ubiquitin-conjugating enzyme 13 (Ubc13) and Uev1a, a Ubc-like protein, to initiate the synthesis of Lys63-linked polyubiquitin chains on target proteins including TRAF6 molecules (Wang *et al*. 2001). The MyD88-IRAK-TRAF6 axis constitutes the basis of the MyD88-dependent pathway. These events lead to the activation of the IκB kinase (IKK) complex. This multi-protein complex causes phosphorylation of IκB, which sequesters the transcription factor NF-κB outside the nucleus. Upon its phosphorylation, IκB is degraded which allows NF-κB to enter the nucleus and activate transcription of pro-inflammatory cytokine genes (Hayden and Ghosh. 2004).

1.4.2 MyD88 independent signalling

As expected, MyD88 deficient macrophages failed to show any activation of NF-κB and AP-1 in response to most TLR ligands. However, treatment of these cells with LPS resulted in the transcription factor activation albeit with delayed kinetics relative to wild type macrophages (Yamamoto *et al*. 2002). This novel discovery pointed towards a MyD88-independent signalling pathway. The key adaptor protein in this pathway is TRIF. TRIF interacts directly with TLR3 while TRAM is required to function as a bridging adaptor for the association between TRIF and TLR4 (Oshiumi *et al*. 2003). Once TRIF is recruited to the receptor, it subsequently interacts with receptor interacting protein 1 (RIP1). The C-terminus of TRIF possesses a RIP homotypic interaction motif (RHIM) and through this RIP-1 is allowed to interact. In the absence of RIP-1, TLR3 and TLR4 mediated transcription factor activation is abrogated highlighting the requirement of RIP-1 for signal transduction (Meylan *et al*. 2004). The recruitment of RIP-1 and TRAF6 facilitates transforming growth factor-β (TGF-β) activating kinase (TAK1) activation which subsequently leads to NF-κB and MAPK induction.

MyD88-independent signalling also leads to the activation of members of the IRF family of transcription factors, IRF3 and IRF7. The recruitment of TRIF, to the receptor, results in the engagement with TRAF3 (Matsumoto and Seya, 2008). Two non-canonical IKKs, inducible IKK (IKKi) and TANK Binding Kinase 1 (TBK1) are subsequently recruited. NF-κB-activating kinase (NAK)-associated protein 1 (NAP1) is implicated in recruiting these kinases (Sasai *et al*. 2005). In order for nuclear translocation to occur, both IRF3 and IRF7 are phosphorylated at key serine/threonine residues by TBK1 and IKKi (Sharma *et al*. 2003). This phosphorylation promotes dimerisation and transport to the nucleus where they interact with their transcriptional co-activators cAMP responsive element binding protein (CBP)/p300 (Gauzzi *et al*. 2010). CBP/p300 acetylates IRF3 homodimers, inducing a conformational change which reveals its DNA binding domain (Lin *et al*. 1999). Once inside the nucleus, IRF3 and IRF7 then bind to the promoter regions, activating the expression of Type I IFNs (Lin *et al*. 1999).

1.5 Transcription factors

The NF-κB transcription factor is a critical regulator of immunity, stress responses, apoptosis and differentiation chiefly due to the vast number of genes it may activate. Mammalian NF-κB has five subunits; RelA (p65), Rel B, c-Rel, p100/52 and p105/p50. NF-κB subunits are located in the cytoplasm, inhibited from entering the nucleus by IκB family members (Hayden and Ghosh 2004). Upon IL-1R or TLR activation, degradation of IκB occurs, freeing up NF-κB to translocate to the nucleus initiating the induction of pro-inflammatory genes. Stimulus induced degradation of IκB proteins is promoted via phosphorylation by the IκB Kinase (IKK) complex which consists of two catalytically active kinases, IKKα and IKKβ, and the regulatory subunit NF-κB essential modulator (NEMO) (Hayden and Ghosh 2004). Phosphorylated IκB proteins are targeted for ubiquitination and degradation by the 26S proteasome, releasing bound NF-κB dimers to translocate to the nucleus.

There are two main NF-κB-activating pathways which occur in cells. The more traditional canonical pathway is initiated by most physiological NF-κB stimuli including the signals emanating from cytokine receptors such as the IL-17 receptor and pattern recognition receptors such as the Toll-like receptors (Moynagh 2005). It is completely reliant upon the functions of IKKβ and NEMO and results in phosphorylation of IκBα and nuclear translocation of mainly p65-containing heterodimers. The non-canonical pathway involves the processing of p100 to p52 by NIK and IKKα. These kinases phosphorylate p100 which results in the cleavage of p100. The resulting protein p52 then forms dimers with RelB to mediate gene expression (Moynagh 2005). This pathway of NF-κB is only activated by a tiny number of ligands, such as CD40 ligand (CD40L), B-cell activating factor belonging to the TNF family (BAFF), ligands for the lymphotoxin B receptor (LTbR) and TNF-related weak inducer of apoptosis (TWEAK) (Mon Thu and Richmond. 2010).

TLR signalling also results in the induction of a number of interferon regulatory factors (IRFs). There are nine members of the IRF family (IRF1-9) (Moynagh 2005). IRF3 and IRF7 are key regulators of type I IFN gene expression in response to invading viruses. Type I IFNs are a family of cytokines which have pivotal roles in antiviral defence, immune activation and cell growth (Goodbourn *et al*. 2000). Type I IFNs inhibit viral replication and stop the spread of infection. This is achieved by IFNs binding to interferon receptors

(IFNARs) present on the cell surface on adjacent infected and uninfected cells (Moynagh 2005). Endoribonucleases are subsequently turned on and these degrade viral nucleic acid. IRF3 and IRF7 are activated by TBK1 and IKKi. Interestingly, TBK1 seems to be the predominant activator of Type I IFN genes in mouse embryonic fibroblasts (MEFs) (Perry *et al*. 2004). In the MyD88-independent pathway, employed by TLR3/4, TBK1 phosphorylates IRF3 and IRF7. However, in plasmacytoid Dendritic Cells (pDCs) IRF7 is activated in a TBK1/IKKi-independent manner through the MyD88 alternative pathway (Hoshino *et al*. 2006).

1.6 RLR signalling

When a viral infection occurs in mammalian cells, the immune system responds in a highly coordinated fashion with the speedy upregulation of antiviral defence proteins, natural killer cells, neutralising antibodies and cytotoxic T cells (Janeway 2001). This process is regulated by key antiviral signalling molecules which include Type I Interferons (IFNα and IFNβ) and the related Type III Interferon (IFNλ) (Chen and Jiang 2012). It is therefore clear that the mammalian host cells possess mechanisms that allow for viral detection. One of these mechanisms occurs through the RIG-I-like receptor (RLR) family of PRRs in response to viral RNA PAMPs generated during the process of virus infection.

There are 3 members of the RLR family namely the retinoic acid inducible gene (RIG-I), melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP-2) (Bruns and Horvath 2012). Located in the cytoplasm, these proteins all share an integral DExD/H box RNA helicase domain. In addition, RIG-I and MDA5 have 2 N-terminal CARDs which function in permitting the activated of RIG-I or MDA5 to interact with the RLR adaptor protein, mitochondrial antiviral signalling (MAVS; also known as IPS-1, VISA and Cardif) which subsequently localises to the outer mitochondrial membrane (Bruns and Horvath 2012). MAVS transduces the signal to kinases such as TBK1 and IKKε which lead to the activation of the transcription factors IRF3, IRF7 and NF-κB (Belgnaoui *et al* 2011). Furthermore, in an a MAVS independent pathway, RIG-I is able to directly turn on the inflammasome (Melchjorsen *et al*. 2010).

1.6.1 RLR ligands

RLRs need to be highly discriminatory as they are localised in the cytoplasm where there are vast numbers of potentially problematic host RNAs (Loo and Gale 2011). However, RLR signalling is only present in virus infected cells. This feat is achieved by RLRs only responding to distinctive RNA with unusual patterns. These patterns may include chemical modifications of RNA, secondary or tertiary conformations or highly specific sequences (Loo and Gale 2011). Interestingly, activation of RLR signalling may be achieved by RNA of viral or cellular origin. Early studies demonstrated that RNA transcription *in vitro* by phage polymerase (IVT-RNA) generates a powerful RIG-I ligand (Pichlmair *et al*. 2008; Hornung *et al*. 2006). These RNAs contain an uncapped 5' triphosphate (5'PPP) that is essential for interferon inducing activity. 5'PPPs are responsible for initiation of binding of RIG-I, promotion of its adenosine phosphatase (ATPase) activity and induction of conformational changes which permit RIG-I dimerisation and exposure of CARDs for interaction with MAVS (Cui *et al* 2008; Takahasi *et al* 2008). Host mRNAs are capped at the 5' end and nuclear processing of ribosomal RNA and transfer RNAs removes or alters the 5'PPP groups before they get to the cytoplasm. Consequently, it has been demonstrated that viral RNA genomes obtained from influenza A or rabies virus particles may trigger RIG-I (Pichlmair *et al*. 2006). These viruses possess a 5'PPP-bearing RNA genome. Additionally, it has been shown that enzymatic removal of the 5'PPP stops its ligand activity (Pichlmair *et al* 2006; Hornung *et al*, 2006). Other viruses, such as Borna disease virus, have a genome that has 5'monophosphate ends and RNA obtained from such viruses does not possess RIG-I stimulating activity (Habjan *et al*. 2011).

The RNA genomes of viruses recognised by RIG-I have complementary 5' and 3' ends and adopt a panhandle conformation. As a result, these provide 2 features detected by RIG-I: 5'PPP and base pairing at the 5' end (Habjan *et al*. 2011). Other features of RNAs which influence RIG-I ligand activity is the inclusion of modified bases such as pseudo-uridine into IVT-RNA which reduces its stimulatory activity (Hornung *et al* 2006). The hepatitis C virus possesses a sequence motif in the 3' non-translated region which along with the 5'PPP end is essential for RIG-I activity (Saito *et al* 2008).

There are also some RNAs without 5'PPPs which activate RIG-1. For example, chemically synthesised dsRNA oligonucleotides and some forms of polyinosinic :

polycytidylic acid (Poly I:C) (Takahasi *et al* 2008; Kato *et al* 2008). Additionally it has been demonstrated that the products of host RNA cleavage by ribonuclease (RNAse L) activate RIG-I (Malathi *et al* 2007). Also, phosphorothioated single stranded DNA oligonucleotides have been suggested to act as RIG-I antagonists (Ranjith-Kumar *et al* 2009). There is significantly less known about the ligands which cause MDA5 activation. Transfection of Poly I:C inside cells induces MDA5-dependent IFN activation (Tamassia *et al* 2008). It's therefore believed that MDA5 detects dsRNA produced during infection, especially as dsRNA builds up in cells infected with viruses detected by MDA5 (Pichlmair *et al* 2006; Kato *et al* 2008; Pichlmair *et al* 2009). A surprising discovery is that RLRs may initiate IFN induction in response to cytoplasmic DNA. When DNA viruses infect cells, some DNA can be sent to the cytoplasm. This is mimicked *in vitro* by transfecting a DNA polymer poly dA: dT into cells. However, poly dA: dT is not able to be detected directly by RLRs. Cytosolic RNA polymerase III transcribes it into uncapped RNA which may then lead to RIG-I activation (Ablasser *et al* 2009; Chiu *et al* 2009). This is the pathway thought to lead to induction of interferons during infection with the bacterium *Legionella pneumophilia* and DNA viruses such as adenovirus, herpes simplex and Epstein Barr virus while Vaccinia virus is believed to produce an RNA agonist for MDA5 (Pichlmair *et al* 2009; Chiu *et al* 2009).

1.7 IL-17 Signalling

TLRs can trigger inflammatory responses but also there is a lot of interest in effector cytokines and their inflammatory effects especially in inflammatory diseases. Interleukin-17 (IL-17 or IL-17A) is a key effector cytokine which can be produced as a result of TLR-induced NF-κB. IL-17 was initially identified as a transcript from a rodent T-cell hybridoma (Rouvier *et al*. 1993). It is the founding member of a group of cytokines called the IL-17 family. The IL-17 family share homology in amino acid sequences showing highly conserved cysteine residues which are important for their conformation (Hymowitz *et al* 2001). To date, there have been 6 members identified, Il-17A-F (Yao *et al* 1995; Li *et al*. 2000; Starnes *et al* 2002). Although this cytokine serves as a key player in host defence during infection, dysregulated expression of IL-17 is implicated in a whole host of inflammatory pathologies

such as experimental autoimmune encephalitis (EAE) and collagen induced arthritis (CIA) and cancer (Ouyang *et al* 2008; Dong 2008). Whereas the signature cytokines involved in the T_H1 and T_H2 cell lineages initiate the JAK–STAT pathway, the IL-17-family cytokines induce signalling through a novel ACT1-dependent pathway, resulting in the activation of pro-inflammatory factors such as NF-κB that are usually associated with innate immune signalling (Gaffen 2011). Consequently, the properties of the IL-17 signalling family lend itself to being a mediator between the innate and adaptive immune system.

1.7.1 IL-17 Cytokine family

IL-17A, the hallmark cytokine of the IL-17 family, is secreted by cells of the activated immune system, including T_H17 cells, γδ T cells and natural killer cells. Human IL-17A is a 21kDA glycoprotein with 155 amino acids and 63% amino acid homology with murine 1L-17A (Rouvier *et al*. 1993). It is a pleiotropic cytokine that exerts its function on numerous cell types augmenting the production of pro-inflammatory molecules. IL-17A coordinates tissue inflammation and mobilizes neutrophils by stimulating the production of cytokines and chemokines, including IL-6, KC and MIP-2 in mice and IL-8 and GRO family members in humans, antimicrobial peptides such as defensins and s100 proteins and matrix metalloproteinases including MMP9 (Iyoda, M. *et al* 2010). IL-17A expression is therefore tightly regulated. IL-17F shares the highest similarity with IL-17A; in fact IL-17A and IL-17F can form heterodimers and perform similar functions to IL-17A and IL-17F homodimers (Chang and Dong 2009). However, IL-17F can play a distinctive role in some circumstances such as during allergic inflammation in the lungs and intestinal inflammation *in vivo* (Hizawa *et al*. 2006; Yang *et al* 2008; Ishigame *et al*. 2009). A striking feature of IL-17A, IL-17F and IL-17A/F is their modest signalling activity. Nevertheless, they display great synergy, with TNF, LTα, IFNγ and IL-1β (Gaffen, 2009). Although the mechanisms involved are not clearly understood, a significant element is the cooperative enhancement of mRNA stability of certain IL-17 target genes. For example, IL-6 and CXCL1 are both induced weakly at the transcriptional level by IL-17 alone, but their transcript half-life is enhanced dramatically by a combination of TNFα and IL-17. IL-17E (IL-25) shares the least homology with IL-17 A. It is responsible for facilitation of T_H2 cell mediated immune responses, where it has a role in allergic disease and defence against helminthes (Barlow and McKenzie 2009). A range of

cellular sources of IL-25 have been identified namely epithelial cells, eosinophils, mast cells and basophils. The roles of the remaining cytokines IL-17B, IL-17C and IL-17D are as of yet not fully elucidated.

1.7.2 IL-17 Receptor Family

The IL-17 receptor family consists of five members (IL-17RA, RB, RC, RD and RE) and each shares significant homology to the first discovered member, IL-17RA (Kolls *et al* 2004; Moseley *et al*. 2003; Aggarwal and Gurney 2002). IL-17 signalling is abrogated in IL-17R (IL-17RA) knockout mice and furthermore soluble IL-17RA or even anti-IL-17RA antibodies have been shown to inhibit the activity of IL-17 in human epithelial cells highlighting the requirement of IL-17R for IL-17 signalling (Toy *et al* 2006; Ye *et al* 2001; McAllister *et al* 2005). IL-17RA is constitutively expressed on a wide variety of tissues whereas IL-17RC is mainly expressed in non-hematopoietic tissues. Despite this ubiquitous expression, IL-17RA is dynamically regulated. Treatment with IL-15 and IL-21 increase expression of IL-17RA in CD8+ T cells and phosphoinositide 3-kinase (PI3K) inhibits IL-17RA expression in T cells (Lindeman *et al* 2008). Interestingly, a major disparity between IL-17RC and IL-17RA is that IL-17RC is found expressed as numerous different isoforms with approximately 11 splice variants (Haudenschild *et al* 2002). IL-17A, IL-17F and IL-17A–IL-17F all signal through the same receptor subunits, IL-17RA and IL-17RC, which together form a heteromeric complex (Haudenschild *et al* 2002) . Another IL-17 receptor, IL-17RB, has been identified as the receptor for IL-25 (IL-17E). Ligation of IL-15 with IL-17RB results in the major tissue infiltration of eosinophils and upregulation of Th2-related molecules such as the production of IL-4, IL-5 and IL-13. Also, in lung epithelial cells there is increased Th2 cytokine and mucus production resulting in impaired clearance of parasitic helminthes in IL-25 knockout mice (Fallon *et al* 2006). Detailed bioinformatic analysis has shown that members of the IL-17R family all possess a “similar expression to FGF/IL-17R” (SEFIR) domain. In actual fact, this SEFIR domain bears a striking resemblance to the functional TIR domain within the TLR family.

In addition to the SEFIR domain, all receptor subunits contain an extracellular fibronectin III-like domain (Novatchkova *et al* 2003). IL-17 signals through the IL-17R complex to activate MAPK, NF-κB, phosphoinositide-3-kinase (PI3k)-Akt and

CCAAT/enhancer binding protein (C/EBP) (Gaffen 2011). In addition to this, IL-17R signal transduction shares certain similarities with TLR signaling in that they both employ the ubiquitin E3 ligase, TRAF6. However, while there are certain similarities between the biological functions of IL-1 signalling, IL-17 signalling does not require TIR domain containing adaptors such as MyD88 or TRIF or downstream IL-1 signalling components such as IRAK 4 (Gaffen 2011). In fact, a novel adaptor protein, Act1 (transcription factor NF-κB activator 1) has been shown to bind to IL-17RA, initiating TRAF6 recruitment allowing for the IL-17 signal transduction to occur (Gaffen 2011).

1.7.3 IL-17 signal transduction

As previously alluded to, IL-17A signaling culminates in activation of NF-κB and MAP kinases through IL-17RA. However, in TRAF6 knockout mouse embryonic fibroblast (MEFs) there is a loss of NF-κB and MAPK activation as well as IL-6 production (Schwandner *et al*. 2000). Furthermore, TRAF6 has been shown to immunoprecipitate with IL-17RA when both proteins are overexpressed. As TRAF6 is essential in IL-17RA signaling, this suggests a shared similarity with TLR/IL-1R. It has consequently been proposed that IL-17R family belongs to a larger group of proteins called similar expression to fibroblast growth factor and IL-17R; Toll-IL-1R (STIR). However, IL-17RA does not have a TRAF6 binding site, which highlights the need for an adaptor protein which would mediate TRAF6 recruitment. One such protein is Act1 (Gaffen 2011).

Act1 is a cytoplasmic SEFIR domain containing adaptor protein employed by the IL-17R family for activation of NF-κB and other signals (Shen and Gaffen 2008). Initially discovered by a yeast-two hybrid screen based on its interaction with IKKγ, it was found to be a negative regulator of CD40 and BAFF-mediated B cell signaling (Wu *et al* 2012). Act1 interacts directly with the cytoplasmic domain of IL-17RA. In Act1 knockout cells, IL-17 signalling is abrogated which highlights the requirement of the adaptor (Qian *et al* 2008). Also, Act-1 deficient mice display less inflammatory disease in autoimmune encephalomyelitis and dextran sodium sulfate-induced colitis (Qian *et al* 2008). As IL-17F signaling needs IL-17RA, IL-17F signaling is also lost in Act-1 knockout cells. Furthermore, Act1 has been shown to associate with IL-17RC and the interaction increases on IL-17A treatment (Yang *et al* 2008; Ho *et al* 2010). The SEFIR domain of IL-17RA is necessary for

the homotypic interaction with Act1. SEFIR and the TIR-like loop (TILL) domains of IL-17RA are needed for NF-κB and MAPK induction. The TILL motif has also been declared to confer structural integrity to the IL-17RA while an extended area beyond the SEFIR and TILL is also implicated in signal propagation (Gaffen 2009). Act1 is also implicated in IL-17RB signaling. The deletion of the SEFIR domain of Act1 and/or of IL-17RB abolished the interaction thus leading to reduced levels of gene expression (Gaffen *et al* 2009). Recently, a novel TRAF6-independent IL-17 mRNA stability signaling pathway has been identified. Here, IL-17 promotes interactions among Act1, TRAF2 or TRAF5, and the multifunctional RNA-splicing regulatory factor SF2 (ASF) which is a vital component of the splicing process (Sun *et al* 2011).

1.8 IL-17RD regulates immune signalling pathways

Immune signalling pathways require tight control as it is clear that dysregulated signalling can lead to a whole host of quite serious chronic inflammatory disorders such as Inflammatory Bowel Disease, Multiple Sclerosis and Rheumatoid Arthritis. This thesis describes a role for a novel regulator of TLR, RIG-I and IL-17 signalling.

IL-17RD is an orphan receptor from the IL-17 receptor family (Moseley *et al*. 2003). While the immunomodulatory effects of other IL-17 receptors have been identified, the role of IL17-RD remains unclear. IL-17RD is also called similar expression to FGF genes (Sef) as it shows the same expression patterns in zebrafish embryos as Fibroblast Growth Factor genes and it is also a part of the same expression group as *fgf8* and *sprout*. Sef was initially characterised as a negative regulator of FGF signalling (Furthauer *et al*. 2002; Tsang *et al*. 2002). Fibroblast growth factors are secreted proteins which function by activating specific tyrosine kinases receptors, the FGF receptors which transmit the signal by activating different pathways including the Ras/MAP kinase and the phospholipase-C gamma pathways. FGFs are important in regulating many developmental processes including patterning, morphogenesis, differentiation, cell proliferation or migration (Goldfarb 1996). Different targets have been revealed for IL-17RD in FGF signalling. IL-17RD inhibits at the receptor level but also downstream at the level of MEK (Furthauer *et al*. 2002; Kovalenko *et al*. 2003; Tsang *et al*. 2002; Xiong *et al*. 2003). IL-17RD also interferes with the dissociation of ERK from MEK, impeding the translocation of ERK to the nucleus (Torii *et al*. 2004).

A number of studies in the past number of years have highlighted IL-17RD's role in many cancers. For example, loss of IL-17RD expression is associated with high grade and metastatic prostate cancer and IL-17RD inhibits fibroblast growth factor-induced tumourigenic behaviour in prostate cancer cells and is downregulated in aggressive clinical disease (Darby *et al* 2006; Darby *et al* 2009). Additionally, IL-17RD has been shown to reduce the proliferation of endometrial adenocarcinoma cells via inhibiting FGF 2-mediated MAPK/ERK signalling pathway (Zhang *et al* 2011). It has also been implicated as a negative regulator of fiber cell differentiation in the ocular lens (Newitt *et al* 2010). There has been no reported physiological role for IL-17RD in IL-17 signalling, apart from a study in which

overexpressed IL-17RD was shown to co-operate with IL-17RA (Rong *et al*. 2009). While IL-17RD shares a lot of similarity with the IL-17 receptor family, it also contains a partial TIR domain, consisting of boxes 1 and 2 and thus it is part of a larger STIR domain superfamily, the name deriving from both SEFIR and TIR domains which have been shown to be similar in both length and structure (Novatchkova *et al* 2003). Consequently, the study of IL-17RD's role in various immune signaling pathways was considered worthy of investigation

1.9 Project Aims

The overall aims of this project were to:

1) Explore a molecular and physiological role for IL-17RD in TLR signalling

2) Investigate a role for IL-17RD in other innate immune signalling pathways

3) Probe the molecular basis of the role(s) of IL-17RD in innate immunity.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

Reagent	Supplier
Agar	Sigma
Agarose	Promega
Agarose, low melting point	Sigma
Alexa488	Invitrogen
Alexa594	Invitrogen
Ampicillin	Sigma
Antibodies (monoclonal) against -	
β-Actin	Sigma
FLAG M2	Sigma
HA.11	Covance
Myc-Tag (9B11)	Cell Signaling
Myc-Tag (71D10)	Cell Signaling
Antibodies (polyclonal) against -	
ERK1/2	Cell Signaling
Phospho-ERK1/2	Cell Signaling
JNK	Cell Signaling
Phospho-JNK	Cell Signaling
IRF3	Cell Signaling
Phospho-IRF-3	Cell Signaling

IκBα	Santa Cruz
Phospho-serine	Abcam
p38	Cell Signaling
phospho-p38	Cell Signaling
p65 (C-20)	Santa Cruz
p65	Cell Signaling
phospho-p65	Cell Signaling
TBK1	Cell Signaling
Phospho-TBK1	BD
APS	Sigma
ASCC1 cDNA clone in CMV expression vector	OriGene
Blasticidin S	InVivo
Boric Acid	Sigma
Bovine serum albumin	Sigma
Bradford reagent dye	Bio-Rad
Bromophenol blue	Sigma
Chloroform	Sigma
Chloroquine	Sigma
CIP	New England Biolabs
Coelenterazine	Insight Biotech
DEPC-treated water	Ambion
DMEM	Invitrogen
DMSO	Sigma

DNA ladder (1Kb) & Loading dye (6X)	Promega
dNTPs	Promega
DTT	Sigma
E.coli - TOP 10 competent cells	Invitrogen
EDTA	Sigma
Ethanol	Sigma
Ethidium bromide	Sigma
FBS	Invitrogen
Glacial acetic acid	Merck
Glycerol	Sigma
Glycine	Sigma
Goat Serum	Vector Laboratories
GoTaq Flexi DNA Polymerase	Promega
High speed plasmid midi kit	Qiagen
HEPES	Sigma
Hoescht stain	Sigma
Human HEK 293 cell line	InVivo
Hydrochloric acid	Merck
Hygrogold	InVivo
Igepal	Sigma
IL-1β (human)	Cell signaling
IRDye 800CW Goat Anti-Rabbit	Licor Biosciences
IRDye 680 Donkey Anti-Mouse	Licor Biosciences

Isopropanol	Sigma
Kanamycin	Sigma
L-glutamine	Invitrogen
Lipofectamine 2000	Invitrogen
Lipopolysaccharide	Alexis
Luciferase substrate	Promega
Magnesium Chloride	Sigma
β-Mercaptoethanol	Sigma
Methanol	BDH
Microlon 96-well plates	Greiner
MMLV Reverse Transcriptase	Promega
Morpholinos	Gene Tools
Nitrocellulose	Schleicher & Schuell
NF-κB IRDye Labelled Oligonucleotides	Licor Biosciences
OptiMEM	Invitrogen
Paraformaldehyde	Sigma
Para-p-phenylendiamine	Sigma
PBS	Oxoid
pcDNA 3.1/Zeo	Invitrogen
Penicillin / Streptomycin / Glutamine	Invitrogen
PMSF	Sigma
Poly(I:C)	InVivo
Polybrene	Sigma

Poly-L-Lysine	Sigma
Ponceau	Sigma
Prestained molecular weight marker	Fermentas
Protease inhibitor mixture	Roche
Protease inhibitor mixture (EDTA free)	Roche
Protein A/G-agarose	Santa Cruz
Proteinase K	Sigma
Protogel	National Diagnostics
Puromycin	Sigma
QIAquick gel extraction kit	Qiagen
QUANTI-Blue	Invitrogen
Random primers	Invitrogen
Reverse Transcription System	Promega
RNase Zap	Ambion
RPMI	Invitrogen
SB203580	Cell Signaling
SDS	Sigma
shRNA	Sigma
siRNA	Invitrogen
Scrambled siRNA	Invitrogen
Skim milk powder	Marvel
Sodium chloride (NaCl)	Sigma
Sodium Deoxycholate ($C_{24}H_{39}O_4Na$)	Sigma

Sodium hydroxide (NaOH)	Sigma
Sodium orthovanadate (Na3VO4)	Sigma
Sulphuric acid	Sigma
SensiMix SYBR Low-ROX-kit	Bioline
Synthetic oligonucleotides	MWG Biotech
TEMED	Sigma
Tissue culture ware	Greiner
TK Renilla	Promega
Tri reagent	Sigma
Tris-base	Sigma
Tris-HCl	Sigma
Triton-X	Sigma
Trypsin/EDTA	Invitrogen
Tryptone	DIFCO
Tween-20	Sigma
Whatmann paper	AGB

2.1.2 Gifts

Cell lines:

- HEK293 cells and HEK293 stably expressing TLR3 or TLR4 - Prof. Douglas T. Golenbock (The University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA).
- U373 cell line – Dr. Sinead Miggin (Biology Department, NUI Maynooth, Ireland).

Constructs:

- Flag-tagged MyD88 – Dr M. Muzio (Mario Negri Institute, Milan, Italy)
- NF-κB-luciferase reporter construct – Prof. Luke O'Neill (Trinity College Dublin, Ireland)
- GFP-tagged IκBL – Dr. Ross McManus (Trinity College Dublin, Ireland)
- Myc-tagged IL-17RD, Flag-tagged TRAF6 – Dr. Mark Mellett (National University of Ireland, Maynooth)
- FLAG-tagged Act1 from Dr. Antonio Leonardi (Federico II University of Naples, Italy)

2.1.3 Commercial Plasmids

- Myc-tagged IL-17RD TIR purchased from Genscript (New Jersey, USA)

2.2 Methods

2.2.1 Cell culture

2.2.1.1 Adherent cell lines.

Human embryonic kidney (HEK) 293 cells stably transfected with the SV40 large T antigen, TLR3 or TLR4 receptors, human U373 astrocytoma cells and murine bone marrow derived macrophage (BMDM) cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), which was supplemented with 10% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in a 37ºC humidified atmosphere with 5% CO_2. Cells were passaged every 2 to 3 days using 1% (w/v) Trypsin/ethylenediaminetetraacetic acid (EDTA) solution in phosphate-buffered saline (PBS). G418 (500 µg/ml) was used to select for the stably transfected TLR cell lines.

2.2.1.2 Isolation of primary murine BMDMs.

To isolate BMDMs, tibias and femurs from either IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were removed using sterile technique and the bone marrow was flushed with fresh medium. To obtain macrophages, cells were plated in medium supplemented with 20 ng/ml M-CSF and maintained at 37 °C in a humidified atmosphere of 5% CO_2 for 4-6 days.

2.2.2 Transient transfection of cells

2.2.2.1 Transfection of cells for luciferase reporter assay

HEK293 T/TLR3/TLR4 cells were seeded at 2x10^5 cells/ml (200 µl DMEM/well) in 96-well plates and allowed to adhere for 24 h to approximately 70% confluency. Cells were transfected using Lipofectamine 2000. For each well to be transfected, 25 µl of OptiMEM was mixed with the DNA. DNA mixes were made up for the appropriate luciferase

construct. Lipofectamine 2000 (0.4 µl) was diluted in OptiMEM (25 µl) per sample and the reaction was mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the DNA/OptiMEM mix (total volume 50 µl per well to be transfected) and the combined reaction was mixed gently and incubated at room temperature for 20 min. The transfection mixture was then added to each well and mixed gently by tapping the side of the plate. Each sample was transfected in triplicate. 24 h after transfection, cells were pre-treated with various agents for indicated timepoints. The supernatants were subsequently removed and stored at -20°C and then assayed for cytokine production using sandwich enzyme-linked immunosorbent assay (ELISA), while cell lysates were generated and used to measure luciferase activity

2.2.3 Luciferase assays

HEK293 TLR3/TLR4 were all seeded at 2 x 10^5cells/ml in 96-well plates (200 µl DMEM/well) and grown for 24 h. All transfections were performed using Lipofectamine 2000 transfection reagents (as described in sections 2.2.2.1). Details of the constructs transfected are given below. 24 h post transfection, cells were treated with various agents. The medium was then removed from the cells and reporter lysis buffer (100 µl, Promega) was added to each well using a multi-channel pipette. The plate was then wrapped in aluminium foil and placed on a rocker for 30 min at room temperature before being placed at -80^{o}C for a minimum of 1 h. After thawing at room temperature, aliquots (40 µl) of each were assayed for firefly luciferase activity using firefly luciferase substrate (40 µl, Promega), while Renilla luciferase activity was assayed using coelenterazine (0.1 µg/ml in PBS). Luminescence was monitored with a Glomax microplate luminometer (Promega).

2.2.3.1 NF-κB assay

To measure activation of the NF-κB pathway, cells were transfected with NF-κB -regulated firefly luciferase reporter plasmid (80 ng) and constitutively expressed Renilla-luciferase reporter construct phRL-TK (40 ng). The total amount of DNA was maintained at 240 ng using pcDNA3.1.

2.2.4 Propagation of DNA

2.2.4.1 Rapid transformation of competent *E.coli* cells

Commercially available TOP 10 competent *E.coli* bacterial cells were used for propagation of plasmids of interest. Plasmids (100 μg/ml) were added to five times as much TOP10 cells, i.e. 1 μl (100 ng plasmid) was added to 5 μl cells. DNA was mixed gently with cells by pipetting gently up and down and allowed to incubate on ice for 30 min. The plasmids were allowed to enter the bacterial cells by heat shocking the mixture at 42^{o}C for 45 seconds. The cell membrane became permeable allowing plasmid entry and cooling on ice for 2 min makes the cell membrane once again impermeable. The transformed cells were then incubated in 1 ml Luria Bertoni (LB) broth (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 85 mM NaCl) at 37^{o}C on a shaker for 1 h. Bacterial cells were centrifuged at 9000 g for 3 min and 850 μl supernatant was removed. The pellet was resuspended in the remaining LB broth and plated out onto LB agar plates (LB broth with 1.5% (w/v) agar) containing ampicillin (50 μg/ml). Plates were inverted and incubated overnight at 37^{o}C. Plates were then stored at 4^{o}C.

2.2.4.2 Small scale preparation of DNA from *E. coli* cells

LB broth (2 ml) containing the relevant antibiotic (50 μg/ml) was inoculated with a single transformed *E. coli* colony from an agar plate. The culture was incubated on a shaker overnight at 37^{o}C. Small plasmid preparations were made using the Qiaprep Spin Miniprep kit from Qiagen Inc. The bacterial cells were centrifuged at 9000 g for 3 min and the supernatant was discarded and the plasmid DNA was extracted as outlined in the manufacturer‘s handbook. DNA was quantified using a Cary spectrophotometer. After diluting the DNA appropriately in Tris-EDTA (TE) buffer, pH 8.0, (10 mM Tris-HCl, 1 mM EDTA) the absorbance of the solution was measured at 260 nm and 280 nm. All samples used had an optical density (OD) 260/OD280 ratio in the range of 1.7 to 1.9. Ratios below 1.7

or above 1.9 indicated RNA or protein contamination, respectively. The concentration was calculated using the formula:

µg/ml DNA = 50 µg/ml/OD260 x (OD260 measured) x (dilution factor).

2.2.4.3 Large scale preparation of DNA from *E. coli* cells

A starter culture of LB broth (2 ml) containing the relevant antibiotic (50 µg/ml) was inoculated with a single transformed *E.coli* colony and incubated on a shaker at 37^{o}C for 6-8 h. This was then added to a larger volume of LB broth (100 ml) containing the relevant antibiotic and incubated at 37^{o}C overnight shaking. Large plasmid preparations were made using the Qiagen high speed plasmid midi kit from Qiagen. The bacterial cells were centrifuged at 3000 g for 40 min and the supernatant was discarded and the plasmid DNA was extracted as outlined in the manufacturer‘s handbook. DNA was quantified as outlined in Section 2.2.4.2.

2.2.5 Generation of supernatants for ELISA

U373 astrocytoma cells were seeded in 96-well plates at a density of 2x10^{5} cells/ml. BMDM cells were seeded in 96-well plates at a density of 5x10^{5} cells/ml. Cells were grown for 24 h. Next day, cells were stimulated with ligands for 24 h and supernatants were collected from each well and stored at -20^{o}C until analysis of cytokine production by ELISA was carried out according to manufacturer’s instruction.

2.2.6 siRNA studies

2.2.6.1 Transfection of siRNA

Pre-designed siRNA targeting IL-17RD was purchased from Invitrogen (sense sequence: 5' – UCUUGAAGUGCUCUGCCUCCUUCUC -3') and a corresponding scrambled siRNA was designed (sense sequence 5' –

GAGAAGGAGGCAGAGCACUUCAAGA - 3') and purchased from Invitrogen. HEK293 TLR3 cells were seeded at 1.5 x 10^5cells/ml in 6-well plates (3 ml DMEM/well). Cells were grown for 24 h to approximately 60 % confluency. The appropriate amount of Sef siRNA or scrambled siRNA (for a final concentration of 25 nM/well) was diluted in OptiMEM (250 µl). Lipofectamine 2000 (4 µl) was diluted in OptiMEM (250 µl) per sample, and the reaction was mixed gently and left at room temperature for 5 min. After the incubation, the Lipofectamine/OptiMEM solution was added to the siRNA mix (total volume 500 µl per well to be transfected) and the combined reaction was mixed gently and incubated at room temperature for 20 min. 500 µl sample was added to each well and mixed gently by rocking the plate back and forth. Cells were incubated for 48 h prior to generation of extracts for Western immunoblotting or extraction of RNA for quantitative real-time PCR.

2.2.7 Co-immunoprecipitations (CoIPs)

HEK293 TLR4 cells were transfected with Lipofectamine 2000 as previously described in section 2.2.2.1 with equal amounts (2 µg) of expression constructs encoding the potential interaction partners. Cell extracts were generated on ice or at 4^{o}C as follows. Cells were first washed with pre-chilled 1X PBS (1 ml) then lysed with pre-chilled CoIP lysis buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.5 % (v/v) igepal and 50 mM NaF, with 1 mM Na3VO4, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor mixture (leupeptin (25 µg/ml), aprotinin (25 µg/ml), benzamidine (1 mM), trypsin inhibitor (10 µg/ml)) for 30 min on a rocker at 4^{o}C. Lysates were scraped into pre-chilled 1.5 ml microcentrifuge tubes and centrifuged at 12,000 g for 10 min at 4^{o}C. Supernatants were removed to fresh tubes (a sample retained for whole cell lysate analysis) and incubated for 30 min with mouse or rabbit immunoglobulin (Ig) G (1 µg) (depending on the primary antibody) and Protein A/G agarose beads (10 µl) on a rotor at 4^{o}C. Samples were centrifuged at 1000 g for 5 min at 4^{o}C to pellet beads with nonspecifically bound protein and supernatants were removed to fresh pre-chilled tubes. Samples were incubated overnight with primary antibody (1 µg). The following day Protein A/G agarose beads (20 µl) were added to each sample and they were again incubated at 4^{o}C overnight. The subsequent day samples were centrifuged at 16,000 g for 1 min. The supernatant was removed and the beads were washed with CoIP lysis buffer (500 µl) and subject to re-

centrifugation. This step was repeated four times. 40 µl of 1X sample buffer (0.125 M Tris-HCl, pH 6.8, containing 20 % (w/v) glycerol, 4% (w/v) SDS, 1.4 M β-mercaptoethanol and 0.0025 % (w/v) bromophenol blue) was added to the beads for 30 min at RT. Samples were centrifuged at 16,000 g for 2 min and the supernatant was subsequently boiled at 100^{o}C for 10 min and analyzed using SDS polyacrylamide gel electrophoresis and western blotting (section 2.2.8).

2.2.8 Western blot analysis

2.2.8.1 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted according to the method of Laemmli (Laemmli 1970), as modified by Studier (Studier 1973). Samples and appropriate prestained (26.6-180 kDa) protein markers were loaded into separate wells. Electrophoresis was performed at 80 V through a 5 % SDS polyacrylamide stacking gel and then through a 10 % SDS polyacrylamide resolving gel at 110 V for 1-1.5 h, depending on the size of the proteins being electrophoresed.

2.2.8.2 Immunoblotting

Following separation by electrophoresis, the proteins were transferred electrophoretically to nitrocellulose membranes in a wet transfer unit at 150 V for 1.5 h using Whatmann and nitrocellulose pre-soaked in cold transfer buffer (25 mM Tris Base, 0.2 M glycine and 20 % (v/v) methanol) for 10 min. Following transfer, non- specific binding was blocked by incubating the nitrocellulose membranes at room temperature for 1 h (or overnight) in TBS (20 mM Tris-HCl pH 7.5, containing 0.05% (v/v) Tween 20 and 0.5 M NaCl) containing 5 % (w/v) skimmed milk powder. The membranes were then washed 3 times for 5 min each in TBS prior to incubation at 4^{o}C overnight with the primary antibodies diluted in TBS containing 5 % (w/v) skimmed milk powder or BSA. The membranes were subsequently subjected to 5 x 10 min washes in TBS prior to incubation with secondary antibody (1:5000 dilution) specific for the primary antibody in question (anti-rabbit or anti-

mouse) in TBS containing 5 % (w/v) skimmed milk powder for 1.5 h in the dark at room temperature. The membranes were then washed a further 5 times for 10 min each in TBS in the dark. The immunoreactive bands were detected using Odyssey Infrared Imaging System from Licor Bioscience, according to the instructions of the manufacturer.

1° antibody	**Dilution**	**2° antibody***
β-actin	1:5000	mouse
Myc	1:1000	mouse
Erk1/2	1:1000	rabbit
Phospho Erk1/2	1:1000	rabbit
Flag	1:1000	mouse
HA	1:1000	mouse
IκBα	1:500	rabbit
JNK	1:1000	rabbit
Phospho-JNK	1:1000	rabbit
IRF-3	1:1000	rabbit
p38	1:1000	rabbit
Phospho-p38	1:1000	rabbit
Phospho-IRF-3	1:1000	rabbit
p65	1:1000	rabbit
Phospho-p65	1:1000	rabbit
Phospho-Serine	1:200	rabbit
RIG-1	1:1000	rabbit
COX-2	1:200	rabbit
TBK1	1:1000	rabbit
Phospho-TBK1	1:1000	rabbit

* All secondary antibodies were used at a dilution of 1:5000.

2.2.9 Lentiviral shRNA-IL-17RD infection

2.2.9.1 Lentiviral production

HEK293 T cells were seeded at 2 x 10^5 cells/ml in 6-well plates (3 ml DMEM/well) and grown for 24 h to approximately 80 % confluency. The cells were transfected as in section 2.2.2.2. The DNA mixture contained packaging plasmid (900 ng), envelope plasmid (100 ng) and the sh-pLKO.1 vector (1 μg). IL-17RD shRNA sequences were as follows:

Sef shRNA 1 5'- CAGGGATACAAGTTCAACATC -3'

Sef shRNA 2 5'- AGGTCAGATTCAGAGGAAGAG -3'

A control shRNA (CT) was also used in the transfection. The control shRNA is a non-targeting shRNA vector that will activate the RNA-induced silencing complex (RISC) and the RNAi pathway, but it does not target any human or mouse genes. The short-hairpin sequence contains 5 base pair mismatches to any known human or mouse gene. The shRNA IL-17RD and shRNA control plasmids were purchased from Sigma. To remove the transfection reagent, the media was changed 24 h post-transfection and replaced with fresh high serum (30 %) growth media. The cells were then incubated for 24 h. The media containing lentivirus were harvested ~ 40 h post-transfection and transferred to a polypropylene tube for storage at -20°C. The media was replaced with fresh high serum (30 %) growth media and the cells were incubated for 24 h. The virus was harvested one more time and after the final harvest the packaging cells were discarded.

2.2.9.2 Lentiviral infection

U373 cells were seeded at 2 x 10^5 cells/ml in 6-well plates (3 ml/well RPMI or DMEM, respectively). U373 cells were incubated overnight prior to infection to allow the

cells to adhere. The growth media was then removed and replaced with fresh media containing polybrene (8 μg/ml) and 600 μl of virus. The plates were incubated at 37^{o}C. The media was removed 24 h post-infection and replaced with fresh growth media containing puromycin (10 μg/ml) to select for cells transduced with shRNA. The polyclonal population of cells were cultured for 2-3 weeks and used for ELISA and Western Blot experiments.

2.2.10 Isolation of RNA and cDNA synthesis

2.2.10.1 Isolation of total RNA from HEK293, U373 & BMDM cells

In order to minimise RNA degradation, a number of precautions were taken throughout the following procedures. All water and salt based solutions were treated with diethylpyrocarbonate (DEPC) (0.2 % v/v). Certified RNasefree plasticware was used. Before commencing, equipment was wiped with "RNase Zap", an RNase decontamination solution. Gloves were worn at all times and regularly changed. Total RNA was isolated using the Tri Reagent (Sigma) as per manufacturer's instructions. The amount of isolated RNA was quantified by measuring the absorbance at wavelengths of 260 nm and 280 nm on a spectrophotometer, where an absorbance of 1 unit at 260 nm is ~ 40 μg/ml. Pure RNA preparations have an OD_{260}/OD_{280} ratio of 1.6-1.8. Extracted RNA was stored at -80^{o}C.

2.2.10.2 Synthesis of first strand cDNA from mRNA

1 μg of RNA was placed in nuclease-free microcentrifuge tubes and incubated for 10 min at 70^{o}C. The mixture was then chilled on ice and centrifuged briefly. The following components were then added:

MMLV RT	0.25 μl
dNTPs (10 mM)	2 μl
5X MMLV Buffer	4 μl
RNasin (40 U/μl)	0.5 μl
Random Primers (0.5 μg/μl)	1 μl

Nuclease-Free Water	to 20 µl

The reaction mixture was incubated for 10 min at RT allowing the primers to anneal to the RNA. Next, the reaction was incubated at 42°C for 1 h. MMLV RT was then deactivated by heating to 95^{o}C for 5 min followed by cooling to 0-5^{o}C for 5 min. Generated cDNA was stored at 4^{o}C for short-term storage or -20^{o}C for long-term storage.

2.2.10.3 PCR amplification of DNA

PCR products were amplified using either complementary DNA (cDNA) or plasmid DNA as template. Synthesised primers were designed based on deposited GenBank sequences. The GC-RICH PCR System, which contains a mixture of *Thermophilus aquaticus (Taq)* polymerase and a high fidelity proofreading polymerase, was used for the amplification of PCR products. The standard PCR conditions below were used to amplify the target sequence.

Template (100ng cDNA)	1 µL
5X Enzyme Buffer	10 µL
GC-Rich Resolution Buffer	5 µL
Primers (10 pmol/ µL)	2.5 µL each
dNTP mix (10mM each)	4 µL
Enzyme Mix	1 µL
PCR-grade water	50 µL

Each reaction was heated to 94^{o}C for 2 min. Then this step was followed by a cycle of 94^{o}C for 30 s, a target-specific annealing temperature (T_A) for 30 s, and 72^{o}C for a polymerase specific elongation time based on the size of the sequence to be amplified. The appropriate cycle number was experimentally determined for each target to ensure that the reaction was still in the linear phase upon termination. Samples were then incubated at 72^{o}C for 10 min and stored at 4^{o}C if necessary.

2.2.10.4 Quantitative Real Time PCR

Cells were seeded (1.5 x 10^5 cells/ml; 3 ml) into 6-well plates and grown for 24 h to approximately 60 % confluency. Cells were washed with 1X PBS (1 ml) and RNA was extracted, as described in section 2.2.10.1. cDNA was then generated as described in section 2.2.10.2. Samples were assayed by quantitative real time PCR for levels of IL-17RD, IFN-β, KC, IL-6, IL-8 and RANTES cDNA using SensiMix SYBR Low-ROX kit (Bioline). PCR was conducted with the CFB-322001G Opticon thermal cycler (Bio-Rad Laboratories). Reactions were performed using pre-validated primers (Eurofins MWG Operon). Accumulation of gene-specific PCR products was measured continuously by means of fluorescence detection over 40 cycles. Samples were initially heated for 10 min at 95^{o}C. This was followed by 10 seconds at 95^{o}C, 10 seconds at 59^{o}C and 1 min at 72^{o}C for 40 cycles. Gene expression was calculated relative to the endogenous control and analysis was performed using the $2^{-\Delta\Delta CT}$ method.

2.2.11 Determining protein concentration – Method of Bradford

Protein content of BSA standards and cell extracts was measured by the method of Bradford (Bradford 1976). Standards and extracts (10 µl) were diluted in water and mixed with aliquots (190 µl) of Bradford protein reagent (Bio-Rad), diluted 1:5 in distilled water, by pipetting up and down. A blank was prepared using the same dilution of water. Absorbance was measured for each sample in a 96-well plate at 590 nm using ELx800^{TM} microplate reader with Gen5 Data Analysis Software. Six protein standards of BSA (10, 8, 6, 4, 2 and 0 µg/µl) were made and used to construct a standard curve which was subsequently used to determine protein concentration of the cell extracts.

2.2.12 Type I Interferon Blue Assay

B16-Blue™ IFN-α/β Cells (Invivogen) were seeded (1.5 x 10^5 cells/ml; 200 µl) in 96 well plates. 20 µl of supernatant was added to each well and incubated at 37°C in a 5% CO_2 incubator for 20 h. QUANTI-Blue™ was prepared following the manufacturer's instructions.

180 µL of resuspended QUANTI-Blue™ was added to a flat-bottom 96-well plate. 20 µl of induced B16-Blue™ IFN-a/b Cells supernatant was added to each well. The plate was incubated at 37°C for 1-5 h. Absorbance was measured for each sample in a 96-well plate at 620 nm using ELx800™ microplate reader with Gen5 Data Analysis Software.

2.2.13 Mice

2.2.13.1 Generation of Mice

IL-17RD-deficient mice were generated by the German Genetrap Consortium using its proprietary technology (Clone ID: D065E01) (Fig. 3.7a). The genetrap targeting vector, which contains a promoter-less reporter gene downstream of a splice acceptor and a selectable marker gene (*β-Geo* for β-Galactosidase neomycin-resistant fusion gene) was incorporated into the *il-17rd* gene after the first exon. The targeting vector contains loxP, f3 and lox5171 sites allowing conditional mutagenesis or recombinase-mediated cassette exchange. After the targeting vector was transfected into embryonic stem cells, G418-resistent colonies were selected and screened by PCR. Homologous recombinants were microinjected into female C57BL/6 background mice and heterozygous F1 progeny were intercrossed to obtain IL-17RD$^{-/-}$ mice. IL-17RD$^{+/-}$ mice were bred to generate age- and sex-matched IL-17RD$^{+/+}$, IL-17RD$^{+/-}$, IL-17RD$^{-/-}$ mice for experiments.

2.2.13.2 Breeding and maintenance

Breeding, colony expansion and maintenance were performed in the Bioresource facility of National University of Ireland (NUI) Maynooth. All animals were housed in a barrier unit, in IVC cages with HEPA filters. Animals were monitored regularly for microorganisms according to FELASA recommendations. All animal experiments were performed in accordance with the regulations and guidelines of the Irish Department of Health and protocols approved by the Research Ethics committee of NUI Maynooth.

2.2.13.3 Genotyping of mice

Mice were genotyped by PCR analysis of DNA isolated from ear punches using primers **1** 5'-TGTGGTAGCCAAAGACTGCTTCATG-3', **2** 5'-ATAGGTCACTTGCAAATCC-3', **3** 5'-GGACAGGATAAGTATGA CATCATC-3', **4** 5'-CTGGCGTAATAGCGAAGAGG-3' and **5** 5'-CCGCCACATA TCCTGATCTT-3'. PCR analysis using primers 1 and 2 generates a fragment of 449 bp for the WT allele. Primers 1 and 3 generate a fragment of 1103 bp for the knockout allele (Fig. 3.7b). To further confirm the presence of the genetrap cassette primers 4 and 5 amplify a region of the β-Geo insert at a size of 508 bp and this is only found in IL-17RD$^{+/-}$ and IL-17RD$^{-/-}$ mice.

2.2.13.4 Peritoneal and Pulmonary administration of IL-17A

Age- and sex-matched IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were treated with 0.5 μg of IL-17A (R & D Systems) by intraperitoneal injection, or 1 μg of the formyl-Met-Leu-Phe peptide (Sigma) prepared in endotoxin-free PBS and control mice were injected with endotoxin-free PBS. For the exogenous MIP-2 administration experiment mice were subjected to intraperitoneal injection of 0.5 μg of IL-17A or PBS 1 h prior to treatment with recombinant murine MIP-2 (30 ng) for 2 h. The peritoneal cavity was lavaged with 2 ml ice-cold PBS at 0-6 h post-treatment. Peritoneal cells were counted and quantified by flow cytometry and differential cell counting. To induce pulmonary neutrophilia, 0.5 μg IL-17A was introduced intra-nasally to groups of age-matched female IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice. After 4 h, bronchoalvelolar lavage (BAL) was performed and cellular infiltration into the lung was quantified by differential cell counts on BALs. Lung tissue was perfused and fixed in 10% formalin saline. Paraffin-embedded sections were stained with hematoxylin and eosin to assess leukocyte infiltration and pulmonary inflammation.

2.2.14 Flow cytometry

Infiltrating cells were analyzed by surface marker expression by flow cytometry with data collection on a CyAn (Beckman Coulter), as described (Ludwig *et al*. 2010). Data were analyzed using FlowJo software (Tree Star). Cells were stained using PerCP anti-CD11b

(M1/70), PE anti-Ly6G (RB6-8CS) and APC anti-F4/80 (BM8). Flow buffers used contained 2 mM EDTA to exclude doublets. Using appropriate isotype-controls, gates were drawn and data were plotted on logarithmic scale density- or dot-plots.

2.2.15 Slide preparation and differential cell counting

Slides were prepared from BAL fluid or peritoneal lavage (50,000 cells/slide) using a cytospin (Thermo-Shandon). All slides were stained with Wright-Giemsa (Thermo-Shandon) to depict leukocyte subsets. A total of 200 leukocytes were counted per slide.

Chapter 3

Results

3.1 Introduction

The TLR family of PRRs and their associated adaptor proteins have emerged as vital components of the innate immune system which ignite the inflammatory response in reaction to infection from microbial agents. However, the pathways initiated by this family of receptors require numerous levels of regulation to keep in check aberrant inflammatory signals, the initial steps towards chronic inflammation. Focus on TLR signalling has moved from delineating the stepwise pathways to the TIR domain containing proteins which are of interest due to TLRs utilising the different adaptors to tailor specific response to a variety of infections.

IL-17RD has, up until now, been characterised as an antagonist of FGF signalling (Tsang and Dawid 2004). The studies have demonstrated that IL-17RD localises with and is under the control of *fgf* genes, hence the alternative name "Sef" (similar expression to fgf genes). Different targets have been revealed for IL-17RD in FGF signalling. IL-17RD inhibits at the receptor level but also downstream at the level of MEK (Furthauer *et al*., 2002; Kovalenko *et al*., 2003; Tsang *et al*., 2002; Xiong *et al*., 2003). IL-17RD also interferes with the dissociation of ERK from MEK, impeding the translocation of ERK to the nucleus (Torii *et al*., 2004). The regulation of these pathways by IL-17RD is expected to be of physiological importance and this has already been reported in the case of FGF signalling where IL-17RD is downregulated in a variety of carcinomas (Zisman-Rozen *et al*. 2007). In fact, IL-17RD may function chiefly as a tumour suppressor given that a result of its loss is an increase in tumorigenesis. IL-17RD could function in determining cell fate by the inhibition of FGF and NF-κB pathways, which promote cell growth and cell survival and apoptosis (Yang *et al*. 2004). Another reason as to why IL-17RD may play a role in innate immunity could be due to its expression profile which highlights that IL-17RD is found in large amounts in the brain, the ovaries and testes which are immune-privileged sites in the body (Novatchkova *et al*. 2003). An increased amount of IL-17RD in these organs may confer protection against undesired inflammatory responses.

Importantly, IL-17RD was reported to have a TIR-like domain (Novatchkova *et al*. 2003). This TIR-like domain is characterised as a SEFIR (Sef and IL-17Rs) domain as it lacks box 3 of the TIR domain and shares significant homology with other Sef proteins and IL-17Rs (Novatchkova *et al*. 2003). Box 1 and 2 of the TIR domain have been shown to relate to TIR-

TIR interactions while Box 3 is associated with receptor localisation (Slack *et al*. 2000) Over the last 10 years or so, there has been more and more investigation into the proteins that regulate TLR signalling and increasingly it has been shown that this regulation is carried out by a significant number of TIR domain containing proteins. For example, SIGGIR, which contains a putative extracellular immunoglobulin domain in addition to the TIR domain (O'Neill 2003). Given that IL-17RD contains a TIR-like domain we initially looked at its effect on pathways regulated by TLRs with a special emphasis on the NF-κB and IRF pathways and transcription of TLR responsive genes.

IL-17RD, which was first described in Zebrafish, exhibits considerable conservation across species (Moseley *et al*. 2003). A 739 amino acid transmembrane protein, IL-17RD is composed of an N terminal signal peptide, an extracellular fibronectin-III (Fn-III) like domain and the previously mentioned intracellular SEFIR domain. In addition to this Fn-III domain, the extracellular region is also characterised by a putative Ig (Immunoglobulin) domain (Tsang *et al*. 2002; Xiong *et al*. 2003). Tandem Fn domains in the extracellular domain are also shared by the IL-17 receptors and are a common feature of cytokine receptors. The IL-17 family of cytokine receptors share the greatest homology with IL-17RD Novatchkova *et al*. 2003). They also share similar transmembrane domains, SEFIR domains and polar residue-rich C-termini (Novatchkova *et al*. 2003). Whilst recent studies have outlined key immunoregulatory roles for the various IL-17R members, IL-17RD remains an orphan receptor without a physiological role in IL-17 signalling, apart from a report in which overexpression of IL-17RD was shown to co-operate with IL-17RA (Rong *et al*. 2009). This thesis describes, for the first time, an important biological function of IL-17RD in IL-17 signalling. We also propose a possible role of IL-17RD in non-TLR/IL-17 pathways. Overall, the studies described here highlight important regulatory roles for IL-17RD in innate immune signalling pathways.

3.2 Results

3.2.1 Suppression of endogenous IL-17RD expression enhances the activation of TLR signalling components

In order to characterise the physiological role of IL-17RD in TLR signalling, the effects of IL-17RD knockdown on TLR ligand induced phosphorylation of important downstream signalling components were assessed. U373 astrocytoma cell lines were transduced with lentivirus to stably express IL-17RD specific shRNA or control shRNA. Knockdown efficiency was regularly checked in these U373 shRNA stable cell lines and 50-75% knockdown of *il-17rd* mRNA was consistently achieved compared with the control shRNA (Fig. 3.1). Cells were treated with LPS (Fig. 3.2) or Poly I:C (Fig. 3.3) over various timepoints. IL-17RD shRNA was shown to potentiate the phosphorylation of TLR signalling components in cells treated with LPS and Poly I:C. Knockdown of IL-17RD in these cells resulted in augmented LPS induced phosphorylation of IκB and IKK relative to control shRNA cells (Fig 3.2). In the case of Poly I:C, there is increased phosphorylation of TBK1 at earlier timepoints and more prolonged and sustained phosphorylation of IRF3 in IL-17RD knockdown cells relative to control cells (Fig 3.3). Such differences between control and shRNA cells strongly suggest IL-17RD may act as a negative regulator of TLR signalling.

3.2.2 IL-17RD knockdown augments TLR mediated cytokine mRNA induction

Given that the above results highlight a potential role for IL-17RD in TLR signalling, it was necessary to probe the regulatory effects on NF-κB and IRF responsive genes in control and IL-17RD shRNA cells. IL-6 was chosen as a representative NF-κB gene. IL-6 is produced by cells such as macrophages and endothelial cells and is released in response to infection, burns and neoplasia. It has a wide range of functions such as acute phase protein induction and B and T cell growth and differentiation (Ray *et al* 1989; Janeway *et al* 2001; Fernandez – Botran, 1995). As NF-κB activates IL-6 production, we investigated if the absence of IL-17RD would have an effect on its induction. Cells were treated with LPS at 3 hour and 6 hour intervals (Fig 3.4). IL-6 mRNA was quantitated by real time PCR. IL-17RD shRNA led to a modest enhancement of LPS induced expression of IL-6 mRNA relative to control shRNA cells. However, at 6 hours there was a pronounced (~ 50%) augmentation of Poly(I:C) induced expression of IL-6 mRNA in the IL-17RD shRNA cells. The type 1

interferon, IFN β, was chosen as an IRF responsive gene. Interferons are potent antiviral agents, not normally expressed in cells. Viral infection of a cell causes interferons to be produced and released. It subsequently binds to target cells and induces an antiviral state. The expression of IL-17RD shRNA greatly augmented the ability of Poly (I:C) to induced IFN β expression at 6 hours post Poly (I:C) stimulation (Fig 3.5). Consequently, IL-17RD appears to negatively regulate activation of NF-κB and IRFs and production of important proinflammatory genes such as IL-6 and IFN β in response to TLRs. .

3.2.3 Knockdown of IL-17RD increased TLR-induced cytokine production

Since the above studies indicated that IL-17RD negatively regulates the expression of TLR-responsive genes at the mRNA, we were keen to explore the effects of loss of IL-17RD on expression of these genes at the protein level. RANTES, IP-10 and IFN β were chosen as representative proteins that are regulated by NF-κB and IRF3.

In order to assess the effect of IL-17RD on the production of these proteins, U373 cell lines, stably expressing control and IL-17RD shRNA, were treated with Poly (I:C). Supernatants were obtained 24 hours after treatment and used to measure RANTES, IP-10 and IFN β production by ELISA. In IL-17RD knockdown cells, there were marked increases in the production of RANTES (~150%) (Fig 3.6a), IFN β (~50%) (Fig 3.6b) and IP-10 (~125%) (Fig 3.6c) in response to Poly (I:C) when compared with the control shRNA cell lines. These data corroborate the earlier findings and suggest that IL-17RD may function as an endogenous brake in TLR signalling.

3.2.4 TLR signalling is enhanced in cells from IL-17RD-deficient mice

The aforementioned knockdown studies indicated IL-17RD as a negative regulator of TLR signalling. In order to validate the physiological relevance of such a role, it was necessary to generate IL-17RD deficient mice. This was achieved by gene trap technology in partnership with the German Genetrap Consortium. The murine *il-17rd* gene consists of thirteen exons. The targeting vector disrupted the *il-17rd* gene between the first and second exons with a β-geomycin resistant gene cassette (Fig 3.7a). IL-17RD knockout pups were born in an expected Mendelian ratio and showed no apparent phenotype abnormalities which are consistent with previously generated IL-17RD- deficient mice (Abraira *et al*., 2007). BMDM were generated from the IL-17RD mice and used to further explore the regulatory role for IL-17RD in TLR signalling by measuring the phosphorylation status of key TLR signalling components in response to LPS and Poly I:C. IL-17RD- deficient BMDMs were stimulated with either LPS (Fig 3.8) or Poly I:C (Fig. 3.9) over a number of timepoints as indicated and the phosphorylation of important downstream effector proteins was examined by immunoblotting using specific antibodies. In the wild type macrophages, LPS-induced phosphorylation of p65 and IκBα was detectable after 5 minutes and this decreased over time but levels of phosphorylated p65 and IκBα were higher and more sustained in IL-17RD – deficient cells (Fig 3.8). Poly I:C induced phosphorylation of the TBK1 and IRF3 proteins in wild type BMDMs but the kinetics were altered in IL-17RD-deficient cells such that the Poly(I:C)-induced phosphorylation of TBK1 and IRF3 was apparent at earlier time points (Fig 3.9).

These data validate previous results, demonstrating that IL-17RD is capable of inhibiting downstream activation of the main players in TLR signalling pathways, emphasising its role as a negative regulator.

3.2.5 IL-17RD deficient macrophages exhibit augmented expression of TLR responsive genes

We next explored the functional consequences of augmented TLR signalling in IL-17RD knockout cells by measuring the expression of a number of TLR-responsive genes in these cells. The representative NF-κB and IRF responsive genes chosen were murine IL-6 and KC and Type I Interferons respectively. IL-6 is a key NF-κB responsive cytokine. KC is the murine homolog of IL-8 and is produced by keratinocytes, monocytes, macrophages and endothelial cells. When released by these cells, it functions as a powerful chemoattractant of neutrophils, directing these cells to areas of infection to phagocytose invading antigens (Baggiolini *et al*., 1989).

Wild type and IL-17RD deficient macrophages were treated with the TLR ligands LPS (TLR4) , Poly (I:C) (TLR3), Zymosan (TLR2), Flagellin (TLR5) and Pam3Csk4 (TLR1/2) and a number of key pro-inflammatory cytokines including IL-17, IL-1β and TNF. 24 hours post treatment, supernatants were obtained and levels of mIL-6 and KC were quantified by ELISA. LPS potently induced mIL-6 in wild type BMDMs but this was further augmented in IL-17RD knockout cells (Fig 3.10). Additionally, IL-17RD deficient macrophages showed a significant enhancement of mIL-6 production in response to Poly I:C, IL-17, Zymosan, Flagellin and Pam3Csk4 relative to their wild type counterparts (Fig 3.10). Interestingly, IL-17RD deficiency had no effect on the efficacy of TNF, CpG or IL-1β in inducing IL-6. Loss of IL-17RD had similar effects on the expression of KC (Fig 3.11) in that IL-17RD knockout cells showed enhanced expression of KC in response to LPS, Poly I:C, Pam3Csk4, Flagellin and Zymosan relative to wild type cells. Such an increase in NF-κB responsive gene activation support a model in which IL-17RD acts an endogenous braking system for NF-κB and the induction of NFκB-responsive genes.

The functional consequence of IL-17RD deficiency on activation of the IRF pathways was next examined by measuring the production of Type I Interferons and IFNβ in response to Poly I:C in BMDMs from wild type and IL-17RD-deficient mice. IL-17RD deficiency potentiated Poly(I:C) induced expression of Type I Interferon as measured by bioassay (Fig 3.12) and ELISA (3.13). This enhancement in interferon production due to the absence of IL-17RD confirms that the orphan receptor acts as an endogenous regulator of IRF signalling and is capable of targeting the TLR3-TRIF pathway.

3.2.6 IL-17RD deficiency augments TLR induced cytokine mRNA induction

To explore if the negative effects of IL-17RD on TLR-responsive genes operated at the transcriptional level, it was necessary to verify if induction of TLR responsive cytokine mRNA production was elevated in IL-17RD deficient macrophages. The TLR ligands LPS, Poly I:C, Zymosan, Flagellin and Pam3Csk4 were chosen as representative ligands. The mRNA levels in primary BMDMs were quantified using qPCR analysis. Production of KC (Fig 3.14a) and mIL-6 (Fig 3.14b) mRNA, in response to LPS, was enhanced in IL-17RD deficient macrophages relative to WT cells. Such augmentation in IL-17RD knockout cells was also apparent with respect to mIL-6 (Fig 3.15a) and RANTES (Fig 3.15b) mRNA induction in response to Poly I:C. The TLR5 ligand, Flagellin, strongly induced both KC (Fig 3.16a) and mIL-6 (Fig 3.16b) mRNA expression. Augmented induction of these NF-κB responsive genes was displayed in the IL-17RD knockout primary macrophages. Zymosan, a component of the fungal cell wall and a powerful TLR 2 agonist, induced both RANTES (Fig 3.17a) and KC (Fig 3.17b) mRNA in WT cells and this was further increased in IL-17RD-deficient cells. Furthermore, Pam3Csk4-induced KC mRNA fold induction was potentiated in cells lacking IL-17RD (Fig 3.18). Clearly, these results, in conjunction with earlier data above, strongly suggest that IL-17RD functions as a strong negative regulator of TLR signalling pathways inhibiting the production of TLR induced expression of inflammatory genes such as IL-6, KC and RANTES and type I IFNs.

3.2.7 Suppression of IL-17RD expression enhances TLR-induced CREB signalling

It was subsequently of interest to observe how IL-17RD would affect other transcription factors given its strong regulation of the NF-κB and IRF pathways. CREB (cAMP response element binding) is a vital transcription factor involved in mediating the regulation of gene expression in response to the second messenger, cAMP (Johannesen *et al*., 2004). Stimuli that increase cAMP activates cAMP dependent Protein Kinase A (PKA) which in turn phosphorylates CREB at Ser-133 increasing its transactivation potential. Several TLRs also initiate phosphorylation of CREB leading to enhanced transcription of CREB responsive genes such as cyclooxygenase 2 (COX-2) and anti-inflammatory genes such as IL-10 (Eliopoulos *et al*., 2002; Platzer *et al*., 1999). To this end, it was sought to establish the role, if any, IL-17RD had in the CREB activation pathway. Given that a prerequisite for CREB activation is Ser-133 phosphorylation, LPS induced CREB phosphorylation was characterised in both wild type and IL-17RD deficient BMDMs. Both

sets of primary macrophages were stimulated with LPS over various times and the phosphorylation of CREB was examined by immunoblotting using a phosphor-Ser-133 specific antibody. In wild type cells, LPS induced phosphorylation of CREB was detected after 5 minutes and was maintained up to an hour (Fig 3.19). However, in IL-17RD deficient cells, there was much more pronounced and sustained CREB phosphorylation. Additionally, another band, corresponding with the mobility of an approximately 80 kDa protein, was detected. This band shares a similar profile as the phospho-CREB, possibly indicating it is a modified form of phosphor-CREB. Levels of COX-2 were also examined by immunoblotting (Fig 3.19). COX-2 is a key component of the biosynthetic pathway which results in the production of prostaglandins. Elevated levels of COX-2 were observed after 60 minutes of LPS treatment in the wild type cells. This becomes more pronounced at 4 hours. However, there is augmentation in the levels of COX-2 in the IL-17RD deficient macrophages.

To further examine IL-17RD as a potential effector of a novel regulatory mechanism in the CREB pathway, an assessment of the production of the anti-inflammatory IL-10 was performed. Produced by monocytes, macrophages and B and T cells, IL-10 displays pleiotropic effects in immune system regulation and inflammation. It has the ability to inhibit the synthesis of pro-inflammatory cytokines such as IFN-γ, TNF and GM-CSF made by macrophages and T-reg cells. Also, it is capable of downregulating expression of T_H1 cytokines, MHC class II antigens, and co-stimulatory molecules on macrophages. BMDMs were treated with LPS, Zymosan, Pam3Csk4 and Flagellin. Supernatants were then harvested 24 hours post treatment and these were used to measure IL-10 expression by ELISA. In response to each TLR ligand, there is augmented IL-10 production in the IL-17RD deficient macrophages (Fig 3.20). This suggests an important role of IL-17RD in regulating CREB activation and the expression of CREB responsive genes.

3.2.8 Absence of IL-17RD causes increased TRAF6 ubiquitination in response to TLR ligands

We next aimed to probe the mechanistic basis to the effects of IL-17RD on the various transcription factors and TLR-responsive genes. Since other IL-17 receptors and TLRs use TRAF6 as a key signalling intermediate it was interest to probe the effect of Il-17RD on TRAF6. TRAF6 is a key mediator in NF-κB signalling. It is a ubiquitin E3 ligase that can be auto-ubiquitinated and is responsible for the activation of the IKK complex. We thus assessed the effect of IL-17RD deficiency on the ubiquitination of TRAF6. Wild type

and IL-17RD deficient macrophages were treated with LPS, over various timepoints (Fig 3.21). The cell lysates were immunoprecipitated with a specific anti-TRAF6 antibody followed by immunoblotting with specific anti-ubiquitin antibodies. Ubiquitination of TRAF6 was detected transiently in the wild type macrophages at 10 minutes and 30 minutes (Fig 3.21). Such LPS-induced polyubiquitination of TRAF6 was moderately enhanced in the IL-17RD deficient cells and such effects on the ubiquitination of TRAF6 are consistent with increased LPS-induced activation of NF-κB in the absence of IL-17RD. While this study validates the functional data from previous studies, it also furnishes evidence that IL-17RD in fact functions either upstream or at the level of TRAF6. Taken together, these studies confirm IL-17RD acts as a signalling "brake" in TLR signalling.

3.2.9 The SEFIR domain of IL-17RD functions similarly to full length IL-17RD

Previous work on IL-17RD (unpublished work from the host laboratory), has established that IL-17RD interacts with all TIR domain containing adaptor proteins and TRAF6 and it inhibits TLR adaptor driven NF-κB luciferase activity in a dose dependent fashion. It was decided to establish whether a truncation mutant of IL-17RD containing just its SEFIR domain would have the same effects as the wild type. Overexpression of this mutant which just contains residues 354-518 (Fig. 3.22a), like full length IL-17RD, showed no significant induction of NF-κB activity (Fig 3.22b). The importance of the IL-17RD TIR domain and deleted regions for mediating the inhibitory effects of IL-17RD was next examined by analysing the effects of the mutants on TIR-adaptor mediated NF-κB activation. Increasing amounts of IL-17RD full length and IL-17RD TIR were co-transfected with MyD88 and the NF-κB luciferase reporter construct. IL-17RD wild type was employed as a positive control to be able to accurately compare the inhibitory effects, if any, of the mutant. IL-17RD TIR inhibited, with comparable efficacy, NF-κB activation in a dose dependent manner. (Fig 3.24). Such findings strongly implicate an important role for the IL-17RD TIR domain in mediating the inhibitory effects of IL-17RD.

Given that IL-17RD can regulate the ubiquitination of TRAF6, co-immunoprecipitation studies were performed to analyse whether myc-tagged IL-17RD SEFIR could interact with TRAF6. The myc-tagged full length IL-17RD, employed here as a positive control, was demonstrated to be capable of interacting with flag-tagged TRAF6. Interestingly IL-17RD TIR was also shown to be capable of binding to TRAF6, similar to the wild type (Fig 3.23).

3.2.10 IL-17RD differentially regulates RIG-I associated NF-κB and IRF activation

Given the profound effects IL-17RD displays on TLR signalling, the regulatory effects of IL-17RD on another pathway was subsequently examined. It was interesting to observe how IL-17RD would affect the signalling of a non-TLR pathway that also activates NF-κB and IRFs. IL-17RD was assessed for its ability to regulate RIG-I signalling. In order to explore the physiological relevance of IL-17RD in this pathway, IL-17RD deficient BMDMs were infected with Sendai virus (SeV), a potent agonist of RIG-I signalling. SeV is a single stranded RNA virus of the Paramyxoviridae family. Primarily infecting mice, it is also responsible for morbidity among other rodents such as rats and can even cause mortality among swine. RIG-I detects the ssRNA initiating a downstream signalling cascade which is responsible for the induction pro-inflammatory and anti-inflammatory cytokines. Initial studies focused on comparing cytokine induction in wild type and IL-17RD deficient cells. IL-17RD knockout macrophages were infected with SeV and supernatants were harvested 24 hours after treatment. An interferon bioassay was performed to assess the induction of biologically active Type 1 Interferons. SeV induced Type I Interferons in the wild type macrophages (Fig 3.25). Intriguingly, diminished Type I Interferon production was observed in the knockout macrophages relative to the wild type cells, indicating that IL-17RD could play a role mediating RIG-I induced activation of IRFs.

The supernatants were then used to measure IFN β and mIL-6 production by ELISA. Once again, IL-17RD deficient macrophages displayed reduced ability of SeV to induce expression of an IRF responsive gene product, IFN β, relative to wild type cells (Fig 3.26). Interestingly, when production of mIL-6 was investigated, we saw a statistically significant enhanced induction of this NF-κB responsive cytokine in the IL-17RD deficient macrophages relative to the wild type cells (Fig 3.27). These differential effects of IL-17RD on RIG-I induced activation of NF-κB and IRFs were also observed in the phosphorylation status of downstream signalling components. The knockout macrophages displayed elevated phosphorylation of the NF-κB subunit p65 relative to the wild type macrophages in response to SeV infection (Fig 3.28). This result ties in with the previously reported increase in mIL-6 production in the absence of IL-17RD. Furthermore, we demonstrated that IL-17RD deficiency results in impaired IRF3 phosphorylation in response to SeV which corroborates

the reduced Type I Interferon production. These findings confirm that IL-17RD potentially mediates the IRF arm of RIG-I signalling while differentially inhibiting NF-κB activation.

3.2.11 Gene silencing of IL-17RD in human cells enhances IL-17A signalling

Given the striking effects that the loss of IL-17RD appears to have on TLR signalling, it was intriguing to explore a possible functional role for this protein in other immune signalling pathways. As previously outlined, IL-17RD is an orphan receptor of the IL-17 receptor family, with to date, no known physiological function in IL-17 signalling. In order to elucidate a potential role in IL-17 signalling, we studied IL-17A induced NF-κB signalling pathways in the absence of IL-17RD. In addition to lentiviral shRNA knockdown studies, siRNA studies were also utilised to probe the function of IL-17RD in this system. HEK 293T cells were mock transfected or transfected with IL-17RD-specific siRNA or a scrambled control siRNA. Subsequently, qPCR was performed to confirm knockdown of IL-17RD mRNA. Knockdown of 30-60% was consistently achieved with IL-17RD specific siRNA while the scrambled siRNA had no effect (Fig 3.29). The siRNA molecules were then used to determine the effects of *il-17rd* gene silencing on IL-17A induced NF-κB activation. HEK293 TLR4 cell lines were transfected with 20nM of IL-17RD specific siRNA or scrambled siRNA. 48 hours post treatment; cells were treated with IL-17A over various timepoints. IL-17RD siRNA potentiated the activation of downstream components of IL-17 signalling compared to its scrambled siRNA counterpart. Knockdown of IL-17RD in these cells led to enhanced IL-17A-induced phosphorylation of IκB and the MAP Kinases p38 and p42/44 (ERK) (Fig 3.30).

To further validate these studies, the previously generated U373 cell-lines transduced with lentivirus to stably express IL-17RD specific shRNA or control shRNA were once again utilised. Using the same timepoints used for the siRNA studies, the cells were treated with IL-17A. A very similar picture emerged with augmented phosphorylation of p38, ERK, IKK and IκB in the absence of IL-17RD relative to the control shRNA (Fig 3.31). To extend these studies further to investigate the physiological relevance of IL-17RD, it was necessary to probe its regulatory effects on NF-κB responsive cytokines. IL-6 was chosen as a representative gene. IL-6 is produced and secreted by epithelial, endothelial and fibroblastic cells in response to IL-17. Therefore, complementary experiments were performed where we measured levels of IL-6 in response to IL-17 in control and IL-17RD shRNA knockdown U373 cells. These cells were treated with IL-17A and 24 hours post treatment, the

supernatants were obtained and assessed for IL-6 production by ELISA. Knockdown of endogenous IL-17RD expression lead to enhanced IL-6 mRNA (Fig. 3.32) and protein production (Fig. 3.33) in response to IL-17A. These data suggest that IL-17RD negatively regulates IL-17A signalling pathways and the induction of IL-17 responsive genes.

3.2.12 IL-17RD deficiency enhances IL-17A induced signalling in primary macrophages

IL-17A signalling was next examined in cells from IL-17RD deficient mice. IL-17RD knockout murine BMDMs were utilised to elucidate a potential regulatory role for IL-17RD by measuring IL-6 and KC expression in response to the principal IL-17RA cytokine, IL-17A. Primary macrophages were treated with IL-17A over a number of timepoints. Supernatants were harvested post treatment and levels of IL-6 and KC were measured by ELISA. The IL-17A-induced expression of IL-6 expression in wild type cells was further enhanced in IL-17RD deficient macrophages (Fig 3.34). Likewise, loss of IL-17RD resulted in augmented IL-17-induced expression of KC expression (Fig 3.35). We subsequently explored the expression of IL-17A responsive genes at the mRNA level. In addition to IL-6 and KC, we probed another IL-17 responsive gene, murine MMP9. The matrix metalloproteinases (MMPs) are a family of proteases that are important in processes such as tissue remodelling, wound healing, tumour invasion and carcinogenesis (Cousens *et al*., 2002; Sternlicht *et al*., 1999; Vu *et al*., 1998). Aberrant MMP expression is also found in autoimmune diseases such as rheumatoid arthritis. IL-17A is a key inducer of MMPs. Primary macrophages were treated with IL-17A at 6 and 24 h intervals. Real time PCR was carried out to examine the production of IL-6, KC and MMP9 mRNA. IL-17A-induced expression of mRNAs encoding IL-6 (Fig 3.36), KC (Fig 3.37) and MMP9 (Fig. 3.38) was augmented in the IL-17RD knockout cells. Collectively, these data support a negative regulatory role for IL-17RD in IL-17A induced signalling.

It was also of interest to observe how IL-17RD would affect the downstream signalling components of the IL-17 signalling pathway in IL-17RD deficient macrophages. BMDMs were treated with IL-17A over various timepoints and the lysates were subsequently immunoblotted for the specific antibodies. An absence of IL-17RD results in increased IL-17A induced phosphorylation of IκB (Fig 3.39). In terms of MAPK pathways, the most notable IL-17RD-mediated response was a decreased activation of p38 MAPK

phosphorylation. This surprising finding meant that it was possible that there is some sort of differential regulation by murine IL-17RD of the NF-κB and MAPK pathways.

3.2.13 IL-17RD mediates neutrophil infiltration to the peritoneum

Given the above differential effects of IL-17RD on IL-17A-signalling we next probed the physiological relevance of these effects by evaluating IL-17A-mediated neutrophilia in both IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice. Mice were treated with recombinant IL-17A via intraperitoneal injection, sacrificed 3 h post-injection and peritoneal cells were collected from lavage. Flow cytometric analysis was subsequently performed on the peritoneal exudate cells (PECs) to quantitate the neutrophil population (Fig. 3.40a). The administration of IL-17A to wild-type mice induced marked neutrophil infiltration into the peritoneum but this was dramatically reduced in IL-17RD$^{-/-}$ mice. The greatly diminished neutrophil infiltration in IL-17RD$^{-/-}$ mice was observed at various times post IL-17A administration (Fig. 3.40b). The critical mediatory role for IL-17RD in facilitating neutrophil infiltration was selective for IL-17A signalling since neutrophil infiltration trigged by the bacterial chemoattractant peptide formyl-Met-Leu-Phe was unaffected by loss of IL-17RD (Fig. 3.40c). We next measured the expression of IL-6, KC and MIP-2 in PECs isolated from the above treated mice. The *in vivo* administration of IL-17A increased the mRNA levels of all 3 cytokines in PECs from wild-type mice and in keeping with the earlier cell-based models, the induction of IL-6 and KC was further enhanced whilst MIP-2 expression was greatly diminished in PECs from similarly-treated IL-17RD$^{-/-}$ mice (Fig. 3.40d). These findings were further corroborated by measuring KC and MIP-2 protein levels in lavage fluid from IL-17-treated mice. Again administration of IL-17A to wild-type mice caused increased lavage levels of KC and MIP2 with KC being further enhanced and MIP-2 diminished in similarly-treated IL-17RD$^{-/-}$ mice (Fig. 3.40e). Given that IL-17A-induced expression of MIP-2 is greatly suppressed in IL-17RD$^{-/-}$ mice, coupled to the strong chemoattractant activity of MIP-2 towards neutrophils, we investigated if administration of exogenous MIP-2 could overcome the deficiency in IL-17A-induced neutrophilia that we observed in IL-17RD$^{-/-}$ mice. We again treated mice with IL-17A by intraperitoneal injection, which was followed by intraperitoneal injection of recombinant MIP-2 1 h later in order to evaluate if MIP-2 deficiency provided an underlying basis to the phenotypic changes on neutrophilia in IL-17RD$^{-/-}$ mice. As before, the administration of IL-17A to wild-type mice promoted strong neutrophilia and this was not further enhanced by co-administration of MIP-2 indicating that the latter is not a limiting factor in wild-type mice (Fig. 3.40f). However, exogenous MIP-2 significantly enhanced

neutrophilia in IL-17A-treated IL-17RD$^{-/-}$ mice consistent with our hypothesis that deficiency in MIP-2 expression in IL-17RD-deficient mice may be an important limiting factor underlying reduced neutrophilia in these animals (Fig. 3.40f). It is interesting to note that the enhanced KC expression in IL-17RD$^{-/-}$ mice failed to compensate for the reduced levels of MIP-2. However, a previous study indicated MIP-2 to be a more potent neutrophil chemoattractant than KC (Yan *et al*. 1998). Our findings are consistent with this role for MIP-2 being the chief chemoattractant of neutrophils *in vivo* as the diminished IL-17A-induced neutrophil infiltration in IL-17RD$^{-/-}$ mice was associated with loss of MIP-2 expression.

3.2.14 IL-17RD mediates IL-17A-induced pulmonary neutrophilia

The key role of IL-17RD in mediating neutrophil infiltration into the peritoneum in response to IL-17A prompted us to examine a more general role for IL-17RD in mediating tissue neutrophilia. To investigate this, mice were challenged with IL-17A via intranasal injection to elicit pulmonary neutrophilia. Mice were sacrificed after 24 h and cells in the bronchoalveloar lavage (BAL) fluid were analyzed (Fig. 3.41a). Consistent with findings from intraperitoneal injection, IL-17RD$^{-/-}$ mice had significantly less ($p<0.038$, *Student's t test*) lung-infiltrating neutrophils, in response to IL-17A administration, in the BAL fluid relative to wild-type animals (Fig. 3.41a). There was no difference in lymphocyte and monocyte numbers in BAL fluid between IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice demonstrating a specific effect of IL-17RD on neutrophilia. Furthermore, histological analysis of lung tissue showed a significant decrease in neutrophil infiltration in IL-17RD$^{-/-}$ mice compared with IL-17RD$^{+/+}$ mice (Fig. 3.40b). IL-17A-induced expression of IL-6, KC and MIP-2 was also measured in lung tissue from the above mice by qPCR (Fig. 3.41c). IL-17A-induced expression of KC and IL-6 levels was further augmented in lung tissue from IL-17RD$^{-/-}$ mice relative to IL-17RD$^{+/+}$ mice whereas IL-17A-induced MIP-2 expression was greatly reduced in lung samples from IL-17RD$^{-/-}$ mice. This is consistent with the observed absence of neutrophilia in the lung and reaffirms a critical role for IL-17RD as a mediator of neutrophilia.

3.2.15 IL-17RD interacts with key IL-17A signalling components and disrupts the interaction of TRAF6 and ACT1

It was necessary to probe the mechanistic basis for these observed functional effects. Given that IL-17RD contains a SEFIR domain, initial studies probed the ability of IL-17RD to interact with the SEFIR domain containing adaptor proteins and/or key downstream components. Thus, myc-tagged IL-17RD was co-transfected with Flag-tagged ACT1, TRAF5 and TRAF6. Flag-tagged proteins were immunoprecipitated using an anti-flag antibody and immunoprecipitates were probed for the presence of IL-17RD using an anti-myc antibody. Interactions were observed with each protein (Fig 3.42). In order to elucidate the possible mechanism by which IL-17RD differentially regulates NF-κB and MAPK pathways, the interaction of ACT1 and TRAF6 knockdown cells. U373 stably transduced control and IL-17RD shRNA cells were stimulated as indicated (Fig 3.43). This study demonstrated that the interaction of ACT1 with TRAF6 is enhanced in the cells with a lower expression level of IL-17RD which is consistent with a role for IL-17RD in inhibiting the TRAF6-dependent pathway of IL-17 signalling. This suggests that IL-17RD may act as an endogenous braking effector mechanism for IL-17A signalling by inhibiting the binding of ACT1 to TRAF6 and so blocking the TRAF6 dependent pathway.

3.2.16 IL-6 production is synergistically enhanced in IL-17RD deficient macrophages in response to TLR ligand and IL-17A

Recent insights into IL-17 signalling have shed light into its intricate interplay between the innate and adaptive immune systems. IL-17, commonly produced during viral and bacterial infection, has the capacity to specifically augment pro-inflammatory responses by directly synergising with innate immune signalling pathways (Ryzhakov *et al*., 2011). As such, it was intriguing to explore the physiological effects that IL-17RD exerts on synergism between IL-17 signalling and TLR signalling. Murine IL-6 was chosen as a representative gene due to it being induced in response to both IL-17 and TLR ligands. Primary macrophages were treated with either LPS or Poly I:C with and without IL-17A. The supernatants were obtained 24 hours post treatment and the production of mIL-6 was measured by ELISA. As expected, mIL-6 levels were raised in the Poly I:C and IL-17A treated wild type macrophages compared with the Poly I:C or IL-17 alone treated cells (Fig 3.44). Additionally, when the IL-17RD deficient macrophages were treated with both ligands,

there was a synergistic enhancement of mIL-6 levels which exceeded that of the wild type cells. The same pattern of IL-6 induction was achieved when LPS was substituted for Poly I:C (Fig 3.45). These data prove interesting as it displays IL-17RD as a regulator in the interplay of immune signalling pathways.

An overall picture emerges in which IL-17RD can wire various innate immune signalling pathways and can differentially regulate sub-pathways that are triggered by innate immune receptors.

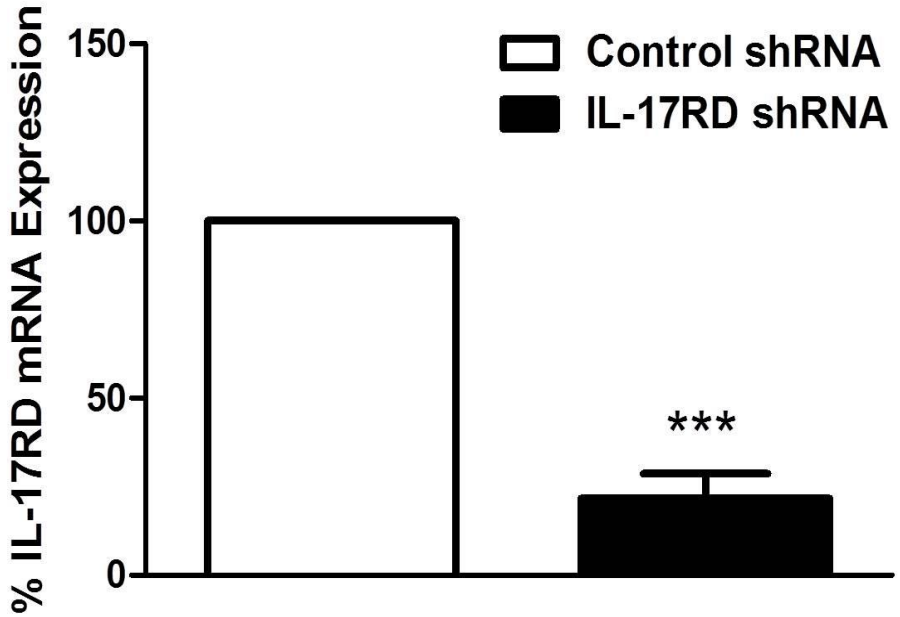

Figure 3.1 Knockdown of endogenous IL-17RD by lentiviral vector-encoded shRNA in U373 cell lines

U373 cells stably transduced with Control or IL-17RD specific-shRNA were cultured in the presence of puromycin (10 μg/ml). Total RNA was extracted and cDNA was generated using RT. Samples were subsequently assayed by quantitative real-time PCR for levels of IL-17RD. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments.

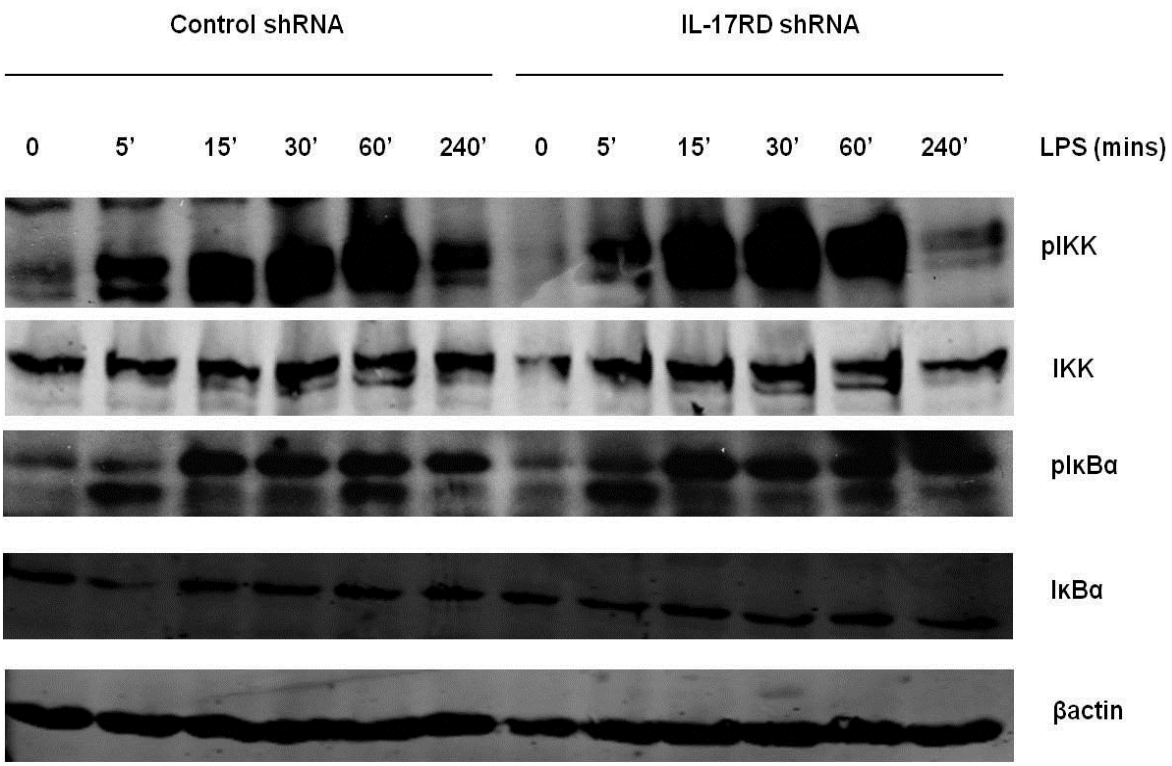

Figure 3.2 Suppression of endogenous IL-17RD expression enhances LPS induced IKK and IκB phosphorylation

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were then stimulated with LPS (100 ng/ml) for the indicated time periods. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out with anti-phospho-IKK, anti-IKK, anti-phospho-IκB, anti- IκB and anti-β-actin antibodies. Blots shown are representative of two independent experiments.

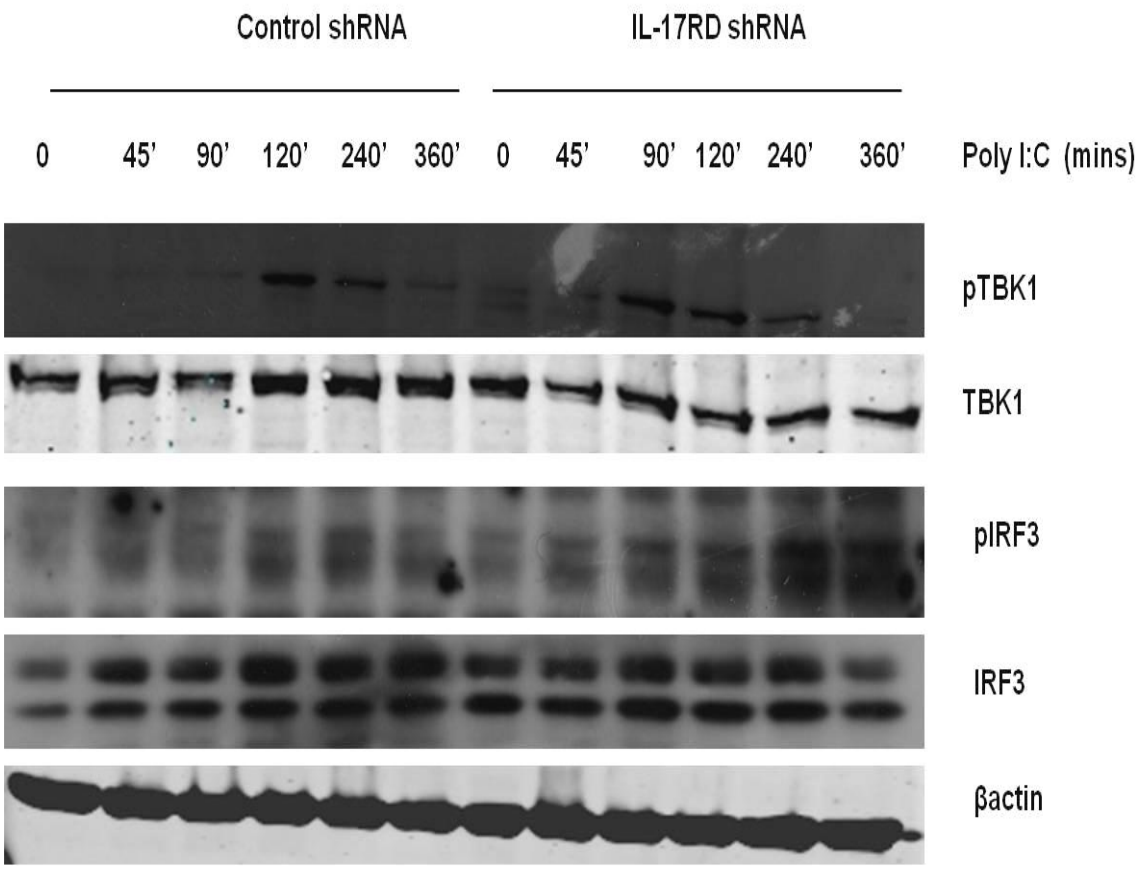

Figure 3.3 Suppression of endogenous IL-17RD expression enhances Poly I:C induced TBK1 and IRF3 phosphorylation

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were then stimulated with Poly I:C (25 μg/ml) for the indicated time periods. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out with anti-phospho-TBK1, anti-TBK1, anti-phospho-IRF3, anti- IRF3 and anti-β-actin antibodies. Blots shown are representative of two independent experiments.

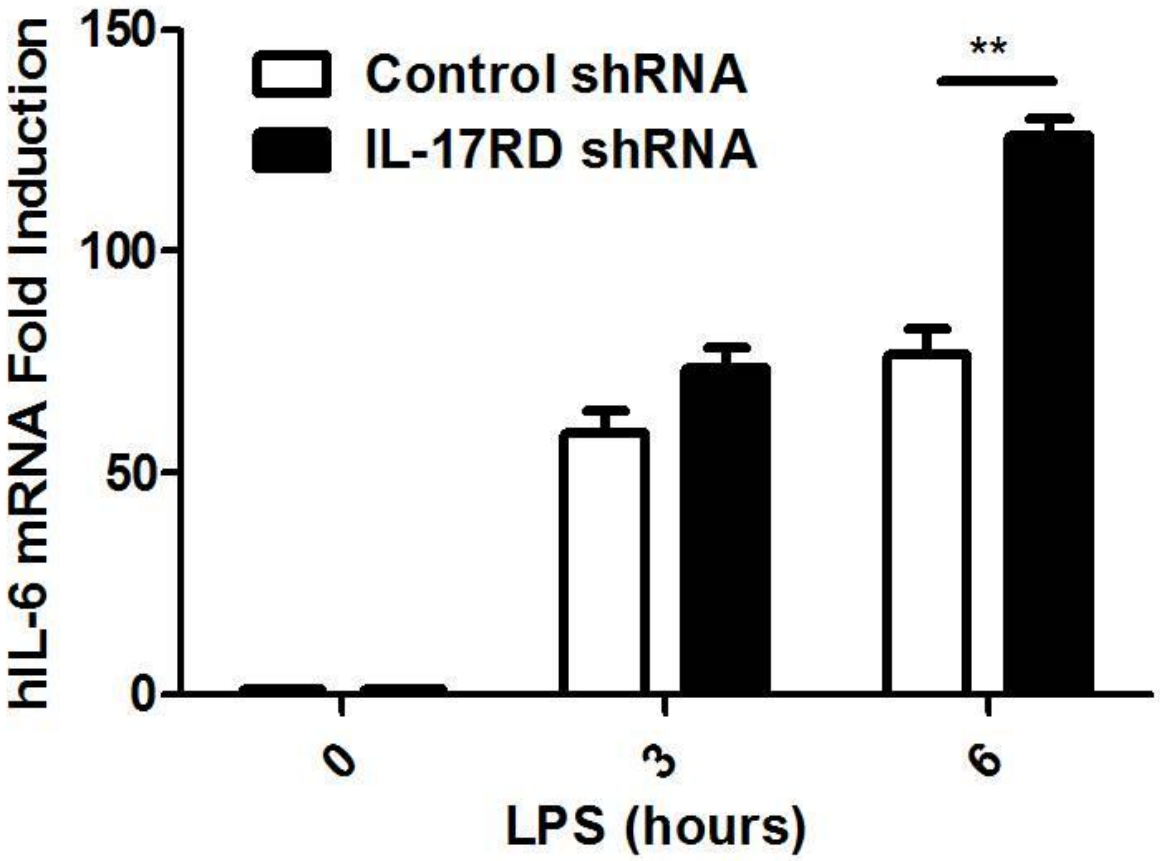

Figure 3.4 IL-17RD knockdown augments LPS induced expression of IL-6 mRNA

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with LPS (100 ng/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of IL-6. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. ** $p < 0.01$, paired t-test, LPS stimulated IL-17RD shRNA cells compared with control shRNA cells.

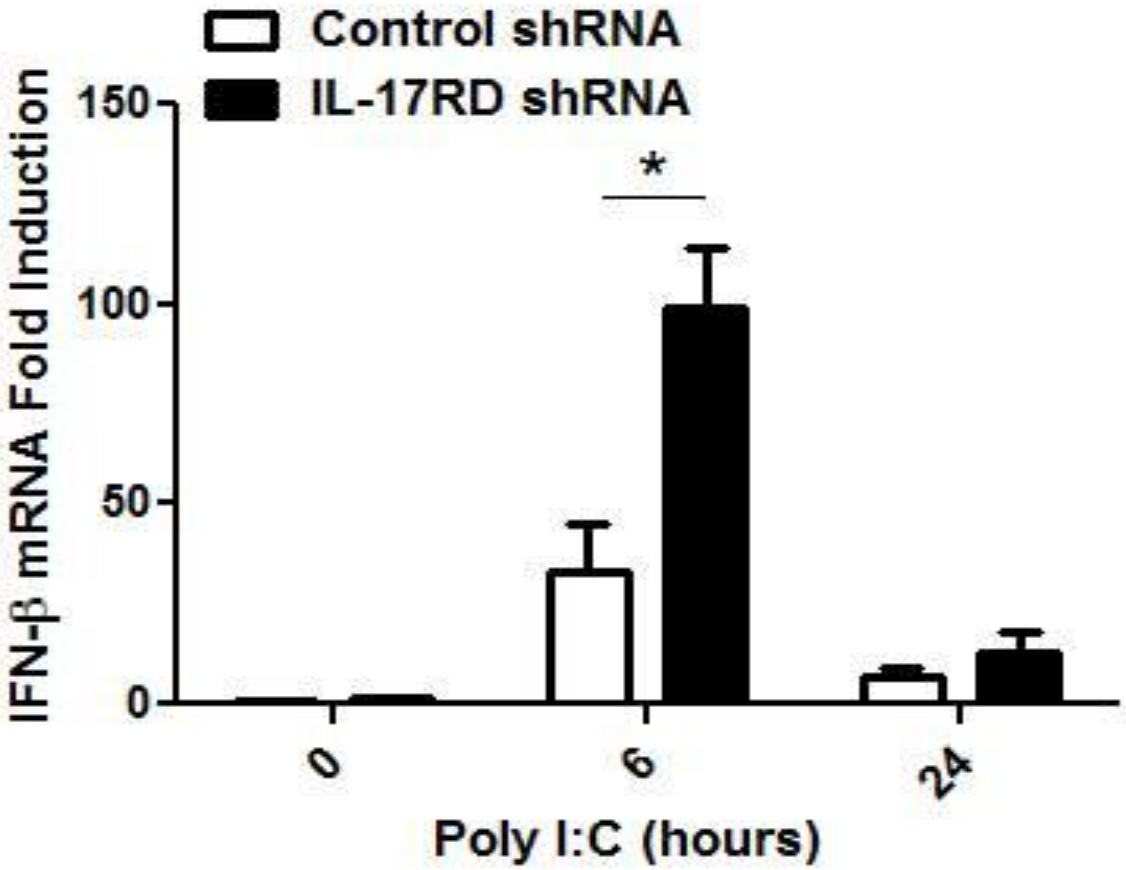

Figure 3.5 IL-17RD knockdown augments Poly I:C induced expression of IFNβ mRNA

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with Poly I:C (25 μg/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of IFNβ. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the 2-ΔΔCT method. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test, Poly(I:C) stimulated IL-17RD shRNA cells compared with control shRNA cells.

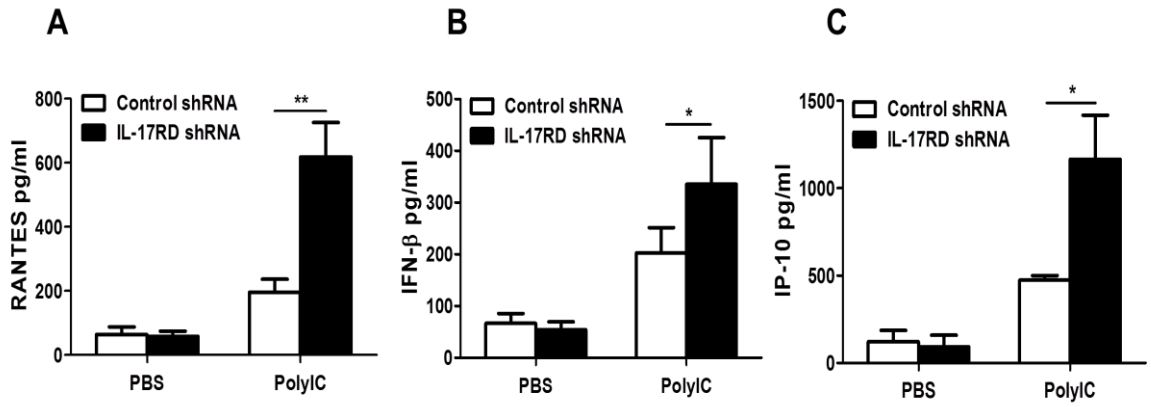

Figure 3.6 Knockdown of IL-17RD augments Poly I:C induced expression of RANTES, IFN-β and IP-10

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 96-well plates for 24 h. Cells were treated for 24 h with Poly I:C (25 μg/ml). Supernatants were obtained and analysed for (a) RANTES (b) IFN-β and (c) IP-10 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, paired t-test, Poly(I:C) stimulated IL-17RD shRNA cells compared with control shRNA cells.

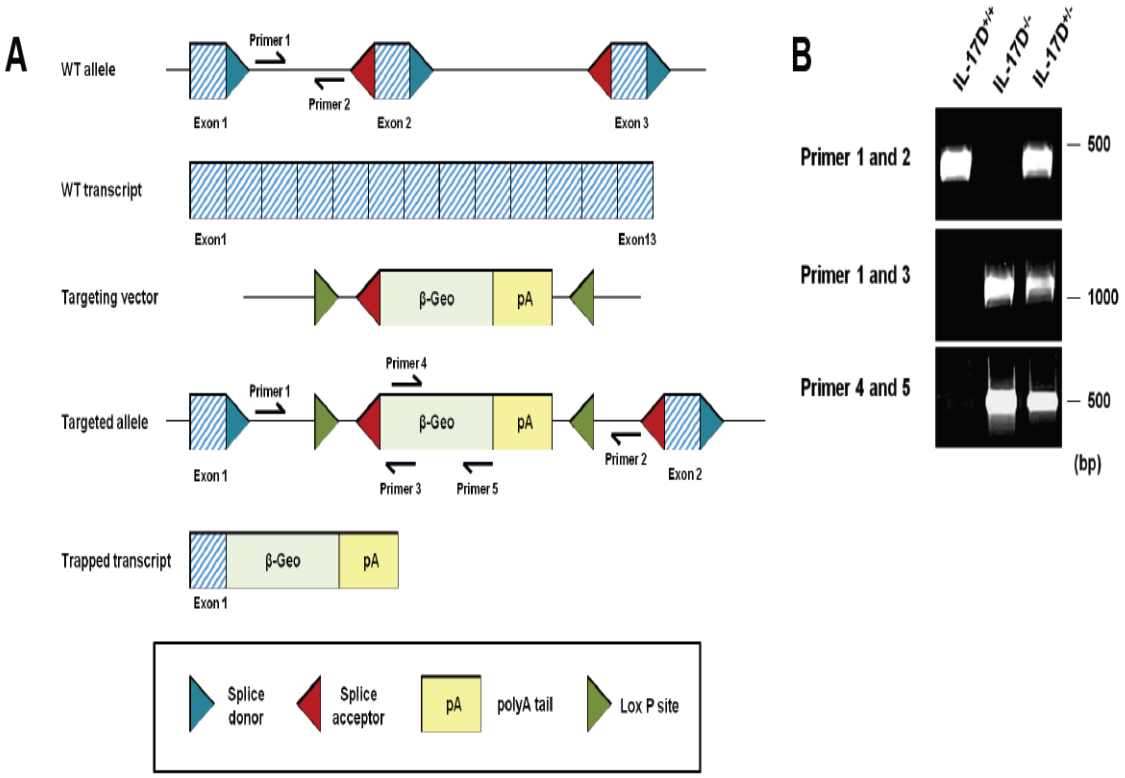

Figure 3.7. Generation of IL-17RD-deficient mice. (A) Schematic shows the murine *il-17rd* gene and the genetrap targeting vector, containing a promoter-less reporter/selectable marker fusion gene (*β-Geo*) downstream of a splice acceptor, which incorporates into the *il-17rd* gene after the first exon. Exons are numbered and regions targeted by genotyping primers are also indicated. The targeting vector also contains frt, f3, lox5171 sites omitted in schematic. (B) Genotyping was performed by PCR on genomic DNA from ear punches. Primer 1 and 2 amplify a fragment from the WT allele of size 449 bp and from the knockout allele due to insertion of the genetrap cassette at a predicted size >7 Kb, however this fragment from the knockout allele is too large to amplify. Primer 1 with primer 3 amplifies a fragment spanning the genetrap cassette of size 1103 bp and differentiates the IL-17RD knockout allele in IL-17RD$^{+/-}$ and IL-17RD$^{-/-}$ mice from IL-17RD$^{+/+}$ mice. To further confirm the presence of the genetrap cassette primers 4 and 5 amplify a region of the β-Geo insert at a size of 508bp, only found in IL-17RD$^{+/-}$ and IL-17RD$^{-/-}$ mice. (Mellett *et al.* 2012)

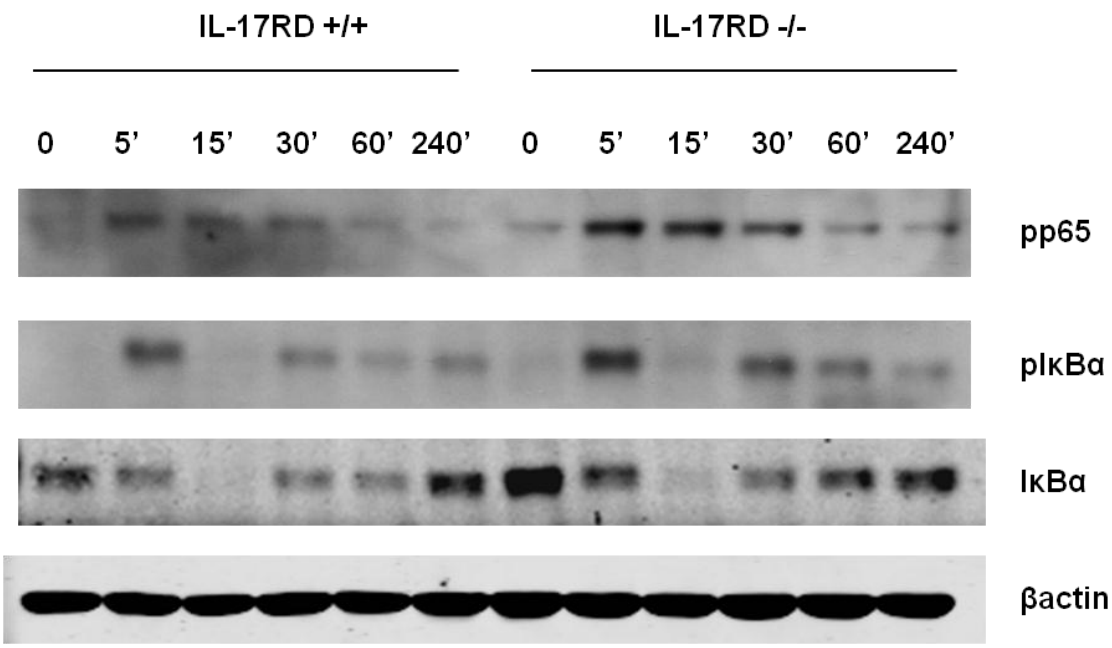

Figure 3.8 IL-17RD deficiency enhances LPS-induced-phosphorylation of IκB and p65

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with LPS (100 ng/ml) as indicated. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out using anti-phospho-p65, anti-p65, anti-phospho-IκB, anti- IκB, anti-COX-2 and anti-β-actin antibodies. Blots shown are representative of three independent experiments.

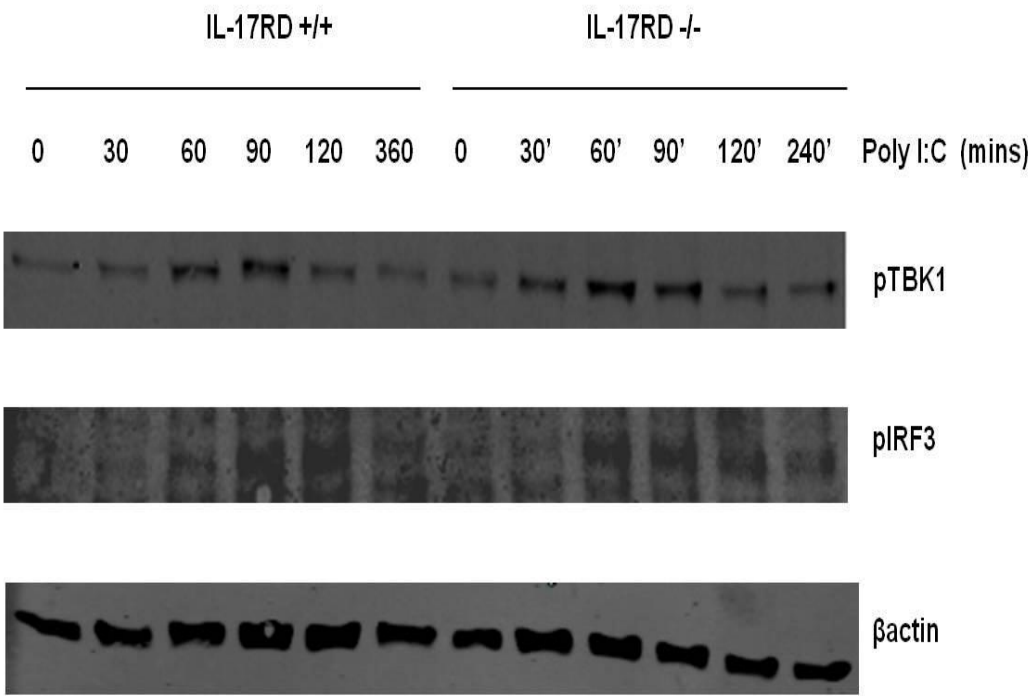

Figure 3.9 IL-17RD deficiency accelerates Poly I:C induced phosphorylation of TBK1 and IRF3

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with Poly I:C as indicated. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out with anti-phospho-TBK1, anti-phospho-IRF3 and anti-β-actin antibodies. Blots shown are representative of three independent experiments.

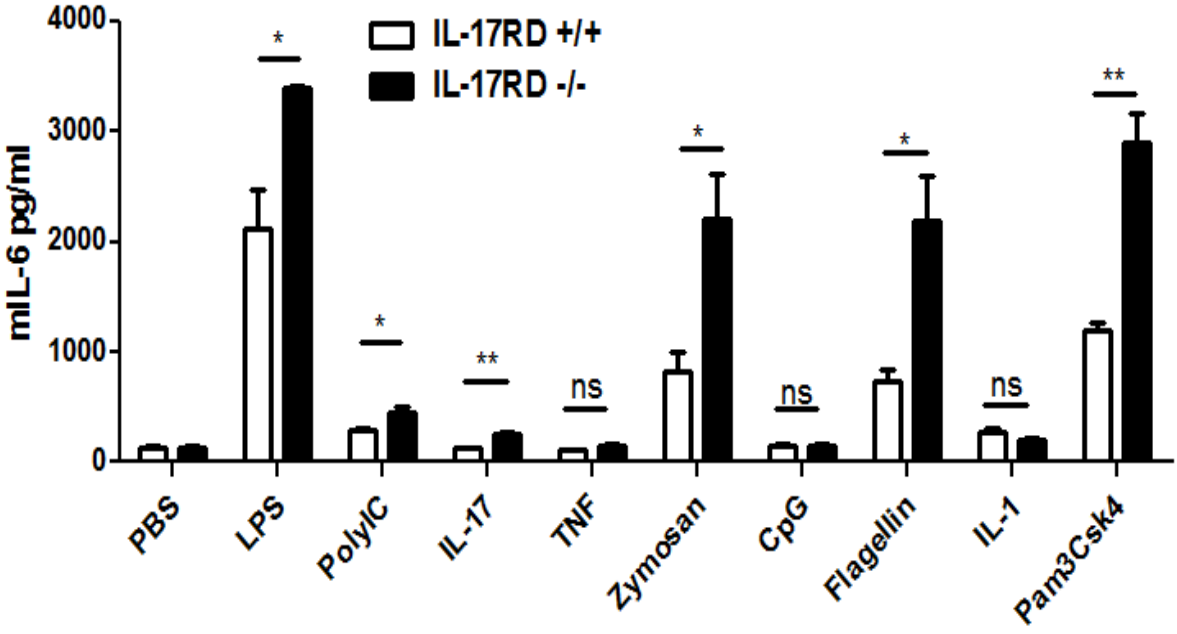

Figure 3.10 IL-17RD deficiency augments TLR induced expression of mIL-6 production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with various ligands as indicated. Cells were treated for 24 h. Supernatants were obtained and analysed for mIL-6 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$,** $p<0.01$ paired t-test.

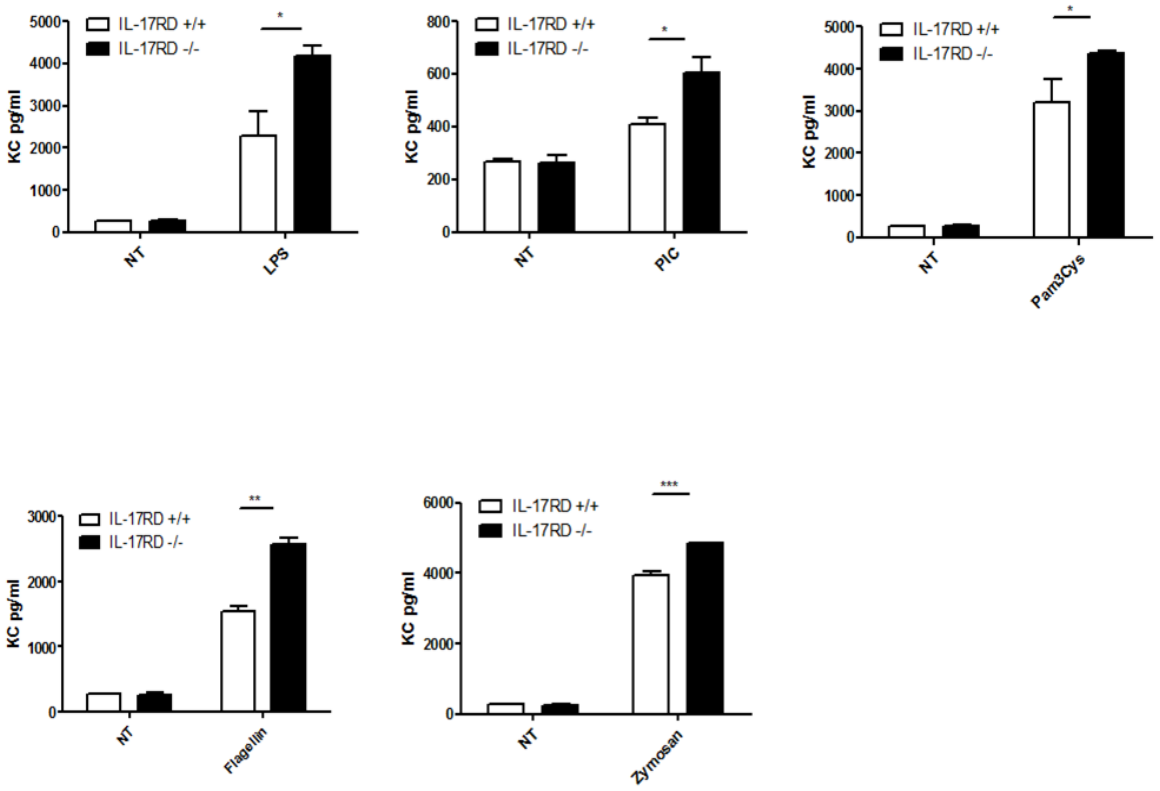

Figure 3.11 IL-17RD deficiency augments TLR mediated KC production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with various ligands as indicated. Cells were treated for 24 h. Supernatants were obtained and analysed for KC production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ paired t-test.

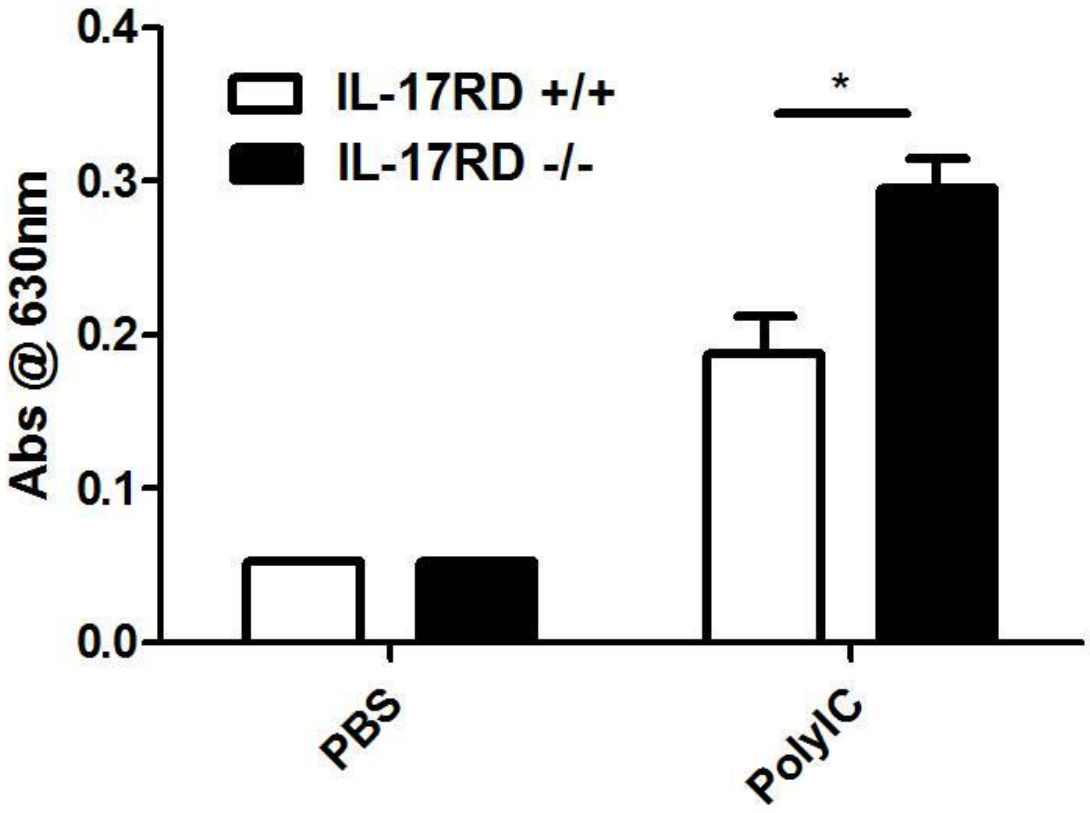

Figure 3.12 IL-17RD deficiency enhances Poly I:C induced Type I IFN production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with Poly I:C. Cells were treated for 24 h. Supernatants were obtained and analysed for Type I interferon production using B16-Blue™ IFN-α/β Cells and the detection medium QUANTI-Blue$^{TM.}$ Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test.

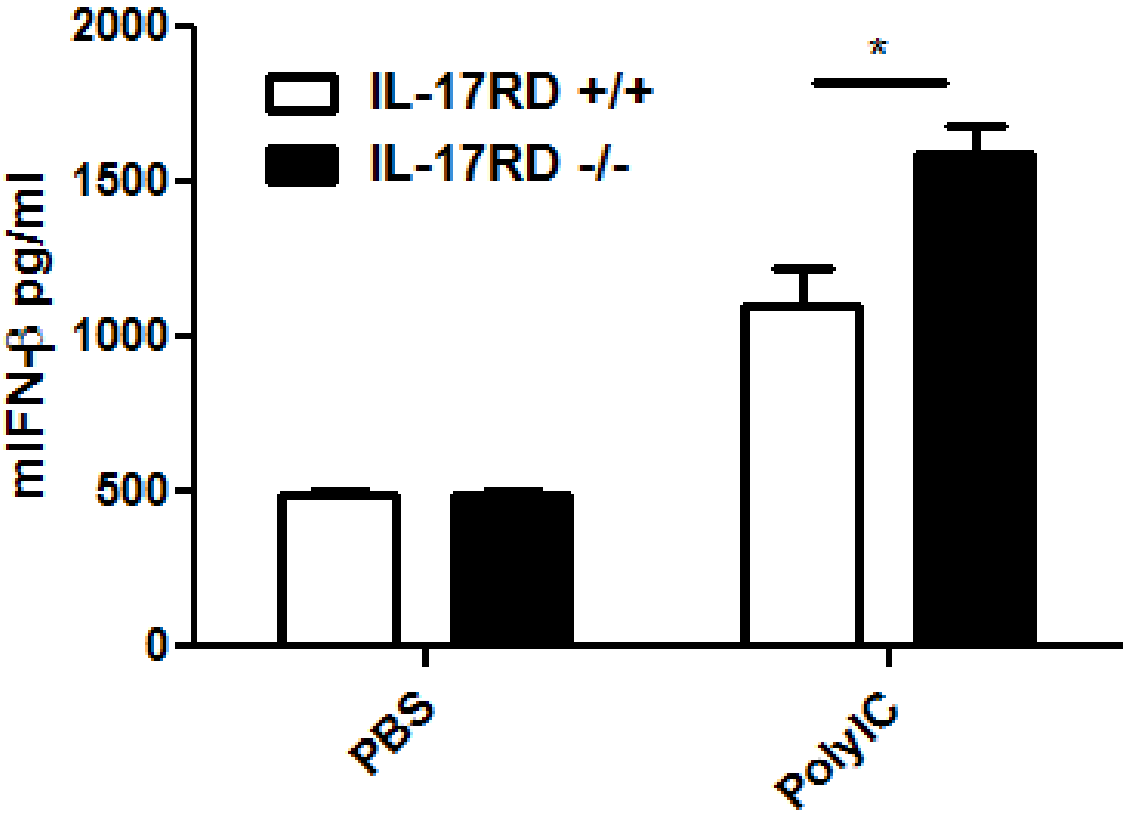

Figure 3.13 IL-17RD deficiency increased Poly I:C induced IFN-β production

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 96-well plates for 24 h. Cells were treated for 24 h with Poly I:C (25 μg/ml). Supernatants were obtained and analysed for IFN-β production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test, Poly(I:C) stimulated IL-17RD shRNA cells compared with control shRNA cells.

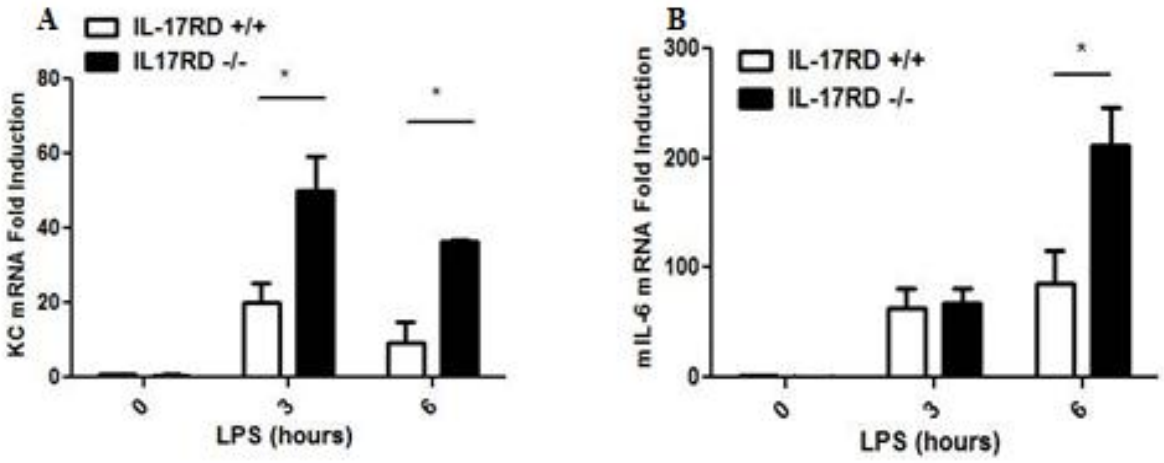

Figure 3.14 IL-17RD deficiency augments LPS induced expression of KC and IL-6 mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with LPS (100 ng/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of KC (a) and IL-6 (b). Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test, LPS stimulated IL-17RD$^{-/-}$ cells compared with IL-17RD$^{+/+}$.

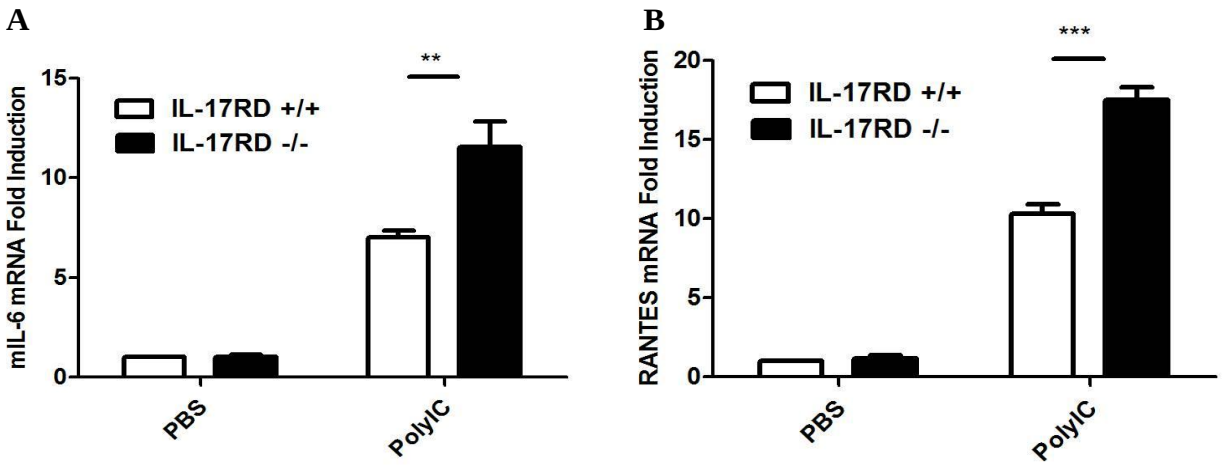

Figure 3.15 IL-17RD deficiency augments Poly I:C induced expression of IL-6 and RANTES mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with Poly I:C (25 μg/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of IL-6 (a) and RANTES (b). Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ paired t-test, Poly I:C stimulated IL-17RD$^{-/-}$ cells compared with IL-17RD$^{+/+}$.

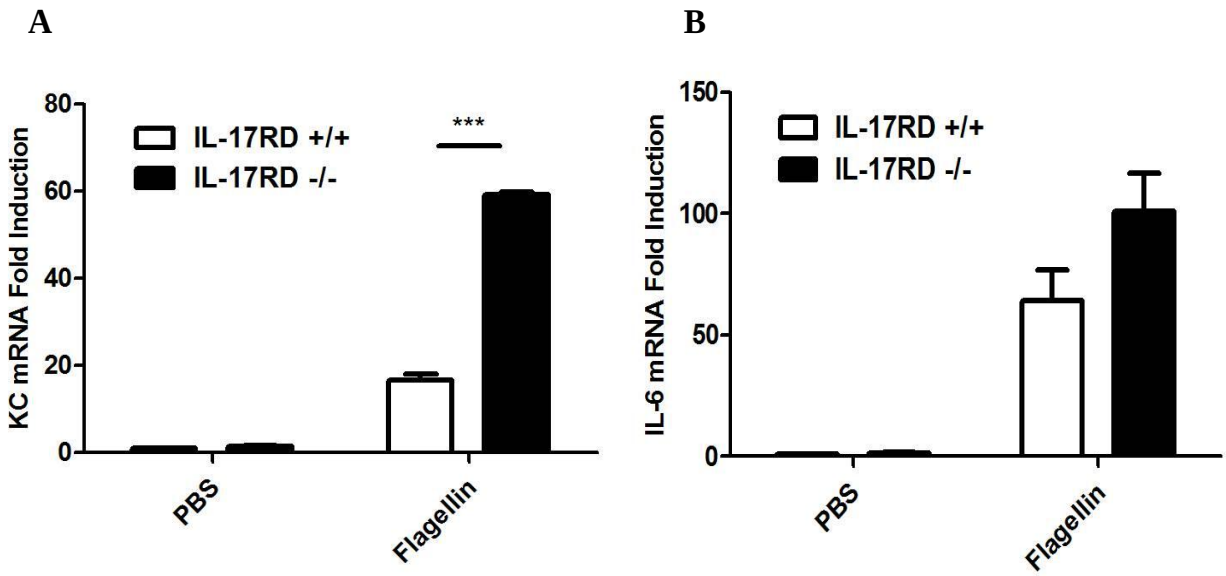

Figure 3.16 IL-17RD deficiency augments Flagellin induced expression of KC and IL-6 mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with Flagellin (100 ng/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of KC (a) and IL-6 (b). Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. *** $p < 0.001$, paired t-test, Flagellin stimulated IL-17RD$^{-/-}$ cells compared with IL-17RD$^{+/+}$.

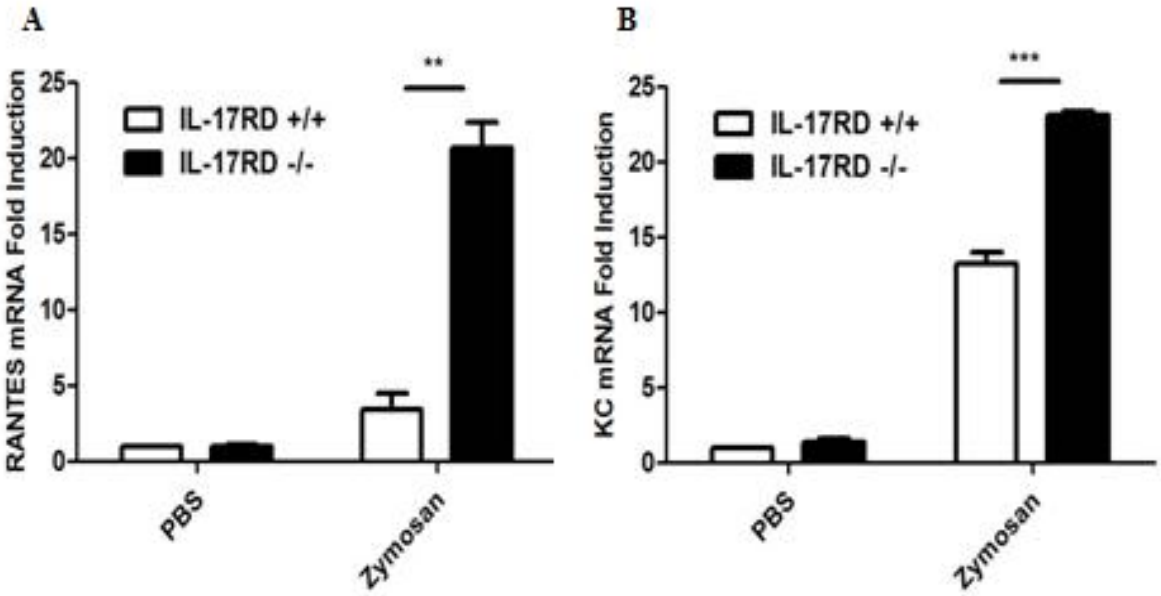

Figure 3.17 IL-17RD deficiency augments Zymosan induced expression of RANTES and KC mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with Zymosan (100 ng/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of RANTES (a) and KC (b). Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. ** $p < 0.01$, *** $p < 0.001$ paired t-test, Zymosan stimulated IL-17RD$^{-/-}$ cells compared with IL-17RD$^{+/+}$.

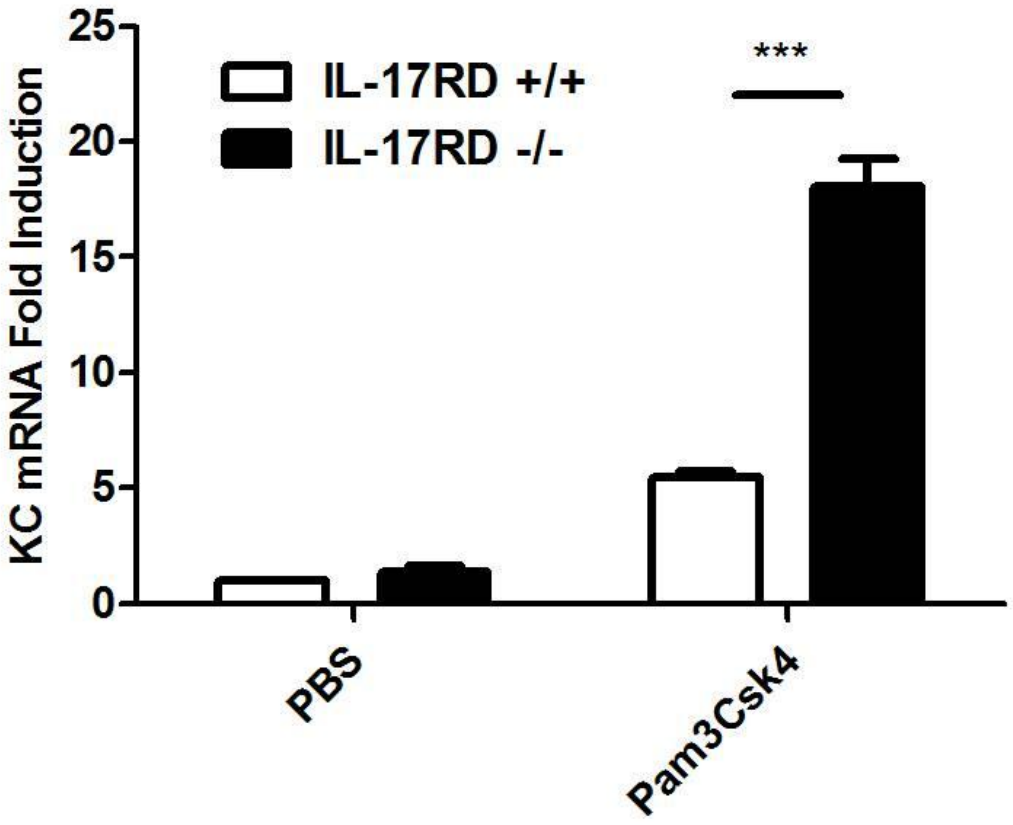

Figure 3.18 IL-17RD deficiency augments Pam3Cysk4 induced expression of KC mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with Zymosan (100 ng/ml). Total RNA was extracted and cDNA was generated by RT. Samples were then assayed by real-time PCR for levels of KC. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments. *** $p < 0.001$, paired t-test, Pam3Csk4 stimulated IL-17RD$^{-/-}$ cells compared with IL-17RD$^{+/+}$.

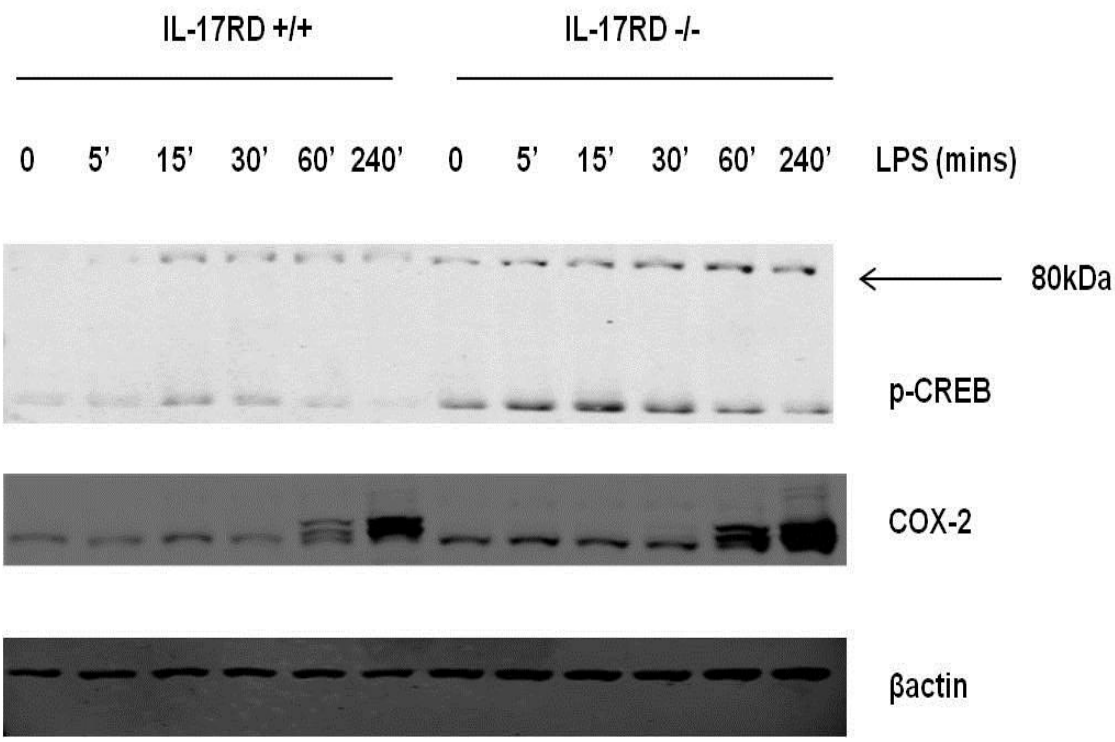

Figure 3.19 IL-17RD deficiency enhances LPS induced CREB activation

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with LPS (100 ng/ml) as indicated. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out probing with anti-phospho-CREB, anti-COX-2 and anti-β-actin antibodies. Blots shown are representative of three independent experiments.

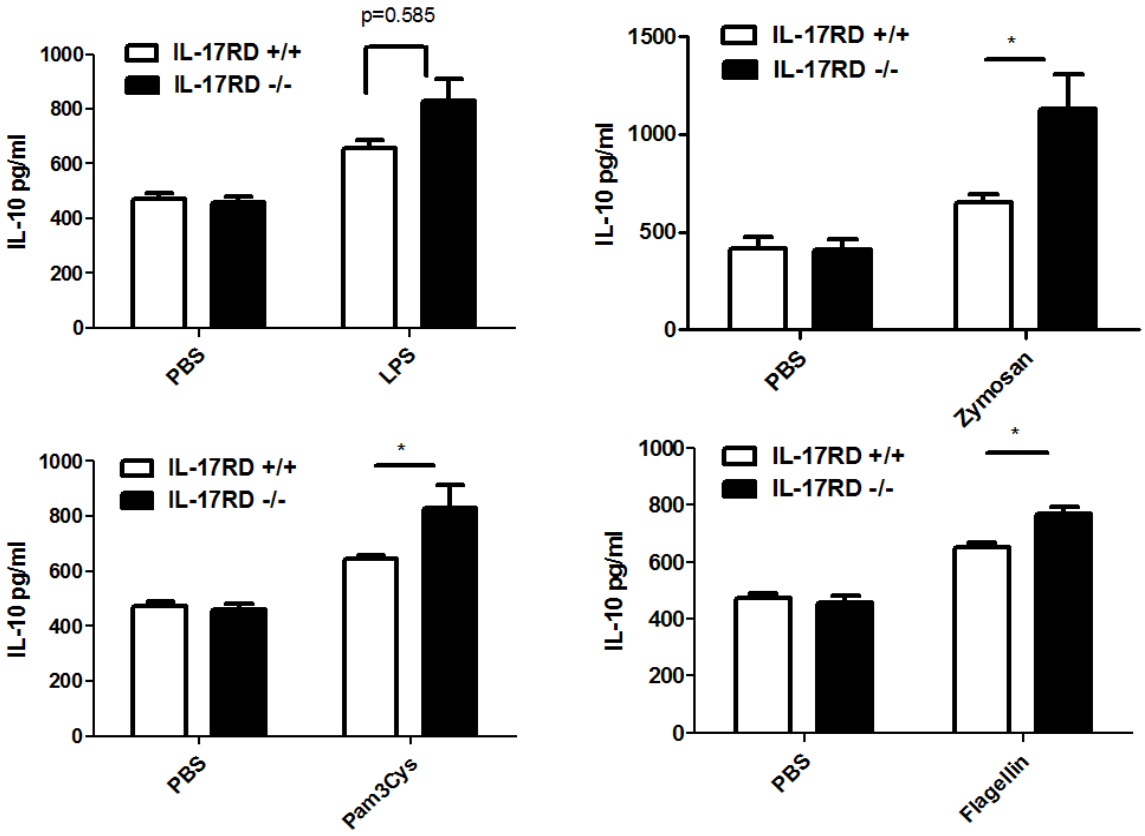

Figure 3.20 IL-17RD deficiency augments TLR mediated IL-10 production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with various ligands as indicated. Cells were treated for 24 h. Supernatants were obtained and analysed for mIL-10 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test.

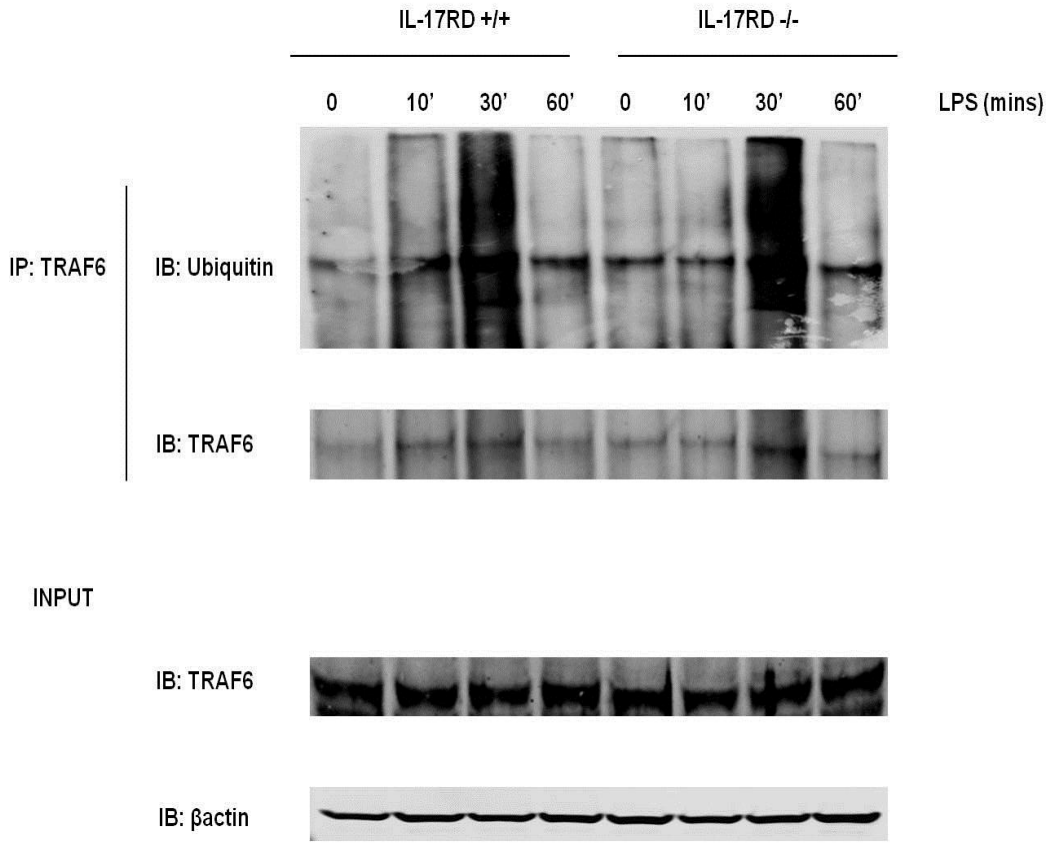

Figure 3.21 IL-17RD deficiency enhances LPS induced ubiquitination of TRAF6

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with LPS as indicated. Cell lysates were generated and incubated at 95^{o}C for 5 min with 0.5% SDS to dissociate interacting proteins. Lysates were then immunoprecipitated with an anti-TRAF6 antibody. Immunoprecipitates and whole cell lysates were then subjected to Western immunoblotting with anti-ubiquitin, anti-TRAF6 and anti-β-actin antibodies. Blots shown are representative of two independent experiments.

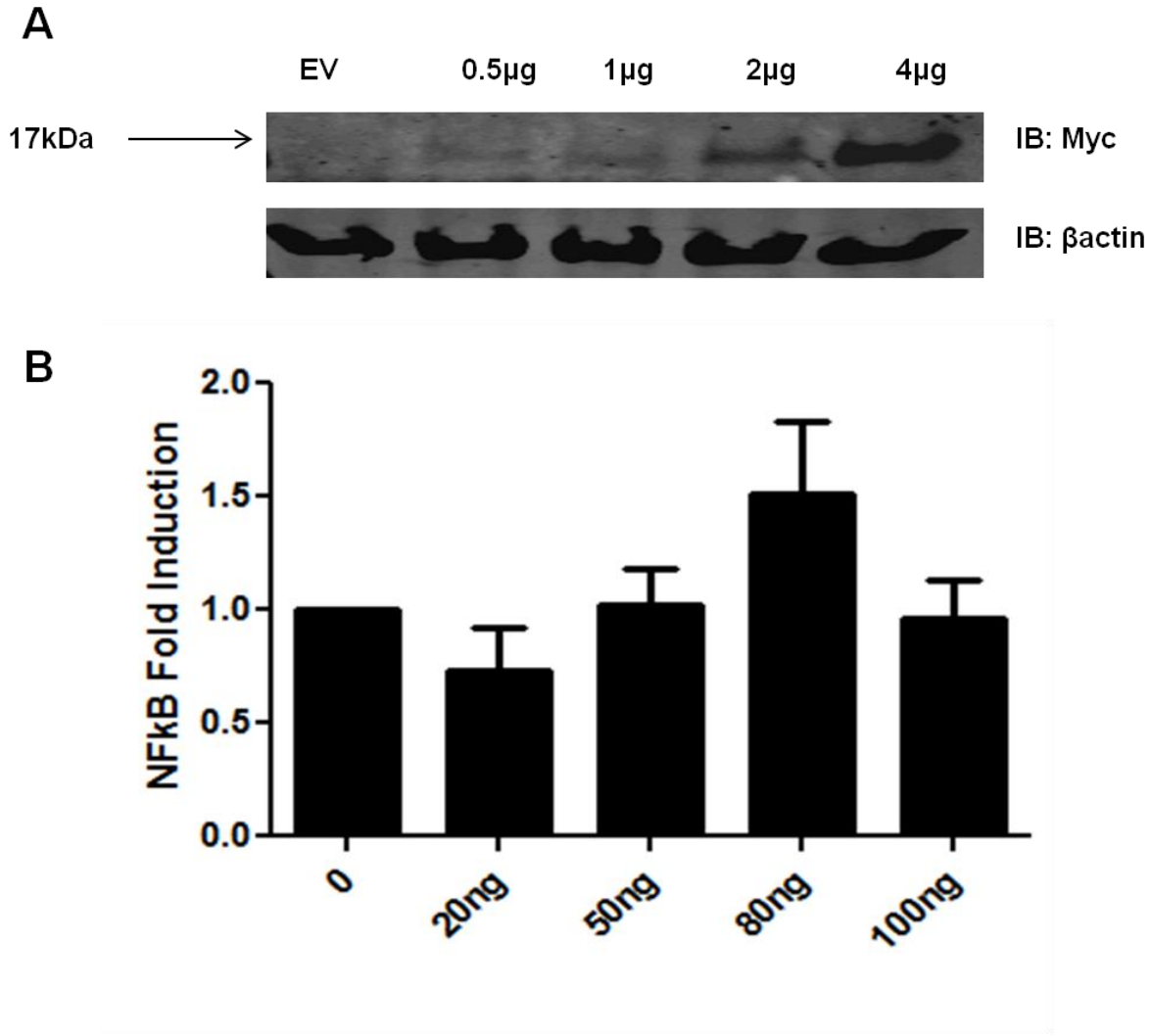

Figure 3.22 SEFIR domain of IL-17RD fails to induce NF-κB regulated luciferase

A. Cells were transfected as above and subjected to Western Blotting using anti-myc and anti-β-actin antibodies. Blots shown are representative of two independent experiments. **B.** HEK293 TLR4 cells were co-transfected with plasmids encoding NF-κB-regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding IL-17RD-myc (20, 50, 80 and 100 ng). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection cell lysates were generated. Lysates were assayed for firefly and *Renilla* luciferase activity. The data presented are mean +/- S.E.M of triplicate determinations from a representative of three independent experiments.

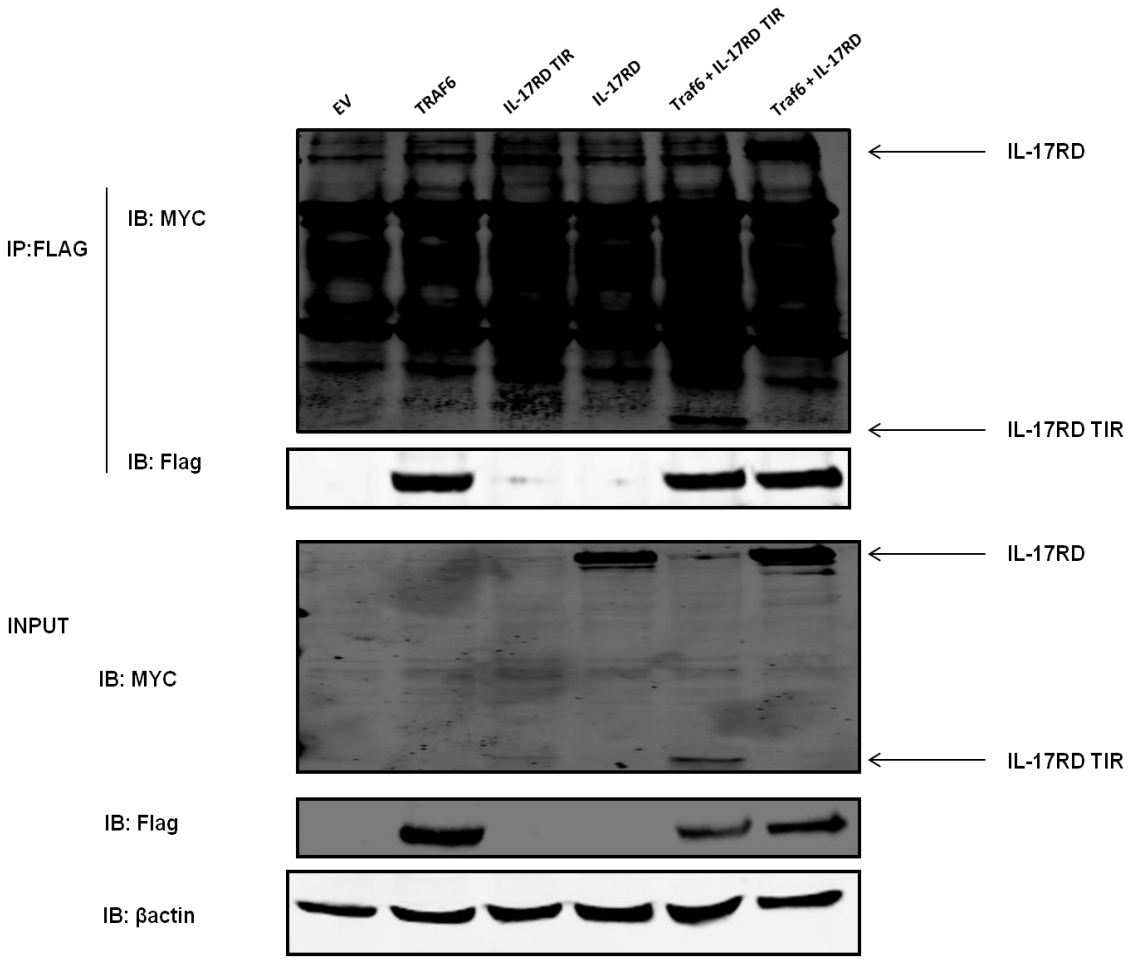

Figure 3.23 SEFIR domain interacts with TRAF6

HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged IL-17RD (2 μg) / IL-17RD TIR and TRAF6-Flag (2 μg). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection lysates were generated and immunoprecipitated with an anti-Flag antibody. Immunoprecipitates and whole cell lysates (WCL)s were subjected to Western immunoblotting using anti-myc and anti-Flag antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

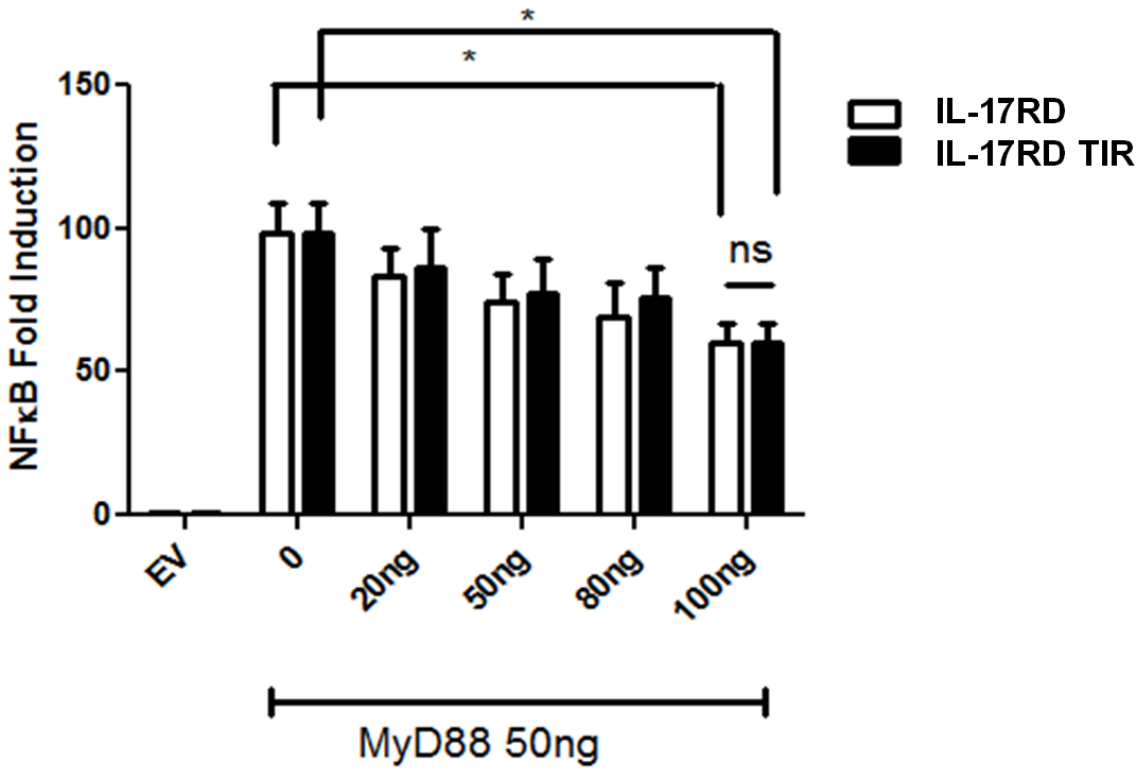

Figure 3.24 SEFIR domain shares similar inhibitory effects as the full length

HEK293 TLR4 cells were co-transfected with plasmids encoding NF-κB-regulated firefly luciferase (80 ng) and constitutively expressed TK Renilla (20 ng) and with or without plasmids encoding IL-17RD-myc (20, 50, 80 and 100 ng) or IL-17RD-TIR-myc (20, 50, 80 and 100 ng) and MyD88-flag (50 ng) or Mal-flag (50 ng). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection cell lysates were generated. Lysates were assayed for firefly and *Renilla* luciferase activity. The data presented are mean +/- S.E.M of triplicate determinations from a representative of three independent experiments. * $p < 0.05$, paired t-test.

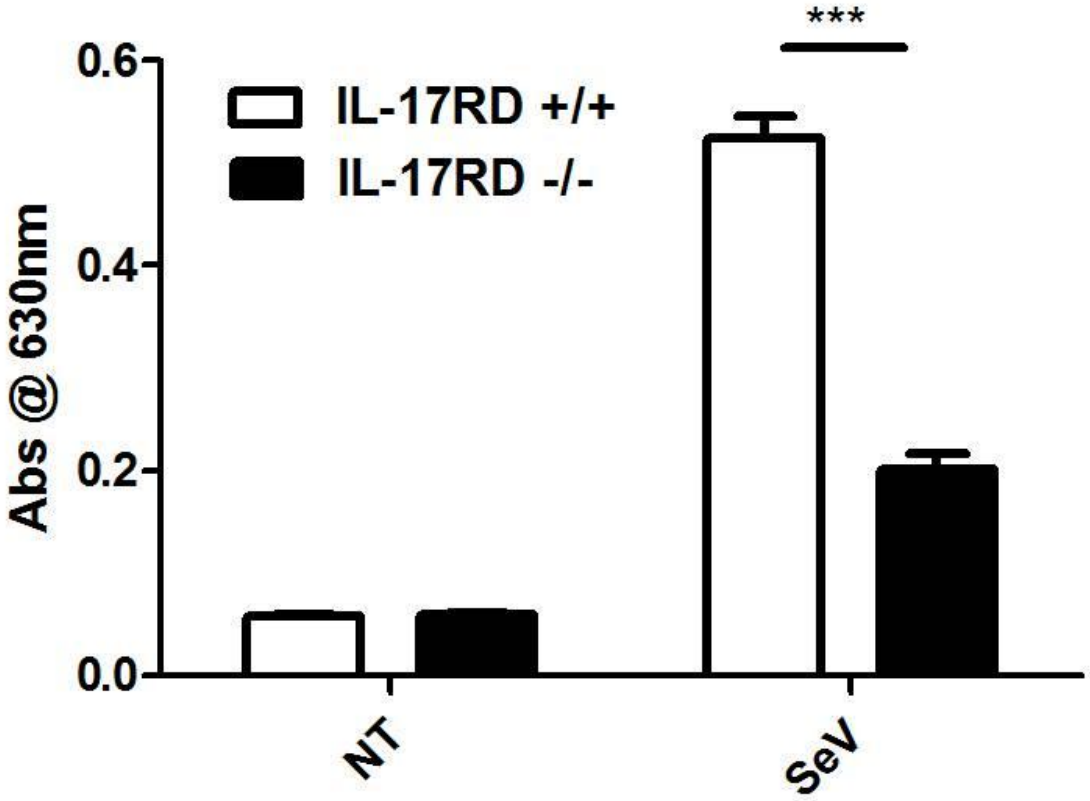

Figure 3.25 IL-17RD deficiency diminishes SeV induced Type I IFN production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were infected with SeV for 24 hours. Cells were treated for 24 h. Supernatants were obtained and analysed for Type I interferon production by Murine Type I IFNs Sensor Cells (Invivogen). Data represent the mean +/- S.E. of three independent experiments. *** $p < 0.001$, paired t-test.

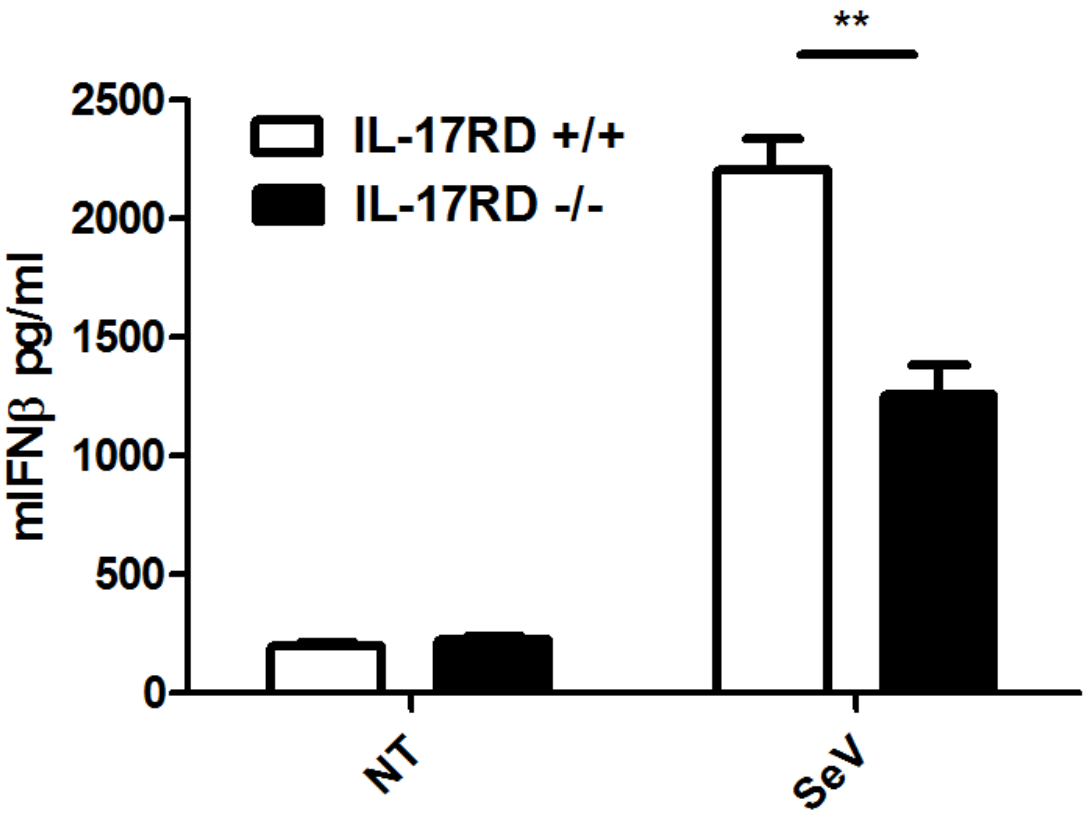

Figure 3.26 IL-17RD deficiency leads to reduced IFNβ production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were infected with SeV for 24h. Supernatants were obtained and analysed for IFNβ production by ELISA. Data represent the mean +/- S.E. of three independent experiments. ** $p < 0.01$, paired t-test.

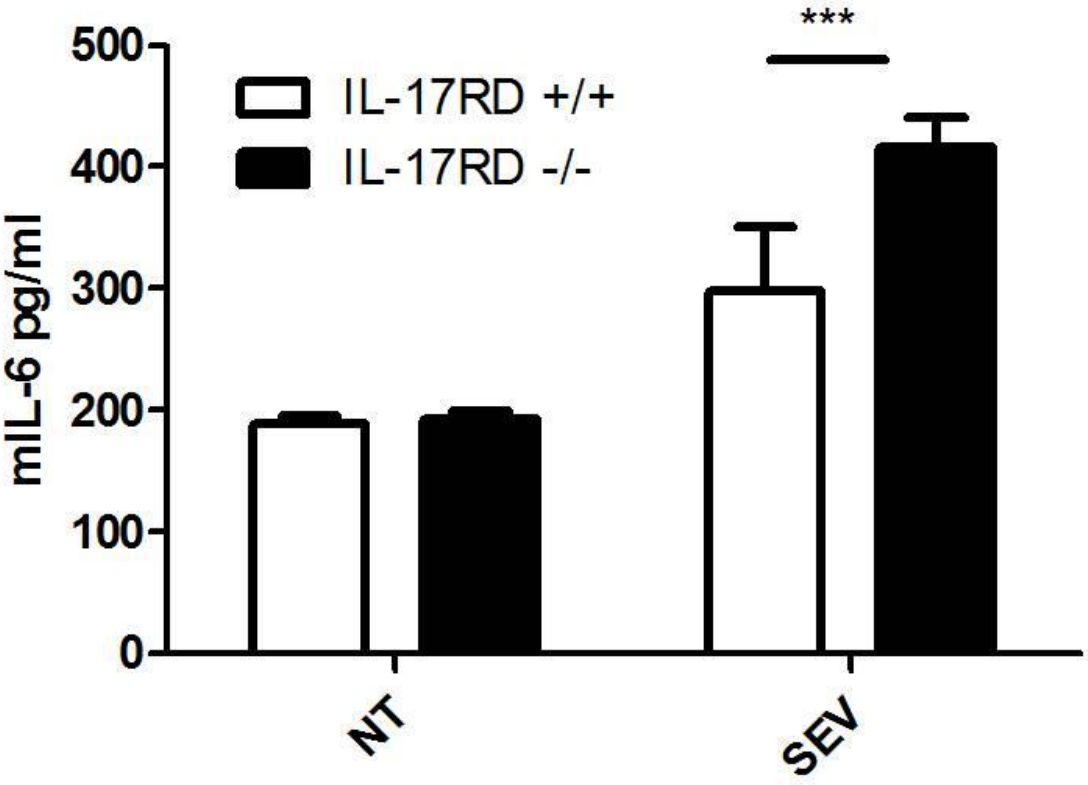

Figure 3.27 IL-17RD deficiency enhances SeV induced IL-6 production.

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were infected with SeV for 24h. Supernatants were obtained and analysed for IL-6 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. *** $p < 0.001$, paired t-test.

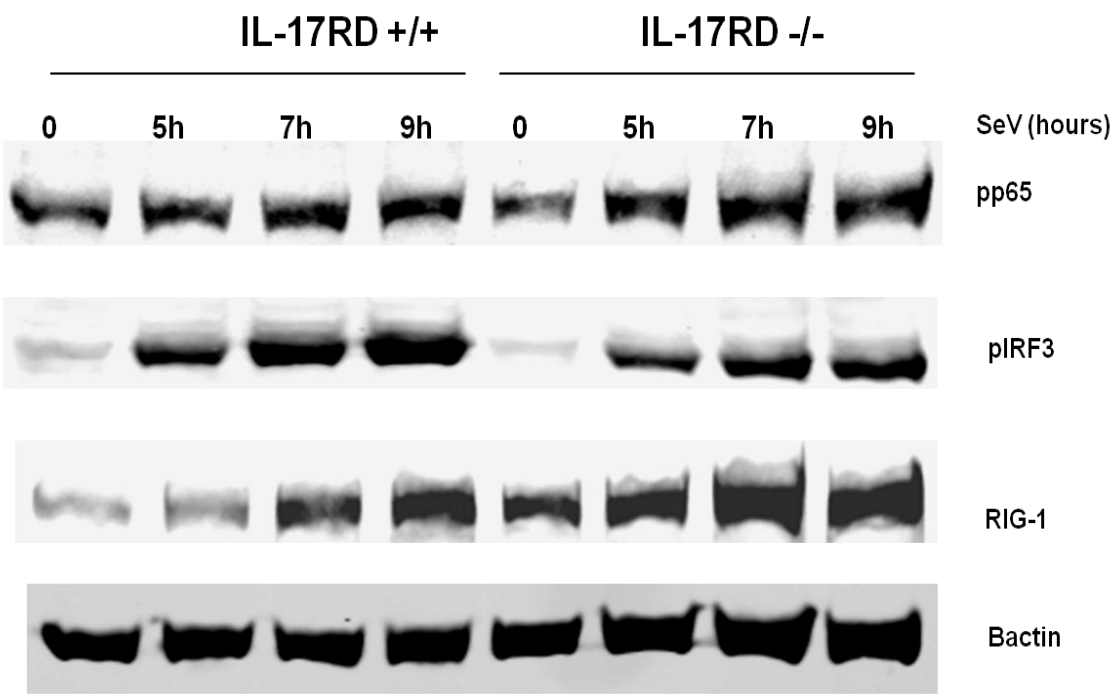

Figure 3.28 IL-17RD differentially regulates SeV-induced NF-κB and IRF signalling

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were infected with SeV as indicated. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out by probing with indicated antibodies. Blots shown are representative of three independent experiments.

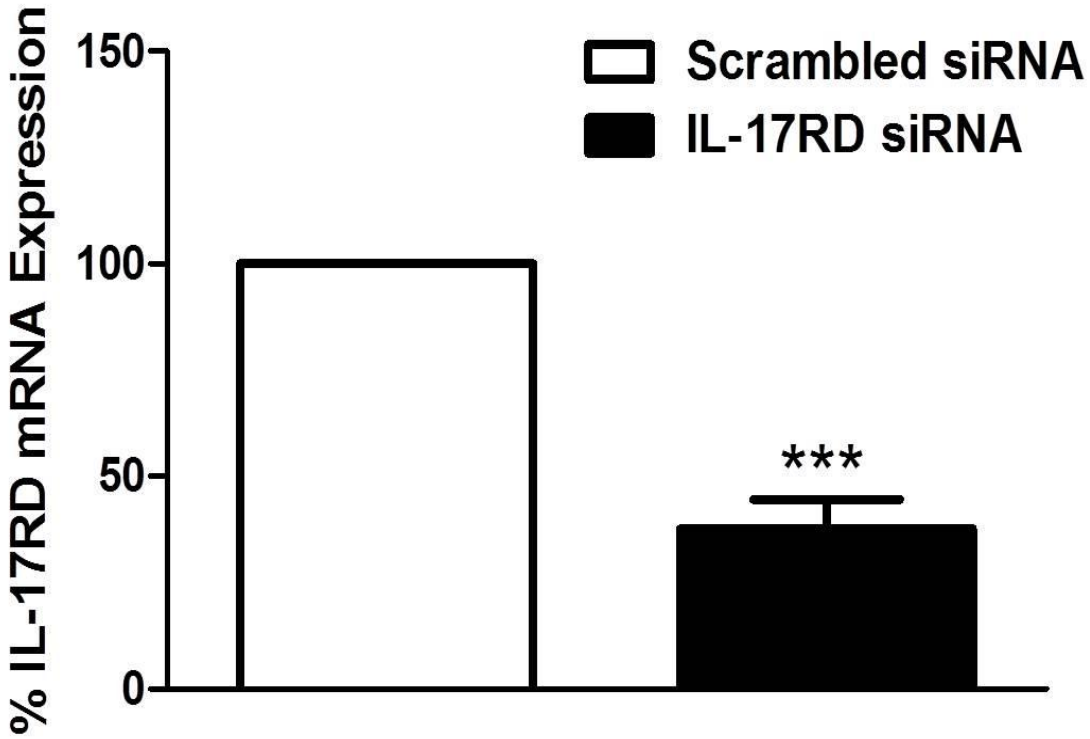

Figure 3.29 IL-17RD-specific siRNA suppresses endogenous IL-17RD expression in HEK293 cells

HEK293 TLR3 cells were transfected with IL-17RD specific siRNA or scrambled siRNA (20 nM). Total RNA was extracted and cDNA was generated using RT-PCR. Samples were subsequently assayed by quantitative real-time PCR for levels of IL-17RD. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Data represent the mean +/- S.E. of three independent experiments.

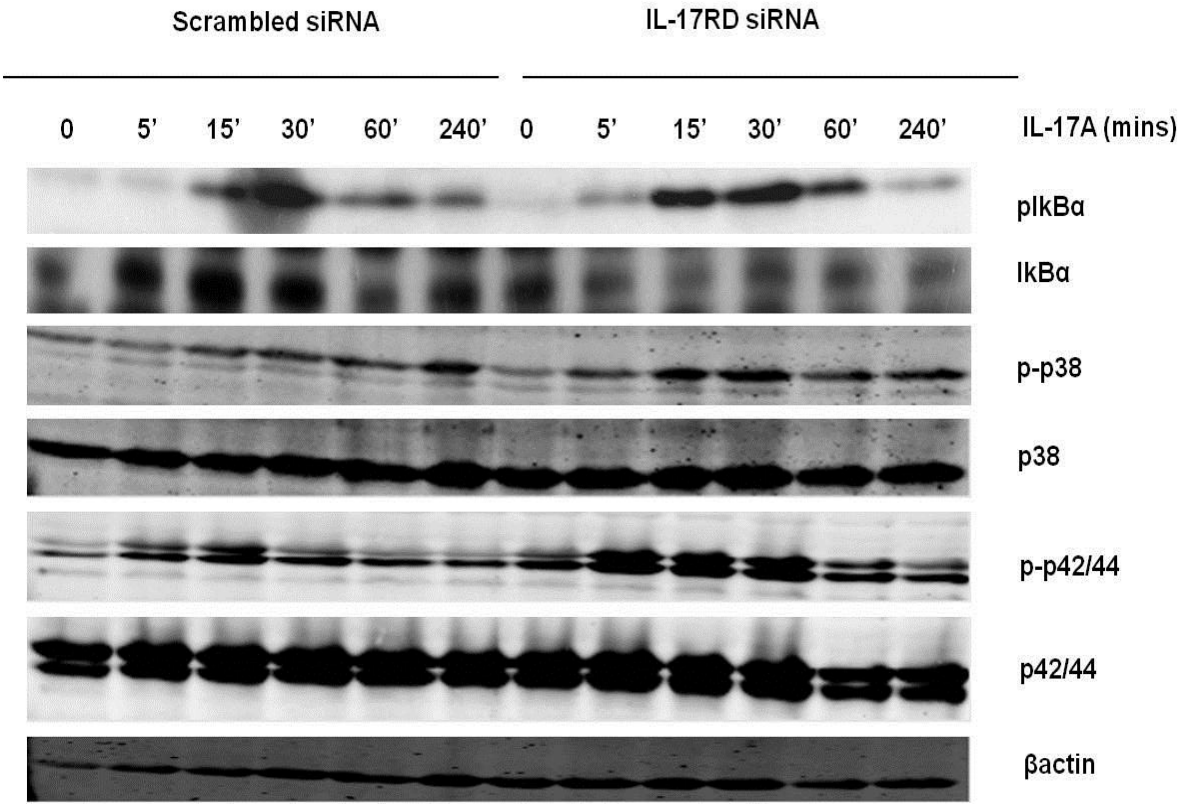

Figure 3.30 Suppression of endogenous IL-17RD expression enhances IL-17A induced signalling

HEK293 TLR4 cells were transfected with IL-17RD specific siRNA or scrambled siRNA (20 nM). Cells were then stimulated with IL-17A (100 ng/ml) for the indicated time periods. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out probing with indicated antibodies. Blots shown are representative of two independent experiments.

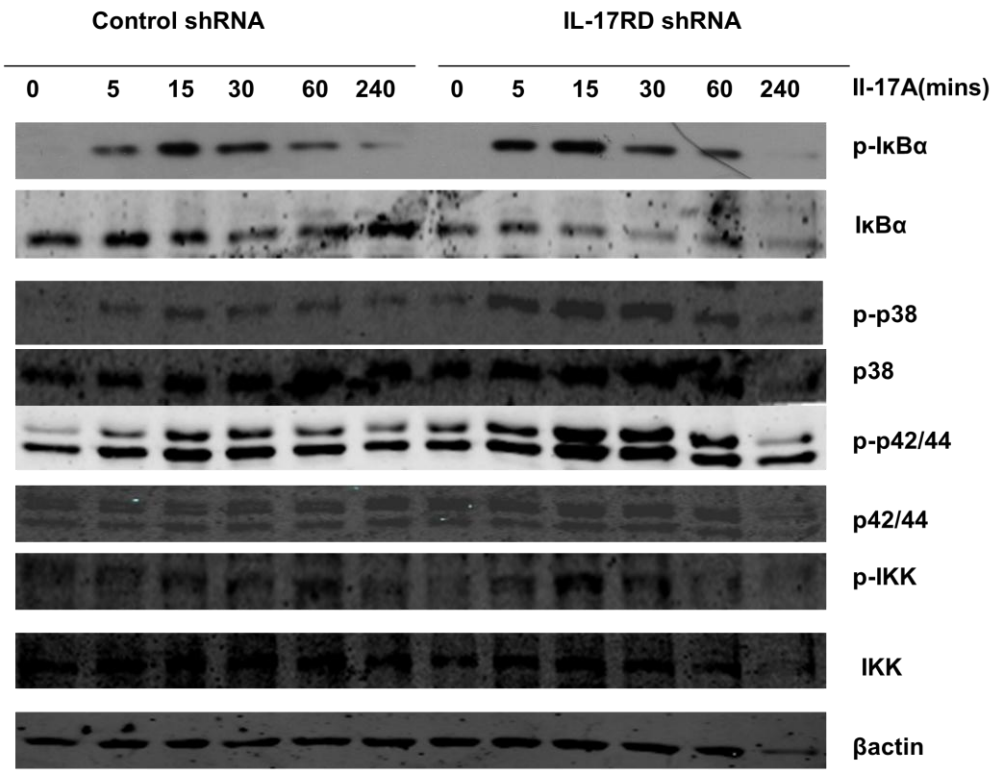

Figure 3.31 IL-17RD shRNA knockdown elevates IL-17A induced signalling

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were then stimulated with IL-17A (100 ng/ml) for the indicated time periods. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out probing with indicated antibodies. Blots shown are representative of two independent experiments.

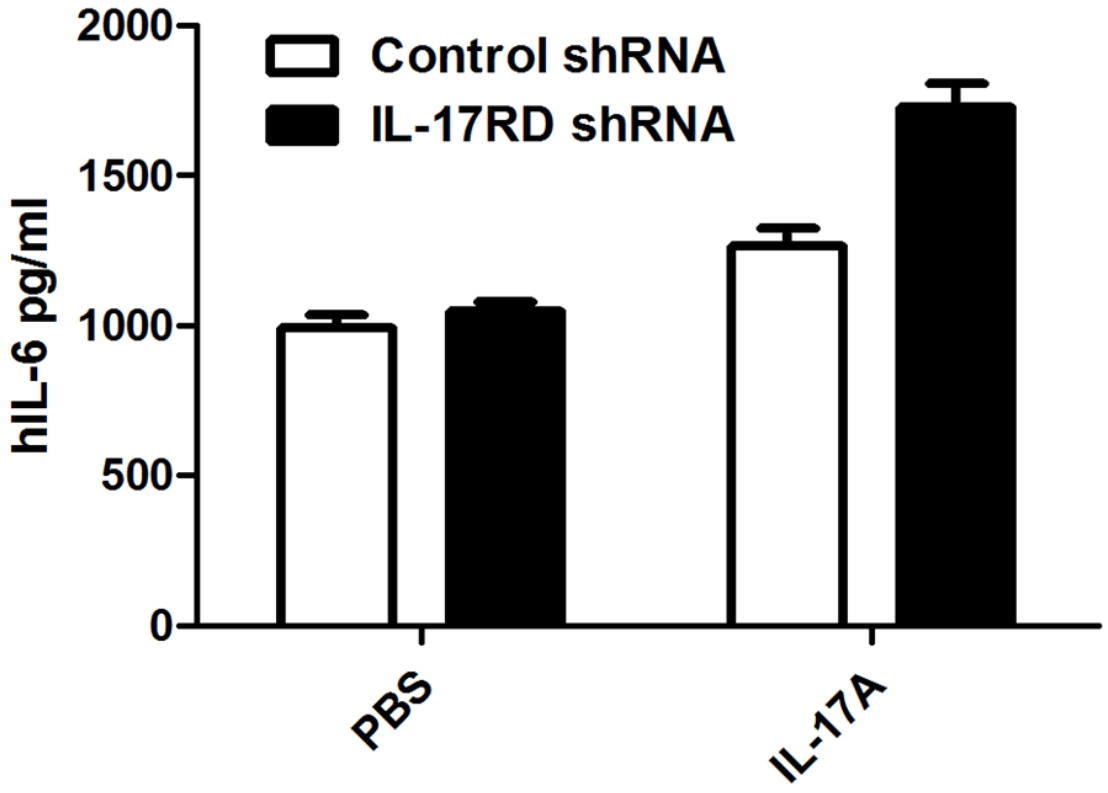

Figure 3.32 IL-17RD knockdown enhances IL-17A induced expression of IL-6

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 96-well plates for 24 h. Cells were treated for 24 h with IL-17A (100ng/ml). Supernatants were obtained and analysed for IL-6 production by ELISA. Result is representative of three independent experiments and the data is mean +/- SD of triplicate determinants.

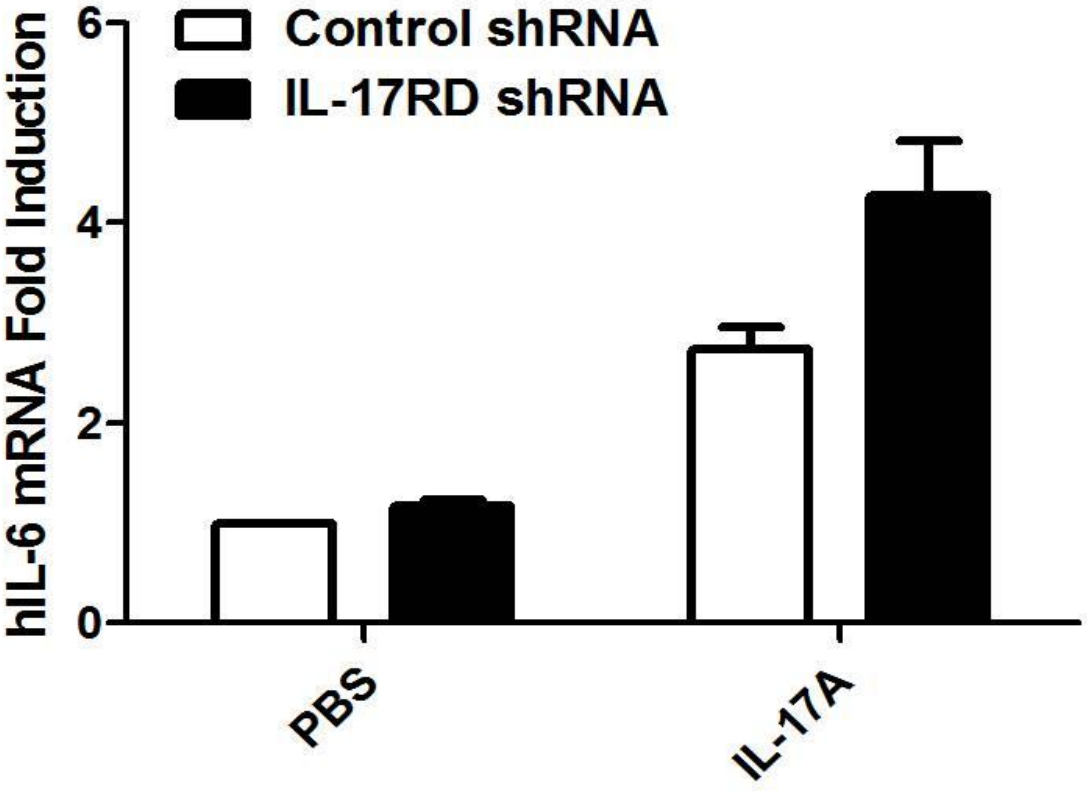

Figure 3.33 IL-17RD knockdown augments IL-17A induced IL-6 mRNA induction

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with IL-17A(100 ng/ml) Total RNA was extracted and cDNA was generated by RT-PCR. Samples were then assayed by real-time PCR for levels of IL-6. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Result is representative of three independent experiments and the data is mean +/- SD of triplicate determinants.

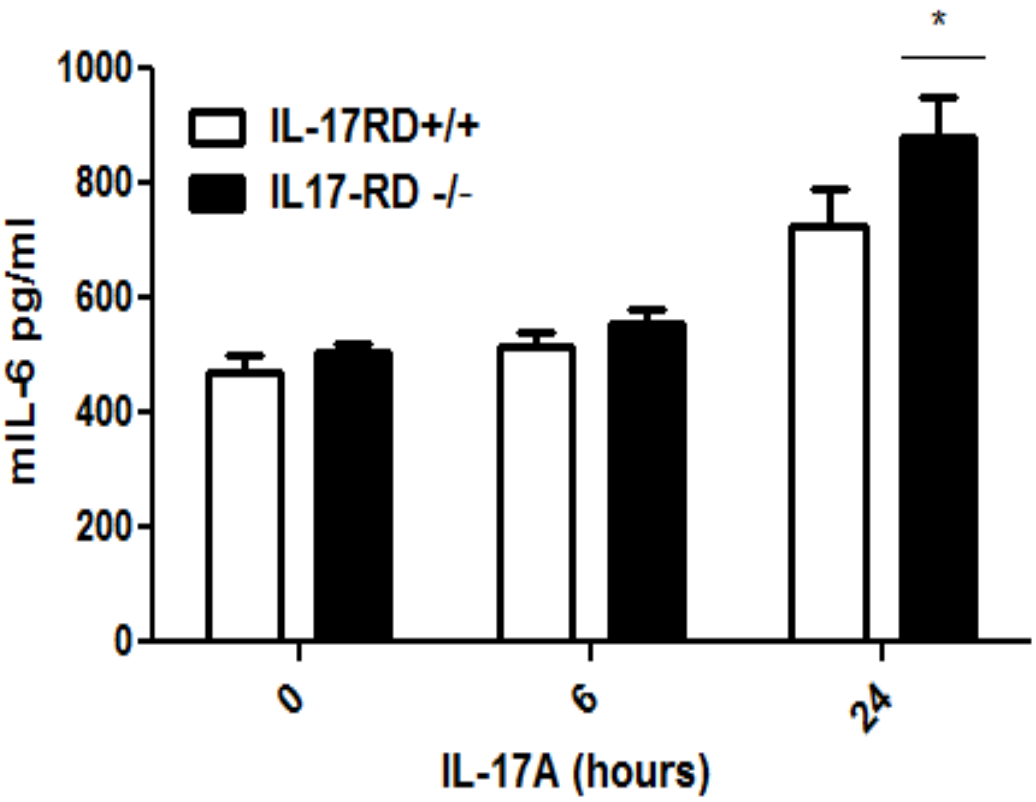

Figure 3.34 IL-17RD deficiency augments IL-17A induced IL-6 production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A as indicated. Cells were treated for various timepoints as indicated. Supernatants were obtained and analysed for IL-6 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. ** $p < 0.05$, paired t-test.

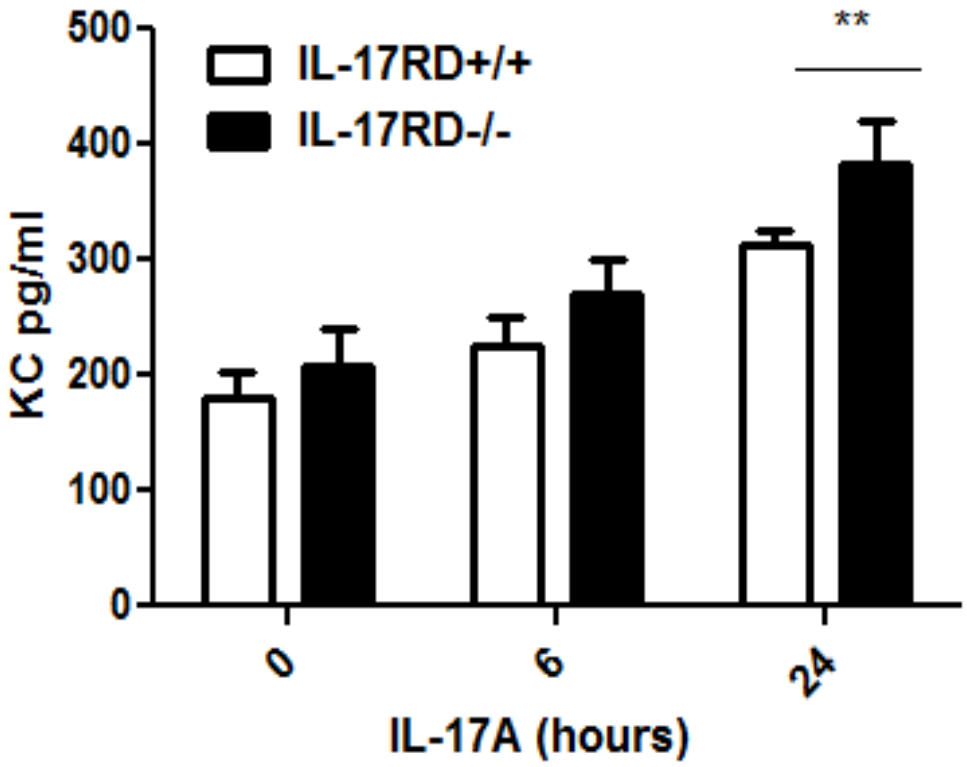

Figure 3.35 IL-17RD deficiency augments IL-17A induced KC production

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A as indicated. Cells were treated for various timepoints as indicated. Supernatants were obtained and analysed for KC production by ELISA. Data represent the mean +/- S.E. of three independent experiments. ** $p < 0.01$, paired t-test.

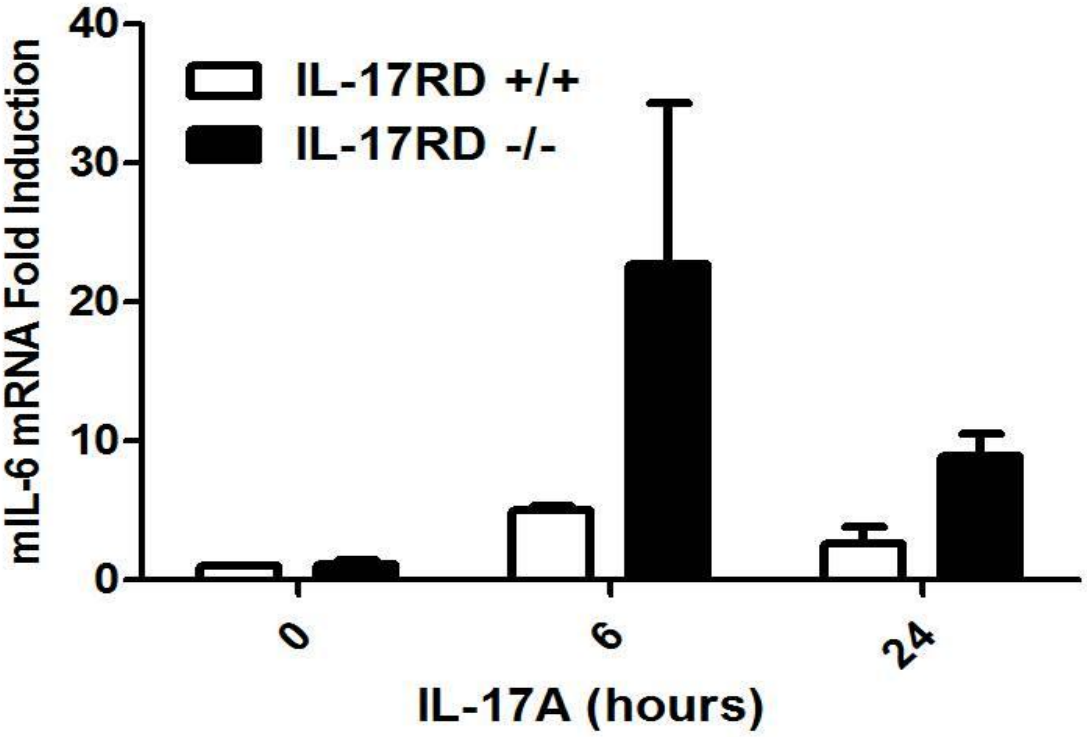

Figure 3.36 IL-17RD deficiency increases IL-17A induced IL-6 mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A as indicated. Cells were treated for the indicated time-points with IL-17A (100 ng/ml) Total RNA was extracted and cDNA was generated by RT-PCR. Samples were then assayed by real-time PCR for levels of IL-6. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Result is representative of three independent experiments and the data is mean +/- SD of triplicate determinants.

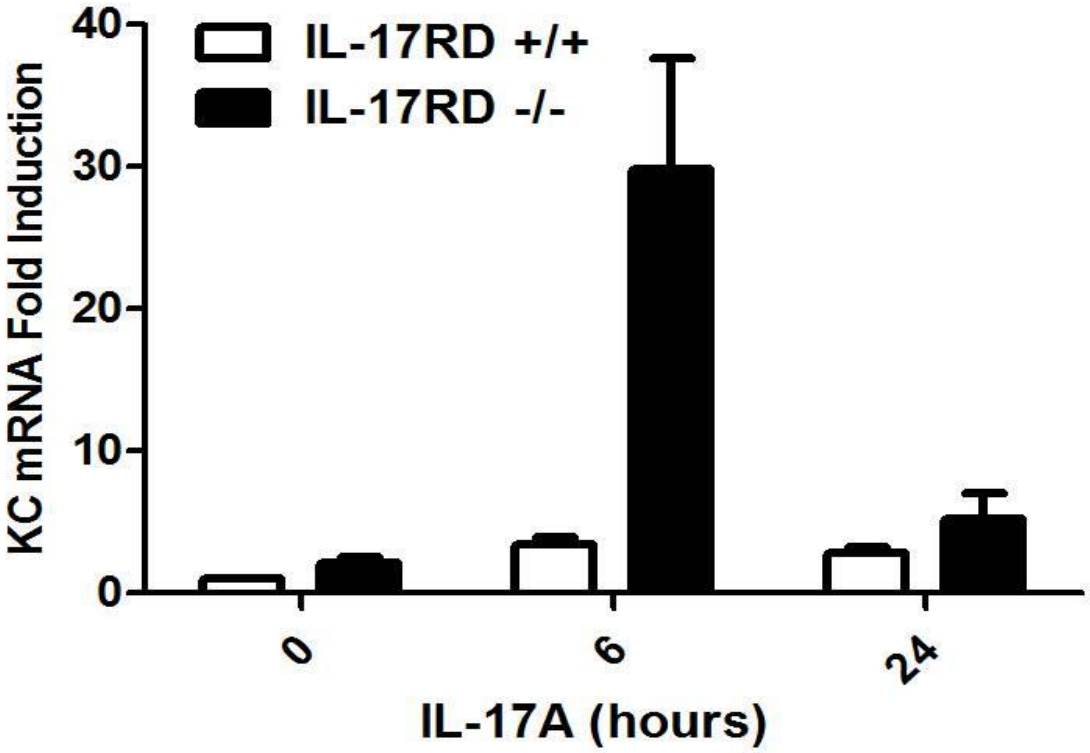

Figure 3.37 IL-17RD deficiency increases IL-17A induced KC mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A as indicated. Cells were treated for the indicated time-points with IL-17A (100 ng/ml) Total RNA was extracted and cDNA was generated by RT-PCR. Samples were then assayed by real-time PCR for levels of KC. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Result is representative of three independent experiments and the data is mean +/- SD of triplicate determinants.

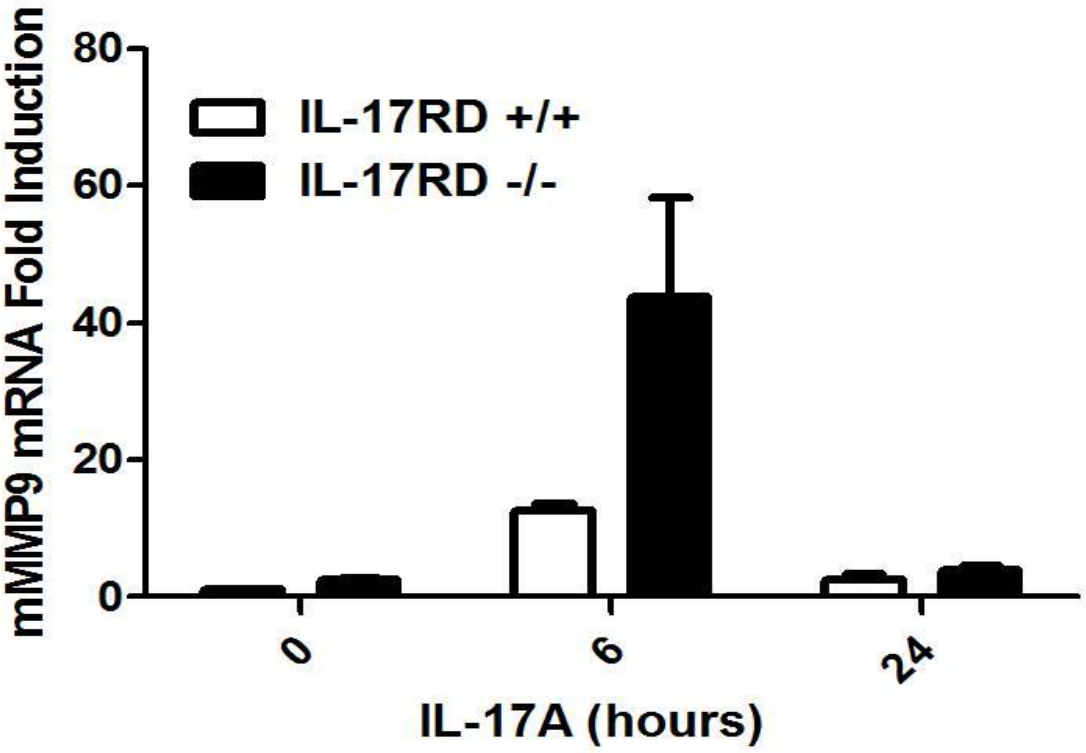

Figure 3.38 IL-17RD deficiency increases IL-17A induced mMMP9 mRNA

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A as indicated. Cells were treated for the indicated time-points with IL-17A (100 ng/ml) Total RNA was extracted and cDNA was generated by RT-PCR. Samples were then assayed by real-time PCR for levels of mMMP9. Gene expression was calculated relative to the housekeeping gene HPRT and analysis was performed using the $2^{-\Delta\Delta CT}$ method. Result is representative of three independent experiments and the data is mean +/- SD of triplicate determinants.

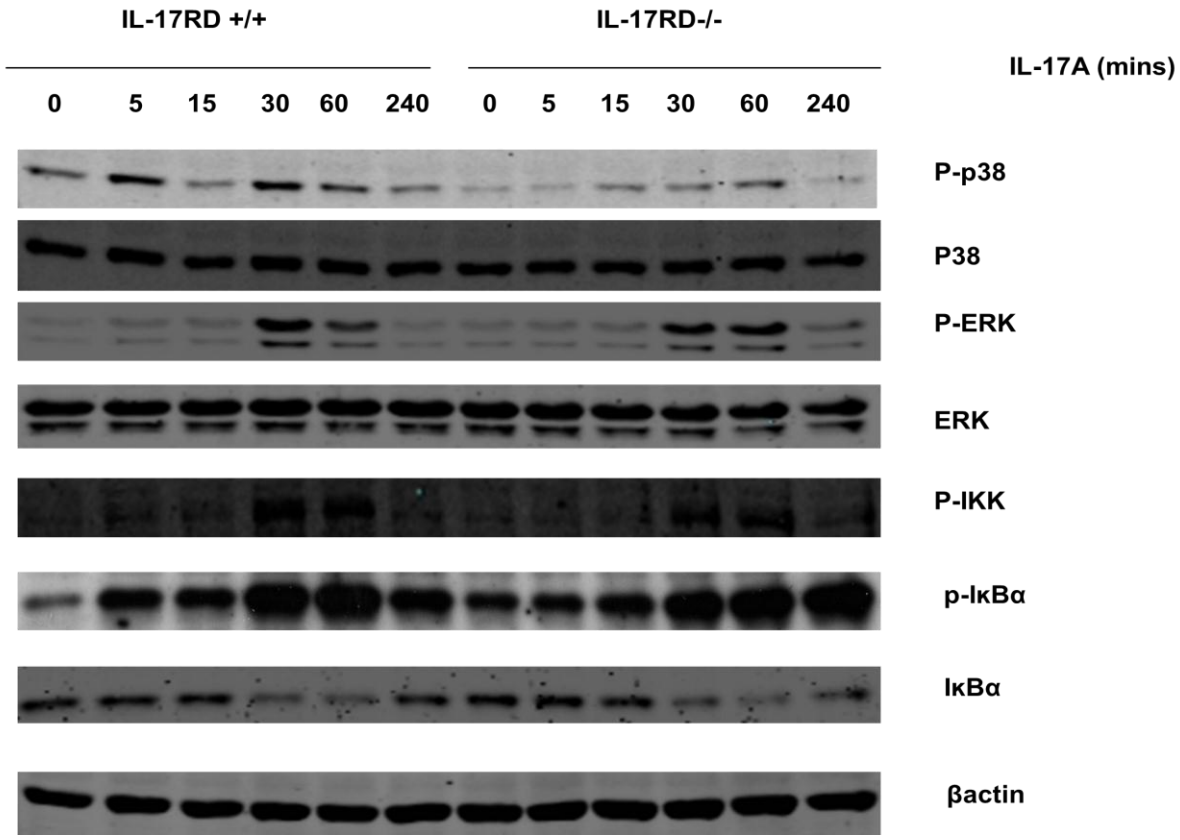

Figure 3.39 IL-17RD differentially regulates IL-17A-induced NF-κB and MAPK activation

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A (100 ng/ml) as indicated. Cells extracts were generated and lysates were resolved by SDS-PAGE. Western blotting was carried out probing with indicated. Blots shown are representative of three independent experiments.

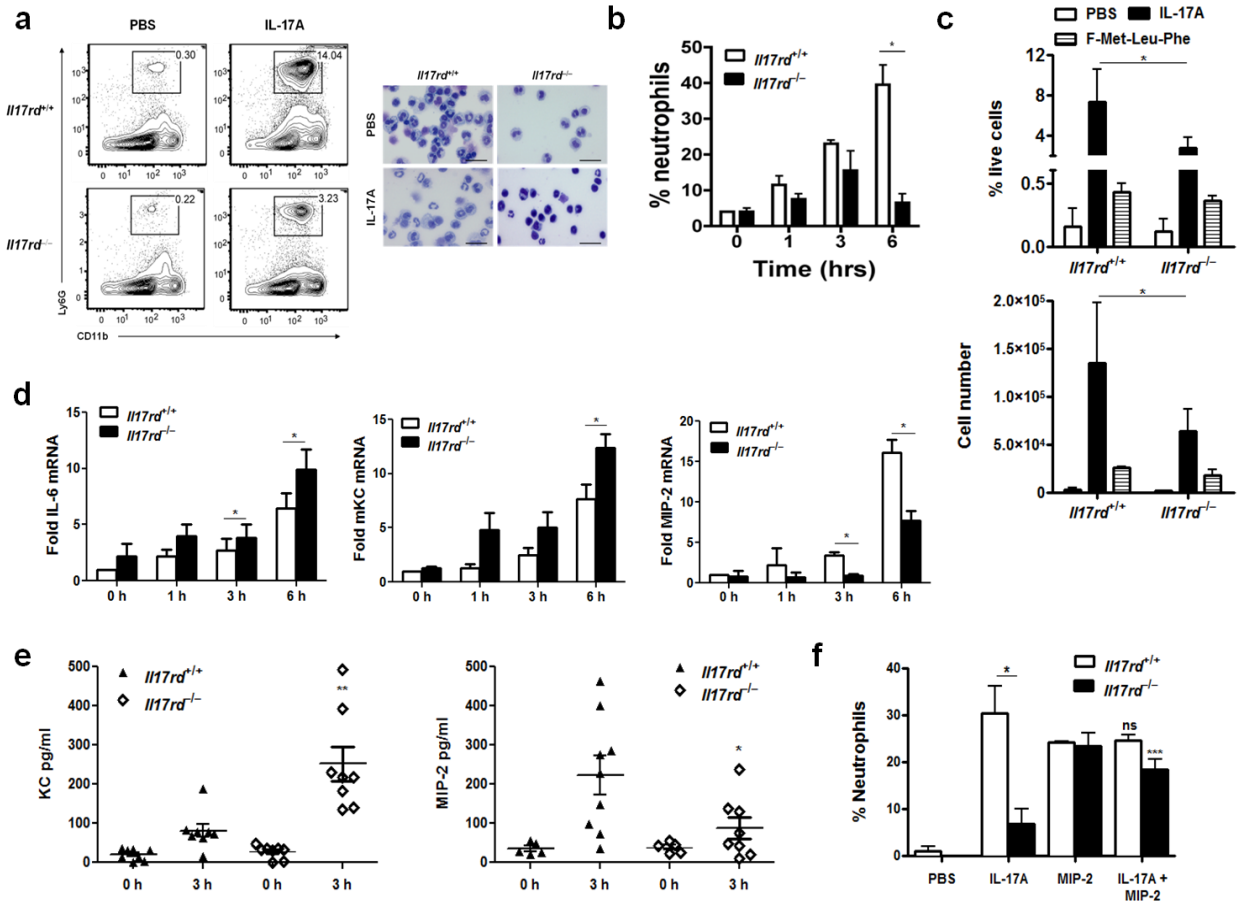

Figure 3.40 IL-17RD deficiency impairs neutrophil recruitment to the peritoneum.(**a, b**) IL-17A (0.5 µg/mouse) or PBS was administered by intraperitoneal injection into IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice. (**a**) Representative flow cytometry plots of neutrophil infiltration, using CD11b and Ly6G expression as markers, 3 h after PBS or IL-17A injection (*left panel*). Wright's staining of infiltrate from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice (*right panel*). (Scale bar, 10 µm). (**b**) Percentages of neutrophils in peritoneal lavages from mice treated with IL-17A as indicated. (**c**) IL-17A (0.5 µg/mouse), formyl-Met-Leu-Phe (1 µg/mouse) or PBS was injected into the peritoneum of IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice. Data indicates percentage (*upper panel*) and absolute numbers (*lower panel*) of neutrophils. (**d**) IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were injected i.p with IL-17A (0.5 µg/mouse). Peritoneal cells were collected from mice treated with IL-17A as indicated. IL-6, KC and MIP-2 mRNA levels were evaluated by qPCR. (**e**) ELISA of MIP-2 and KC levels from peritoneal lavages from mice treated with IL-17A as indicated. (**f**) Percentages of neutrophils in peritoneal lavages from mice treated with IL-17A or PBS for 1 h prior to intraperitoneal injection of murine recombinant MIP-2 (30 ng) for 2 h. Results are mean +/- SEM (n= 4-7 mice per group) and data are representative of three individual experiments (total n= 12-21) (**a-c**), represent mean +/- SEM of three independent experiments (total n=8-9) (**d-e**) or are mean +/- SEM (total n= 4-9) (**f**). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (Student's *t* test) (Mellett *et al.* 2012).

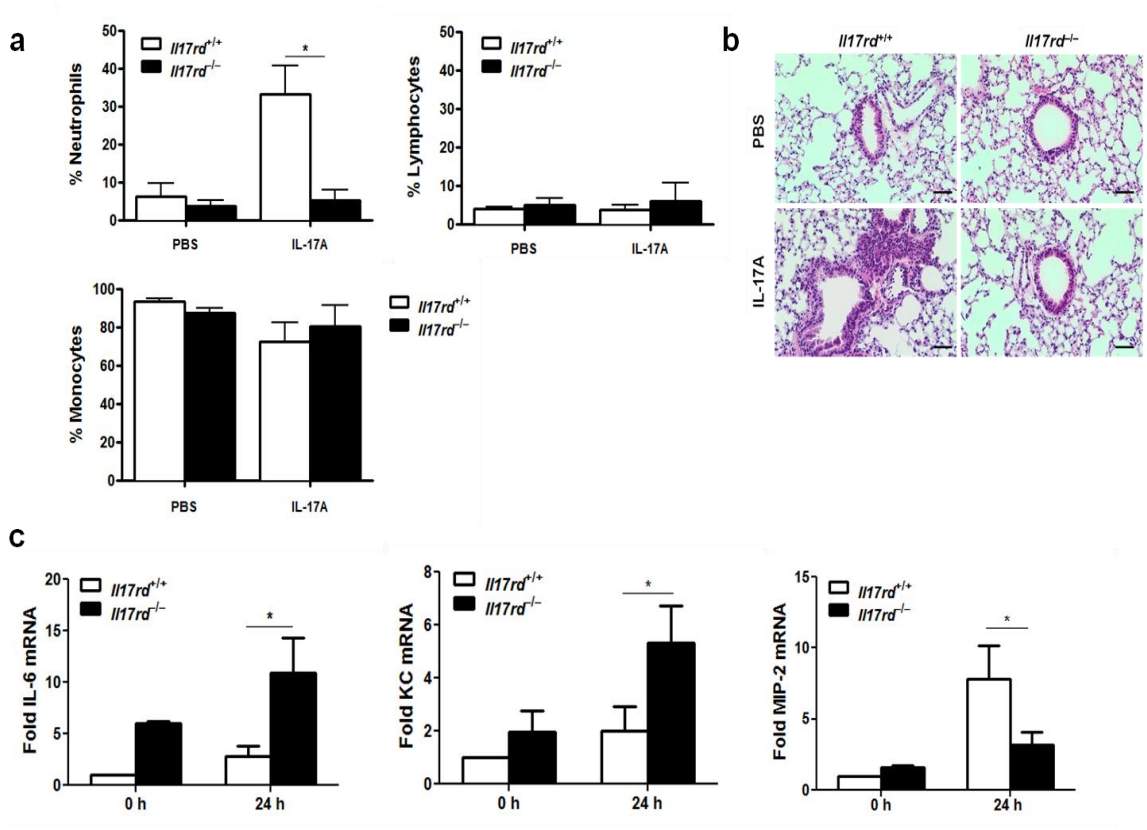

Figure 3.41 IL-17RD regulates IL-17A-induced neutrophil infiltration in lung tissue.

(**a, b**) IL-17A (0.5 µg/mouse) was introduced intra-nasally into IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice. (**a**) Differential cell count data from cytospin slides depicting percentages of neutrophils, lymphocytes and monocytes in BAL at 4 h after IL-17A treatment. (**b**) Representative images of hematoxylin and eosin stained slides from lungs taken from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice after IL-17A treatment. (Scale bar, 50 µm). (**c**) IL-17A (0.5 µg) was introduced intra-nasally for 24 hours and lung tissue was retained to measure mRNA levels of IL-6, KC and MIP-2 by qPCR. Results are mean +/- SEM (n= 3-4 mice per group) and data are representative of three independent experiments (total n= 9-12) (**a, b**) or are mean +/- SEM (n= 4-6 mice per group) (**c**). * $p<0.05$; ** $p<0.01$; ***$p<0.001$ (Student's *t* test). (Mellett *et al*. 2012).

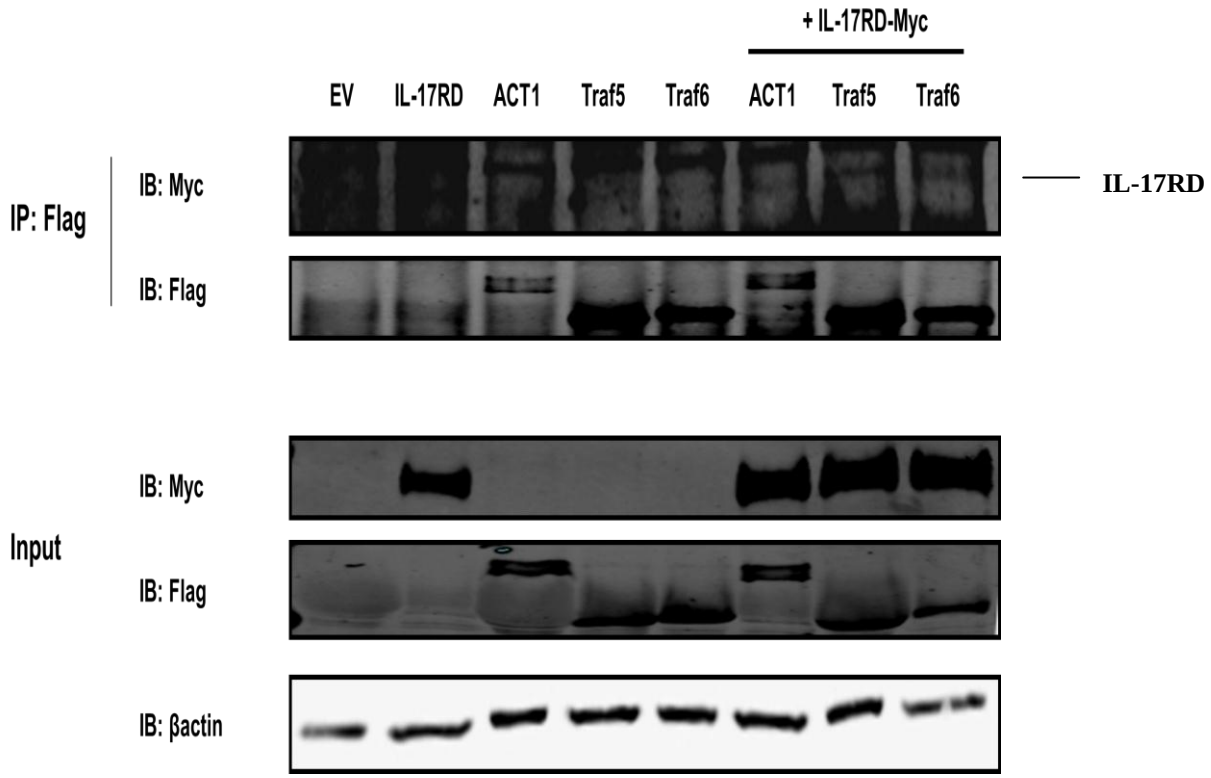

Figure 3.42 IL-17RD interacts with key IL-17A signalling components

HEK293 TLR4 cells were co-transfected with plasmids encoding myc-tagged IL-17RD (2 μg) and ACT1-Flag (2 μg) or TRAF5-Flag (2 μg) or TRAF6-Flag (2 μg). Empty vector pcDNA3.1 was used to normalise the total amount of DNA transfected. 24 h post transfection lysates were generated and immunoprecipitated with an anti-Flag antibody. Immunoprecipitates and whole cell lysates (WCLs) were subjected to Western immunoblotting using anti-myc and anti-Flag antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

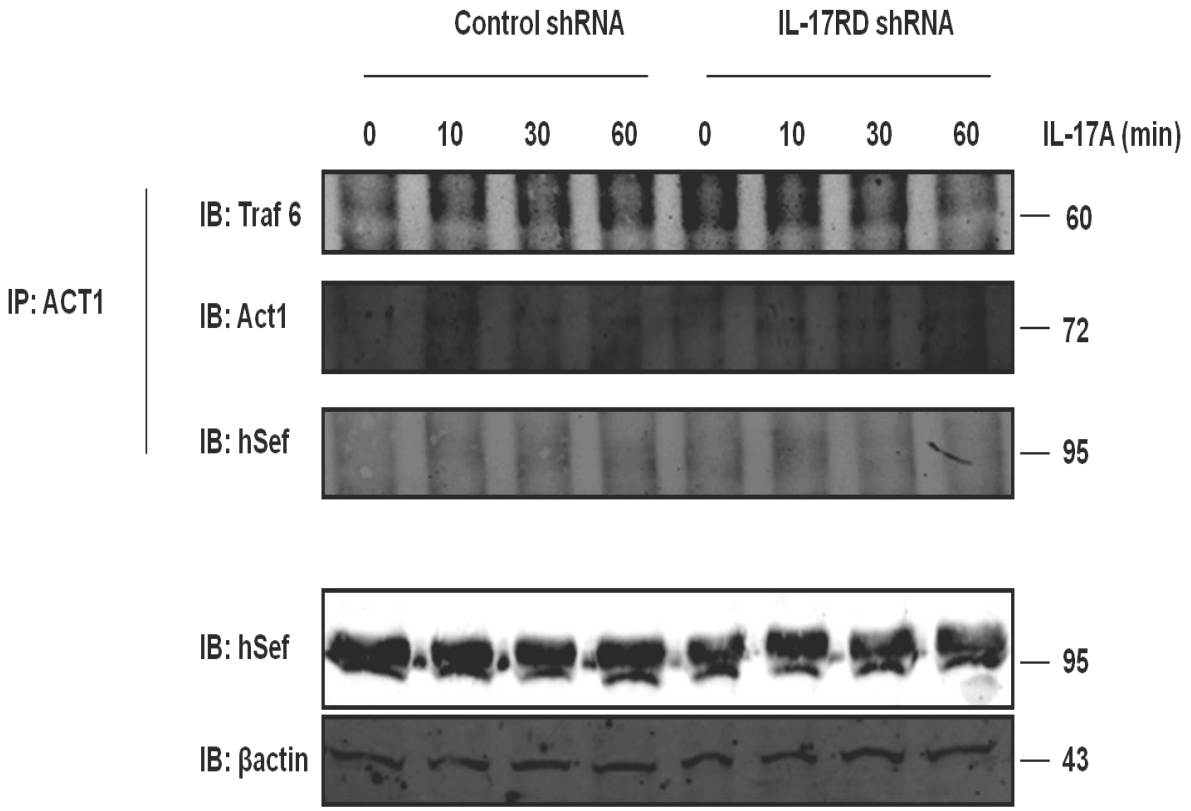

Figure 3.43 IL-17RD disrupts the interaction between TRAF6 and ACT1

U373 cells stably transduced with Control or IL-17RD-specific shRNA were grown in 6-well plates for 24 h. Cells were treated for the indicated time-points with IL-17A (100 ng/ml). Lysates were generated and immunoprecipitated with an anti-Flag antibody. Immunoprecipitates and whole cell lysates (WCLs) were subjected to Western immunoblotting using anti-myc and anti-Flag antibodies. Immunoreactivity was visualised using the Odyssey Infrared imaging system. Results shown are representative of three independent experiments.

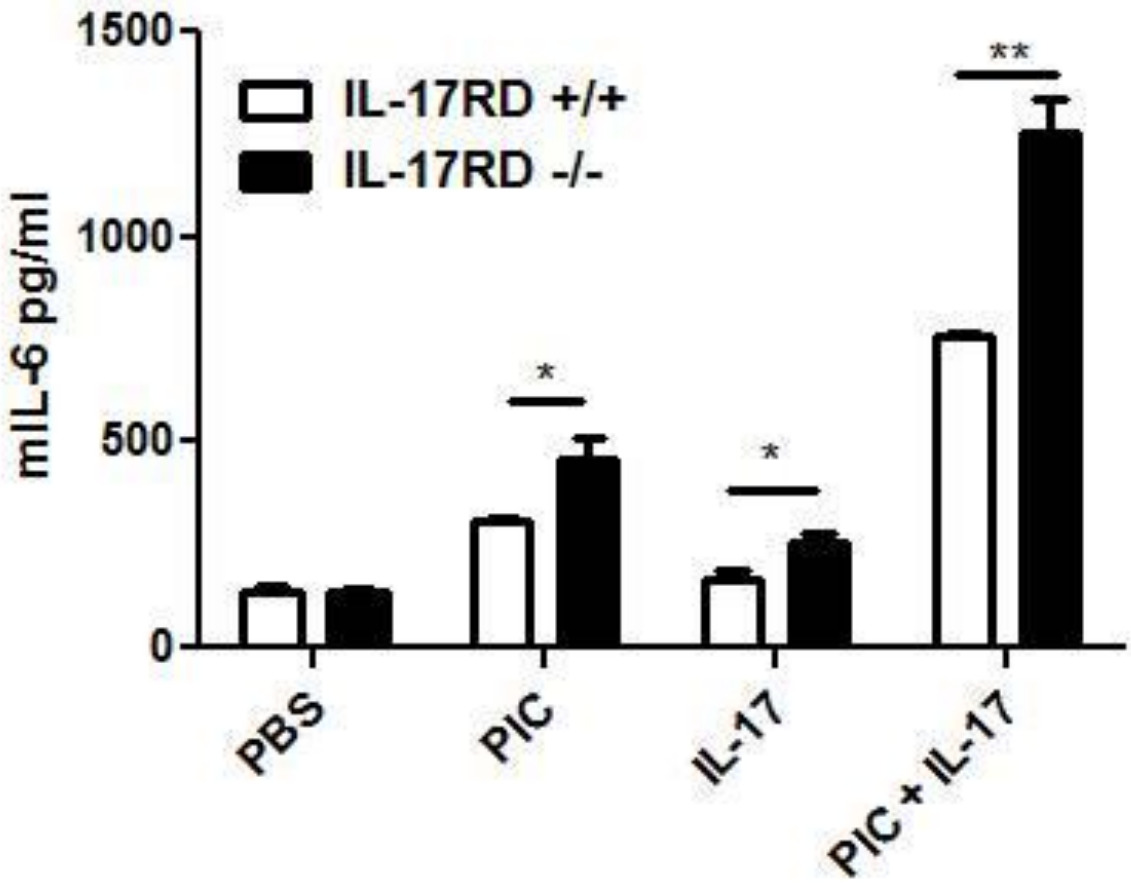

Figure 3.44 IL-17RD deficiency results in enhanced IL-17 and Poly I:C synergism

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A (100 ng/ml) and/or Poly I:C (25 μg/ml) as indicated. Supernatants were obtained and analysed for IL-6 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test.

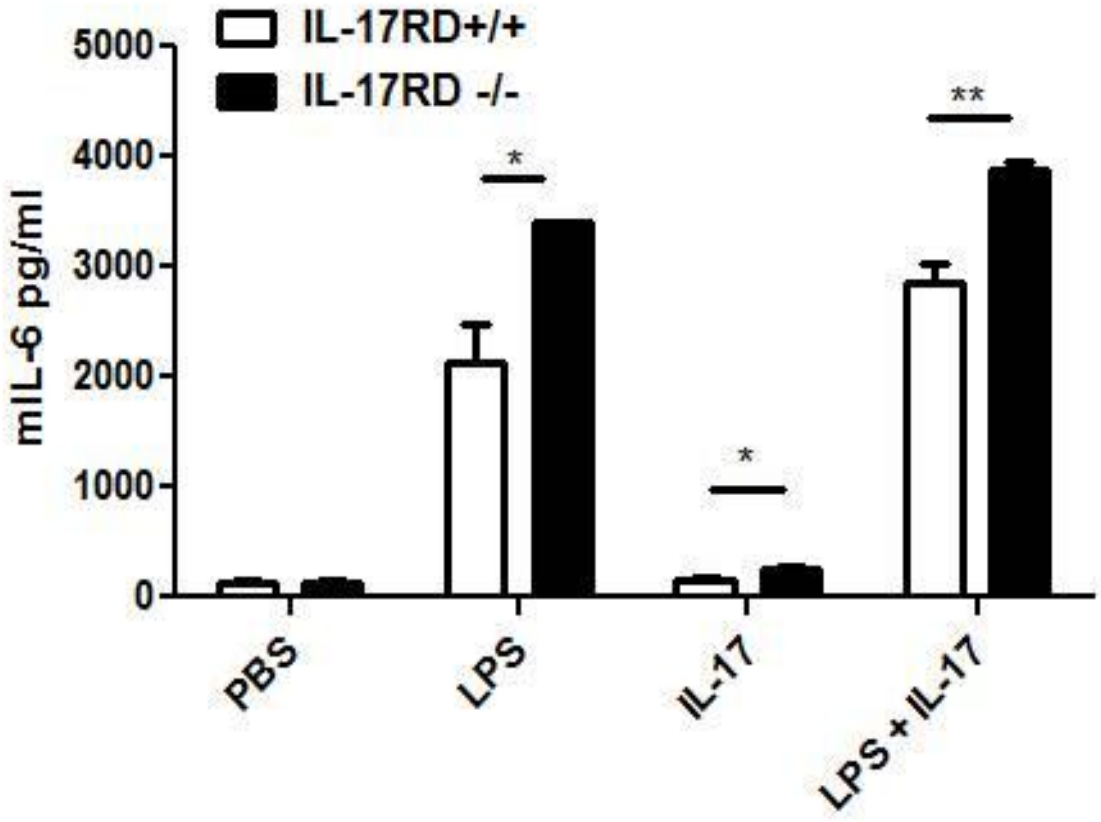

Figure 3.45 IL-17RD deficiency results in enhanced IL-17 and LPS synergism

Primary BMDMs from IL-17RD$^{+/+}$ and IL-17RD$^{-/-}$ mice were stimulated with IL-17A (100 ng/ml) and/or LPS (100 ng/ml) as indicated. Supernatants were obtained and analysed for IL-6 production by ELISA. Data represent the mean +/- S.E. of three independent experiments. * $p < 0.05$, paired t-test.

Chapter 4

Discussion

4.1 Discussion

The initial aim of this body of work was to test whether IL-17RD could affect innate immune signalling pathways as a result of its SEFIR domain which shares structural similarity to the TIR domain of TLR signalling. Most TIR domain containing adaptor proteins induce pro-inflammatory signals by activating NF-κB. Some adaptor proteins including MyD88 and TRIF also have the capacity to induce IRF signalling. On the other hand, several TIR domain containing proteins are inhibitors of immune signalling. ST2, a TIR domain containing protein, can interact with MyD88 and Mal to inhibit TLR2, TLR4 and TLR9 signalling (Brint *et al*., 2004) while SIGGIR obstructs TLR4 and TLR9 and IL-1 signalling (Wald *et al*., 2003).

We began by looking at the effect of IL-17RD on pathways regulated by TLRs. Unlike SARM, for example, IL-17RD was demonstrated to inhibit each of the adaptor proteins from activating NF-κB. IL-17RD inhibited TLR3 and TLR4 ligands from activating NF-κB. In terms of TLR signalling, IL-17RD also inhibited Flagellin, Zymosan and Pam3Csk4 mediated activation of NF-κB. The inhibition of downstream signalling proteins such as TRAF6, IκB and p65 indicates that IL-17RD could target multiple components. This might explain why IL-17RD has the ability to interfere with non-TLR pathways. IL-17 and RIG-I share common components with the TLR signalling pathways such as TRAF6 and IKKs, all of which are vulnerable to the regulatory effects of IL-17RD. As well as targeting NF-κB signalling, IL-17RD disrupts activation of the IRFs by TRIF. The IRFs play a fundamental role in Type I interferon activation.

The subcellular location of IL-17RD may play an important role in its regulatory effects. Several reports have demonstrated that IL-17RD is dispersed throughout the cell. It translocates to the Golgi in the spatial regulation of ERK signalling (Torii *et al* 2004). It's been demonstrated to be primarily localised to the cytoplasm and to intracellular vesicles but relocates to the plasma membrane upon FGF treatment (Ren *et al*. 2006). Due to Ras signalling consisting of endocytotic recycling, it was proposed that IL-17RD targeted Ras signalling at both the plasma membrane and the endocytic vesicles (Roy *et al*. 2002). Crucially, IL-17RD's distribution at the plasma membrane and the endosomal vesicles is consistent with the localisation of the TLRs. Recently, endosomal IL-17RD has been shown

to inhibit IL-1 and TNF signalling (Fuchs *et al*. 2012). In IL-17RD expressing cells, both IκBα and IL-17RD regulate cytoplasmic sequestration of NF-κB. Upon IL-1 stimulation, IκB is swiftly degraded while IL-17RD remains bound to large amounts of NF-κB and thereby impedes NF-κB nuclear translocation. IκB and IL-17RD, both targets of NF-κB, function cooperatively to terminate the NF-κB response. Nascent IκBα enters the nucleus and exports NF-κB to the cytoplasm while newly synthesized IL-17RD sequesters the exported IκBα/NF-κB complex (Fuchs *et al*. 2012).

Previous studies in the laboratory consisted of overexpression of IL-17RD and analysis of its functional effects. However, overexpression of signalling proteins generates the potential for artefacts. Consequently, siRNA and shRNA approaches were taken to specifically look at the functional consequences of knocking down endogenous IL-17RD. The results in this body of work, generated with siRNA and shRNA technology, confirmed what was previously demonstrated in over expression studies. NF-κB activation was enhanced in knockdown cells. The physiological relevance of IL-17RD of the inhibitory effects of IL-17RD was also shown by the generation of IL-17RD deficient mice. In the absence of normal endogenous IL-17RD, cytokine induction was enhanced in response to IL-17A and TLR ligands. IL-17RD dampens down the inflammatory response by decreasing the production of chemokines such as IL-8 (and its murine homolog, KC), RANTES and IL-6. These findings strongly indicate that IL-17RD is an important endogenous regulator of inflammatory signals. The reason why an FGF antagonist has such an impact on innate immune signalling pathways is unclear. IL-17RD is important in regulating early developmental signalling during embryogenesis and also been linked with nervous system development (Tsang *et al*. 2002; Abraira *et al*. 2007). Interestingly, TLRs are intricately involved in early development; in fact, Drosophila Toll was initially demonstrated to be responsible for dorsoventral patterning in developing larvae (Anderson *et al*. 1985). As a result, IL-17RD may have evolved along with the TLRs to become an immune signalling regulator.

Mutational analysis was carried out to probe a possible mechanism by which IL-17RD exerts its inhibitory effects on TLR mediated NF-κB and IRF activation. The TIR domain has been shown to be an important mediator of IL-17RD's inhibitory effects given how it seems to be sufficient to maintain the level of inhibition achieved by the full length IL-17RD. In addition, the interaction between IL-17RD and TRAF6 is maintained when everything except the SEFIR domain is lost implying that once again, the SEFIR domain may

be sufficient to enable TRAF6 interact with IL-17RD. However, while the SEFIR domain of IL-17RD is important for the function of IL-17RD in TLR signalling, IL-17RD is also able to affect non-TLR pathways. Possession of both TRAF6 and TRAF5 binding site may confer on IL-17RD the ability to disrupt pathways that encompass these downstream effector proteins.

Based on structural similarity the IL-17 receptor family is constituted by 5 members (Gaffen 2009). To date, direct functional data has demonstrated a role for only 4 of the IL-17 receptors (A, B, C and E) in IL-17 signalling. Despite sharing structural features with these other family members, IL-17RD remains an orphan receptor with no available data indicating a physiologically relevant role for IL-17RD in IL-17 biology. We now report for the first time, a regulatory role for IL-17RD in IL-17A-induced neutrophilia and activation of MAPK and NF-κB signalling pathways.

Previous findings have demonstrated that IL-17RA, -B, -C and –E, but not IL-17RD, are upregulated upon IL-17A stimulation (Zhang *et al* 2010). Instead basal IL-17RD expression is relatively high compared with IL-17RA and this is consistent with our proposed role for the orphan receptor acting as a basal "braking system" that needs to be relieved by IL-17A stimulation in order to manifest activation of NF-κB and induction of pro-inflammatory cytokines and chemokines. Importantly, IL-17RD appears to act to differentially regulate various IL-17-responsive genes. Whilst IL-17RD negatively regulates IL-17-induced expression of pro-inflammatory genes such as IL-6 and KC, it also fulfills a key mediatory role in facilitating IL-17-induced expression of other pro-inflammatory proteins like MIP-2. Indeed in an inflammatory context, the latter positive role of IL-17RD in IL-17 signalling, appears to be the dominant one at least in the context of IL-17A-induced neutrophilia, since in the present study IL-17RD-deficient mice show greatly diminished neutrophil recruitment in tissues challenged with IL-17A. We also provide a mechanistic basis to the differential effects of IL-17RD on IL-17-responsive genes. We propose that IL-17RD negatively regulates NF-κB by sequestering Act1 and preventing its interaction with IL-17RA and TRAF6 thus inhibiting ubiquitination of the latter and downstream activation of NF-κB and induction of genes such as IL-6 and KC. Intriguingly, IL-17RD also appears to have a contrasting mediatory role in IL-17A-induced phosphorylation of p38 MAPK and induction of MIP-2. The latter is especially important in terms of a critical physiological role for IL-17A in mediating tissue neutrophilia. Such findings also emphasize the complexity of the IL-17A signalling pathway. To date IL-17 signal transduction has been divided into TRAF6-dependent and TRAF6-independent pathways, the former assumed to trigger

activation of NF-κB and MAPKs while the latter regulates mRNA stability. However, the present study clearly shows that IL-17RD can differentially regulate both the NF-κB and p38 MAPK pathways.

In addition to these findings, data which could prove particularly therapeutically informative are results which demonstrated that loss of IL-17RD leads to synergistic augmentation of pro-inflammatory cytokines in response to TLR ligands and IL-17. This may be physiologically relevant given the role of IL-17, PAMPs and DAMPs in a variety of chronic inflammatory diseases, most notably Rheumatoid Arthritis. IL-17, for example, has been shown to enhance the production of inflammation inducing cytokines in synovial fluid as well as the destruction of cartilage tissue (Kuligowska and Odrowaz-Sypniewska 2004). A mechanism, by which attenuation of synergistic induction of these cytokines by DAMPs and IL-17, could prove particularly useful as a potential target for therapies.

The effect of IL-17RD on RIG-I signaling proved intriguing in that it differentially regulated the activation of NF-κB and IRF3. While elevated levels of IL-6 were demonstrated in the absence of IL-17RD in response to SeV infection, Type I interferon production was diminished. This identifies a possibly divergence in mechanism between the two arms of signaling and could prove useful in the delineation of the mechanism of IL-17RD function in other pathways. Common elements in the TLR activation of NF-κB and IRF pathways which are targeted by IL-17RD may be absent or have an opposite function in the IRF wing of RIG-I signaling which would account for the differential regulation.

IL-17RD is a multifunctional protein with a large variety of roles in negatively regulating growth factor signaling, in early development, in cell cycling and also, now, in the immune system. Although IL-17RD has an important role in FGF signaling, particularly in early development, IL-17RD deficient mice proved to be healthy and viable with no obvious clinical features (Lin *et al*. 2005). As a result, these mice proved to be an essential tool in the study of IL-17RD functioning in innate immune signaling pathways in order to confirm the physiologically relevant nature of the knockdown and overexpression studies. This is despite the fact that it has been reported that IL-17RD deficiency in humans has been implicated in a number of cancers (Zisman-Rozen *et al*. 2007). IL-17RD was highly expressed in normal epithelial cells but its expression was abrogated in the equivalent carcinomas. As of yet, there has been no signs of abnormal tumour growth in the IL-17RD$^{-/-}$ mice (Mellett *et al*. 2012). Of interest to the immune functions of IL-17RD are the elevated expression levels of IL-17RD in

epithelial cells. Epithelial cells have the ability to show tolerance to foreign microbes and IL-17RD could reasonably have a function in this process.

Given the versatile nature of IL-17RD and the fact that it impacts upon a variety of pathways, this may make IL-17RD a viable candidate as a potential therapeutic target. What could prove most insightful is discovering the ligand for IL-17RD which may potentially have a therapeutic application in limiting tumour formation and also be of value therapeutically in staving off unwanted immune mediated disease. The use of agonistic or antagonistic antibodies for IL-17RD could be one way in which the therapeutic value of IL-17RD could be exploited. For example, there has been much work into the use of anti-TNF antibodies in autoimmune disease particularly in rheumatoid arthritis (Feldmann and Maini 2003). In fact, by the end of the last decade, anti-TNF therapies were worth upwards of $22 billion (Pappas *et al*. 2009). There are a host of chronic inflammatory disorders which could be tackled by curbing dysregulated inflammatory cytokine induction and knowledge of how the inflammatory signaling is regulated could lead to treatments for the millions of sufferers. Characterising negative regulators like IL-17RD could provide novel treatment targets or at least inform us in more detail of how the inflammatory response operates on a basic level.

As more information is reported, it is becoming apparent that there is a network of crosstalk between inflammatory pathways. Originally, studies of these pathways focused on identifying the components of these pathways which led to transcription factor activation. More recently there has been an increasing focus on negative regulators of these pathways since this allows for homeostasis in innate immune signalling (Germain 2012). These regulators, rather than having an all or nothing effect, in fact function together at different strata in the signalling cascades to tweak the inflammatory response. These diverse, stratified mechanisms include anatomical barriers like the skin, innate wound responses like anti-thrombin III, PAMP sequestration mediators like soluble TLR2, PAMP signalling inhibitors like A20 and transcription factors such as Bcl-6 (Murray and Smale 2012). This thesis, by characterizing the physiological relevance of IL-17RD in innate immune signaling pathways, proposes that we now add IL-17RD to the growing list of regulators which function to keep the immune system in check and advances it as potential target for immune mediated disease therapies.

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