

The role of CYLD in macrophages



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Abstract

Macrophages are essential components of our innate immune system involved in several biological processes, including sensing and clearance of invading pathogens. IFN- γ is a required cytokine to promote macrophage activation. The regulation of this signalling pathway is therefore crucial for successful pathogen clearance and for the engagement of the adaptive immunity.

The aim of this study was to investigate the role of the deubiquitinase CYLD in macrophage activation. CYLD is a negative regulator of the NF- κ B signalling pathway. Consistent with this, CYLD-deficient mice present higher production of pro-inflammatory cytokines and increased clearance when challenged with pathogens. However, the molecular mechanisms and the role of macrophages underlying this protective process have not yet been investigated.

I identified CYLD to regulate and be regulated by the IFN- γ signalling pathway. CYLD regulation of the IFN- γ signalling pathway occurs through its deubiquitinase activity. In line with this, CYLD-depleted macrophages present an enhanced IFN- γ transcriptional response, with upregulation of genes involved in macroautophagy and xenophagy, such as NOD2, RIPK2 and OPTN. Furthermore, these cells display an increased phagocytic and lysosomal activity, as well as higher autophagic flux. Deregulation of the IFN- γ signalling pathway leads to an enhanced ability to restrict intracellular gram-positive and gram-negative bacteria growth in CYLD-depleted macrophages. This novel role of CYLD provides insight into the regulation of macrophage activation, where CYLD directly controls NF- κ B and IFN- γ cellular responses and resultant macrophage functions.

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List of Abbreviations

A	Alanine
aa	Amino acid
AMPK	Adenosine monophosphate kinase
AP1	Activator protein 1
APC	Antigen-presenting cell
APS	Ammonium peroxide sulphate
ATF	Activating transcription factor
ATG	Autophagy-related
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
BMDMs	Bone marrow derived macrophages
BSA	Bovine serum albumin
C-	Carboxy-
CAP-Gly	Cytoskeleton-associated protein glycine-rich
CARD	Caspase recruitment domain
Cas	CRISPR associated
CBP	CREB-binding protein
CD	Cluster of differentiation
CFUs	Colony formation units
cIAP	Cellular inhibitors of apoptosis
ChIP-Seq	Chromatin immunoprecipitation and sequencing
CLR	C-type lectin receptor
Conc.	Concentration
CRISPR	Clustered regularly interspaced short palindromic repeats
CSFR	Colony stimulating factor receptor

CUE	Coupling of ubiquitin conjugation to endoplasmic reticulum degradation
CYLD	Cylindromatosis
CXCL	chemokine (C-X-C motif) ligand
DAMPs	Danger-associated molecular patterns
DAP	l-Ala- γ -d-Glu-meso-diaminopimelic acid
DC	Dendritic cell
DD	Death domain
ddH₂O	double-distilled H ₂ O
DED	Death effector domain
DISC	Death inducing signalling complex
DMBA	7,12-Dimethylbenzanthracene
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DR	Death receptor
DSS	Dextran sulfate sodium
DTT	Dithiothreitol
DUB	Deubiquitinating enzymes
E1	Enzyme 1 (Ubiquitin-activating)
E2	Enzyme 2 (Ubiquitin-conjugating)
E3	Enzyme 3 (Ubiquitin-ligase)
e.g.	Exempli gratia (for example)
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra acetic acid
ERK	Extracellular-signal regulated kinase
et al.	Et alia
EV	Empty vector
EZH2	Enhancer of zeste homolog 2
FACS	Fluorescence assisted cell sorting

FADD		Fas-associated protein with death domain
FBS		Foetal bovine serum
FITC		Fluorescein isothiocyanate
FLIP		FLICE-inhibitory protein
GFP		Green fluorescent protein
GFP-K63-SUB		Green fluorescent protein - Lys63 - Subunit
GFP-M1-SUB		Green fluorescent protein - Met1 - Subunit
GLUE		Gram-like ubiquitin-binding in Eap45
GM-CSF	. . .	Granulocyte-macrophage colony-stimulating factor
GO		Gene ontology
GSEA		Gene set enrichment analysis
h		Human
HECT		Homologous with E6-associated protein C-terminus
hg19		Homo sapiens (human) genome assembly GRCh37
HIF		Hypoxia-inducible factor
HOIL-1		Heme-oxidised IRP2 ubiquitin ligase 1
HOIP		HOIL-1 interacting protein/RNF31
HRP		Horseradish peroxidase
hrs		Hours
HSCs		Hematopoietic stem cells
IAP		Inhibitor of apoptosis
IBD		Inflammatory bowel disease
IBR		In-between RING
IDO		Indoleamine 2,3-dioxygenase
iE-DAP		γ -D-glutamyl-meso-diaminopimelic acid
IFN		Interferon
IFNAR		Interferon α receptor
IFNG		Interferon γ gene
IFNGR1	. . .	Interferon γ receptor 1
IKK		I κ B kinases
IL		Interleukin

IP	Immunoprecipitation
IRF	Interferon responsive factor
ISG	Interferon stimulated genes
IκBα	Inhibitor of kappa B alpha
JAK	Janus kinase
JDP	Jun dimerization protein
JNK	c-Jun N-terminal kinase
K	Lysine (Lys)
KEGG	Kyoto encyclopedia of genes and genomes
kDa	Kilodalton
KO	Knock out
IN₂	Liquid nitrogen
L	Leucine
LAMP	Lysosome-associated membrane protein
LB medium	Luria-broth medium
LC3	Microtubule-associated proteins 1A/1B light chain 3
LLO	Listeriolysin-O
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LUBAC	Linear ubiquitin chain assembly complex
Lys	Lysine
m	Murine
mRNA	Messenger RNA
M	Methionine
M-CSF	Macrophages colony stimulating factor
MAMP	Microbe-associated molecular pattern
MAP1LC3	Microtubule-associated proteins 1A/1B light chain 3
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MDP	Muramyl dipeptide

MDP	Macrophage dendritic progenitor cell
MDSC	Myeloid-derived suppressor cells
MEF	Mouse embryonic fibroblast
Met	Methionine
MHV	Mouse hepatitis virus
MINDY	Motif interacting with Ub-containing Novel DUB
mins	Minutes
NKLAM	Natural killer lytic-associated molecule
MLKL	Mixed lineage kinase domain-like
MRC1	Mannose receptor c-type 1
MS	Mass spectrometry
mTOR	Mammalian target Of rapamycin
N-	Amino-
NEDD	Neural precursor cell expressed developmentally downregulated
NEMO	NF κ B essential modulator protein/IKK γ
NES	Nuclear export sequence
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
NLR	Nod-like receptor
NLS	Nuclear localisation sequence
NOD	Nucleotide-binding oligomerisation domain-like
NOS	Nitric oxide synthase
NOX2	NADPH oxidase 2
NRAMP1	Natural resistance-associated macrophage protein 1
NTHi	Haemophilus influenzae
NZF	Npl4 zinc finger
OPTN	Optineurin
OTU	Ovarian tumour protease
OTULIN	Ovarian tumour deubiquitinase with linear linkage specificity
P	p-value or probability value
PR	Proline-rich

P/S	Penicillin/streptomycin
PAGE	Polyacrylamide gel electrophoresis
PAMP	Pathogen-associated molecular pattern
PAZ	Polyubiquitin-associated zinc finger
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline + 0.1% Tween 20
PC	Precursor cell
PCR	Polymerase chain reaction
PELI3	Pellino E3 ubiquitin protein ligase family member 3
PIM	PUB-interacting motif
PKC	Protein kinase c
PMA	Phorbol 12-myristate 13-acetate
PNG	Peptidoglycan
PRR	Pattern-recognition receptor
PTM	Post-translational modification
PUB	N-terminal PNGase/UBA or UBX containing protein domain
qRT-PCR	Quantitative real time PCR
RAP80	Receptor-associated protein 80
RBR	RING-in-between-RING
RFU	Relative fluorescent units
RHIM	RIP homotypic interaction motif
RIG	Retinoic acid-inducible gene
RING	Really interesting new gene
RIPK	Receptor interacting protein kinase
RLR	RIG-I-like receptor
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT	Room temperature
S	Serine

SCV	Salmonella-containing vacuoles
SDS	Sodium dodecyl sulphate
SDS-PAGE . .	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Seq	Sequencing
Sh	Short hairpin
SHARPIN . .	SHANK-associated RH domain interactor
SLAP	Spacious listeria-containing phagosome
SMAC	Second mitochondria-derived activator of caspases
SOC	Super optimal broth with catabolic repressor
SOCS	Suppressor of cytokine signaling
SPATA2	Spermatogenesis-associated protein 2
SPI	Salmonella Pathogenicity Island
SPIC	Salmonella pathogenicity island C
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-like modifier
T	Threonine
TAB2/3	TAK1 binding protein 2/3
TAK1	TGF activated kinase 1
TAM	Tumour-associated macrophages
TBK1	TANK-binding kinase 1
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.1% Tween 20
TCR	T cell receptor
TGF	Transforming growth factor
Th1	T-Helper 1
Th2	T-Helper 2
TLR	Toll-like receptor
TMM	The trimmed mean of M-values normalisation method
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TNF-RSC . .	Tumour necrosis factor receptor signalling complex

TPA	12-O-tetra-decanoylphorbol-13 acetate
TRADD	TNFR type 1 - associated death domain protein
TRABID	TRAF-binding domain-containing protein
TRAF	TNF-receptor associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TSS	Transcription start site
TUBE	Tandem Ubiquitin Binding Entity
TYK	Tyrosine kinase
Ub	Ubiquitin
UBA	Ubiquitin associated
UBAN	Ubiquitin binding in ABIN and NEMO
UBC	Ubiquitin-conjugating
UBD	Ubiquitin binding domain
UBE	Ubiquitin-activating enzyme
Ubl	Ubiquitin-like protein
UCH	Ubiquitin C-terminal hydrolase
UIM	Ubiquitin-interaction motif
ULK	Unc-51 like autophagy activating kinase
USP	Ubiquitin-specific protease
VEGF	Vascular endothelial growth factor
VHS	Vps27/HRS/STAM
VSV	Vesicular stomatitis virus
WT	Wild type
WB	Western blotting
XIAP	X-linked inhibitor of apoptosis protein
Y	Tyrosine
ZnF	Zinc finger

Symbols and Units

α	Alpha
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β		Beta
γ		Gamma
ϵ		Epsilon
γ		Gamma
$^{\circ}\text{C}$		Degree centigrade
g		RCF or G-force
hr		Hour
hrs		Hours
L		Litre
m		10^{-3}
M		Molar
min		Minutes
O/N		Overnight
RT		Room temperature
sec		Second
secs		Seconds
μ		10^{-6}

1

Introduction

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1.1 The ubiquitin system

Ubiquitin (Ub) is a 76-amino acid (aa) protein that is highly conserved in all eukaryotes, but absent in bacteria and archaea. It is named after its ubiquitous expression in eukaryotic cells, where it represents 5% of all proteins [1]. *De novo* synthesis of Ub depends on four Ub-coding genes: *UBB*, *UBC*, *UBA52* and *RPS27A*. *UBA52* and *RPS27A* encode a single Ub molecule fused to the ribosomal proteins L40 and S27A, respectively, while the *UBB* and *UBC* genes generate polyubiquitin precursors (Ub chains) that need to be processed prior to cellular use [2, 3].

Ub works as a post-translational modification; it can be covalently attached to substrate proteins in order to modulate their activity or fate. In the mid-1970s, it was the first protein to be discovered to act as a post-translational modification. Since then, other Ub-like Proteins (Ubls) have been described, such as Small Ub-like Modifier (SUMO) and Neural Precursor Cell Expressed Developmentally Downregulated (NEDD) 8, which have similar three-dimensional structures to Ub [1, 4]. All Ubls can be attached to substrate proteins via their C-terminal glycine, and Ub in particular targets the ϵ -amino group of Lysine (Lys, K) residues, resulting in an isopeptide bond between Ub and the substrate protein [2, 4].

1.1.1 Ubiquitin cascade

Despite the initial identification of ubiquitination as a post-translational modification leading to substrate protein degradation by the proteasome, it is clear that this modification is also involved in non-proteolytic processes [4]. Ubiquitination of substrate proteins is a three-step enzymatic reaction involving Ub-activating (E1), Ub-conjugating (E2) and Ub ligase enzymes (E3).

E1 enzymes are responsible for the adenylation of Ub's C-terminus using Adenosine Triphosphate ATP and its subsequent interaction with a cysteine in the E1 active site, forming a thioester bond. Following this activation step, Ub is passed to the active site cysteine of an E2 enzyme, ready to be conjugated to a lysine residue of the substrate protein, with the aid of an E3 protein ligase. The conjugation of a single Ub to a substrate is known as monoubiquitination (Figure 1.1) [4, 5].

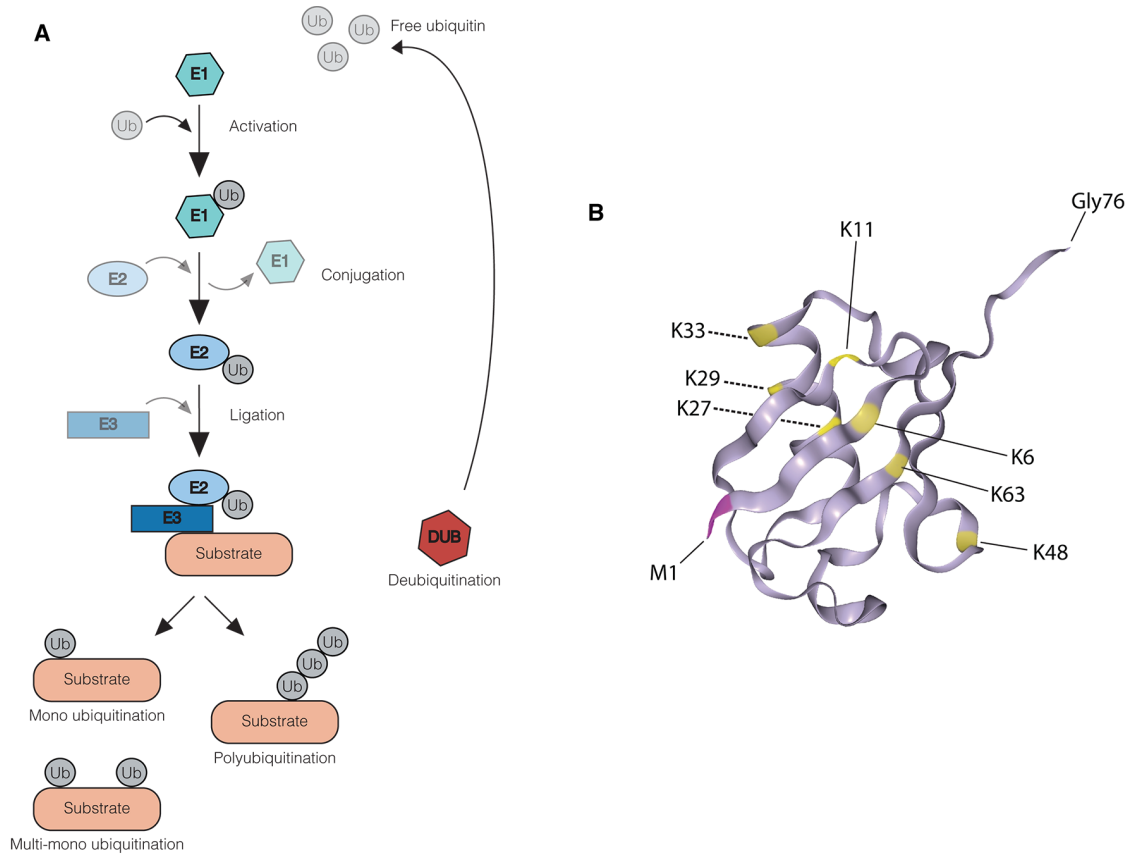


Figure 1.1: Ubiquitin's enzymatic cascade and structure. (A) Schematic representation of the Ub enzymatic cascade, including: the activation of Ub by an E1, conjugation with the E1 causing transfer of the Ub to the active site cysteine of an E2, and ligation with an E3 facilitating the transfer of Ub to a lysine residue on the target protein. Ubiquitinated substrates can be modified in various ways, including the addition of a single (mono) Ub on one or more (multi-mono) sites, or conjugation to an existing Ub molecule to generate polyubiquitin chains. Additionally, a group of enzymes collectively known as Deubiquitinating Enzymes (DUBs) facilitate the removal of Ubs on substrate proteins. Subsequently, free Ub is recycled for conjugation to another substrate in a separate reaction. (B) Structure of Ub showing the C-terminal glycine residue important for conjugation, and the seven lysines (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) plus the methionine (Met1) involved in Ub chains formation. Structure from Derek Leske.

In addition to the above, the Ub molecule itself presents seven lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys48 and Lys63) and an N-terminal methionine residue (Met1) that can serve as acceptor sites to conjugate further Ub molecules. Following the conjugation of a single Ub molecule on a substrate protein (known as the proximal Ub), this first Ub can initiate the generation of polyubiquitin chains. These polyubiquitin chains can be gradually built up by subsequent enzymatic reactions on the proximally bound Ub, and can involve all seven lysines plus the methionine residue. Ub chains generated on a substrate protein can be homotypic (conjugated systematically on the same residue) or heterotypic (conjugated on different residues) [4, 5]. The structures of these eight different Ub linkages depend on the residues engaged during chain elongation. As an example, the structures of Lys63- and Met1-linked Ub chains suggest an open configuration, which is easily accessible by Deubiquitinating Enzymes (DUBs) or interacting proteins, and have been compared to the structure of beads on a string. In contrast, other polyubiquitin chain configurations are more compact, restricting access to important surfaces such as the hydrophobic patch, which contain the isoleucine 44 (I44) residue that is involved in protein interactions (Figure 1.2) [6].

1.1.2 Cellular functions of ubiquitin chains

The variety of Ub modifications that can decorate substrate proteins enables their specific regulation and different biological functions [7]. In particular, Lys48-linked Ub chains have been extensively reported to adorn proteins that are directed towards proteasomal degradation, therefore they are essential for homeostatic functions. In addition, within an immune context, Lys48 proteolytic function is important, together with the non-proteolytic activity of Lys63- and Met1-linked Ub chains, as Pattern Recognition Receptors (PRRs) rely on these modifications for signalling (Figure 1.2) [7, 8].

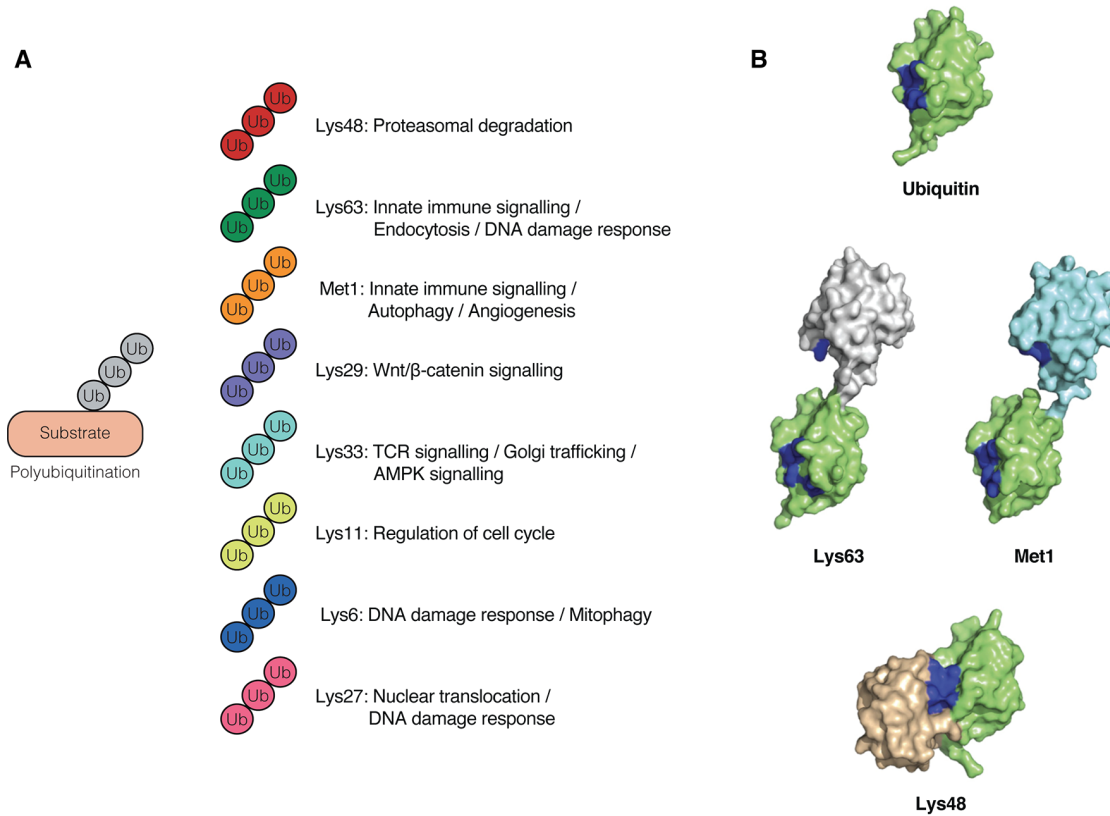


Figure 1.2: Ubiquitin linkages and cellular functions. (A) Schematic representation of the eight different Ub linkages with reported cellular functions. (B) Structural representation of Ub and Lys63-, Met1- and Lys48-linked Ub chains. The hydrophobic patch (I44) is blue in all molecules, and the proximal Ub is green, while distal Ubs are grey, light blue and brown [6]. T cell Receptor (TCR), Adenosine Monophosphate Kinase (AMPK)

1.1.3 Deubiquitinating enzymes

Ubiquitination is a reversible post-translational modification. Accordingly, in addition to the E1, E2 and E3 enzymes that are responsible for conjugating this polypeptide to substrate proteins, Ub can be hydrolysed from target proteins or from other Ub molecules (in case of Ub chains) via DUBs. Approximately 100 DUBs are encoded in the human genome, which can be classified into six different families based on their proteolytic mechanisms. Among these six families, five represent thiol-proteases, while the sixth contains metalloproteases [5, 9].

The thiol-proteases group consist of the Ub-specific Proteases (USPs) family, the Ovarian Tumour Proteases (OTUs) family, the Ub C-terminal Hydrolase (UCH) family, the Josephins and the recently discovered Motif Interacting with

Ub-containing Novel DUB family (MINDY). These five families of cysteine proteases present different catalytic domain structures. Conversely, the JAB1/MPN/MOV34 (JAMM) family of metalloproteases have a different reaction mechanism, relying on zinc [6]. DUBs can hydrolyse monoubiquitin from substrates or they can specifically cleave polyubiquitin chains, and this latter reaction can occur either from the distal Ub of the linkage or from within the chain, defined as *exo-* or *endo-deubiquitination*, respectively [10]. The USP family is the largest, and exhibits little specificity for Ub linkages apart from a few members, such as the Cyldromatosis (CYLD) gene, which preferentially cleaves Lys63- and Met1-linked Ub chains. Conversely, members of the OTU family seem to preferentially cleave defined Ub linkages, e.g. OTU Deubiquitinase with Linear Linkage Specificity (OTULIN), which is specific for Met1-linked Ub chains, or Tumour Necrosis Factor (TNF)-receptor-associated Factor (TRAF)-binding Domain-containing Protein (TRABID), which hydrolyses Lys29- and Lys33-linked Ub chains only [6].

1.1.4 Ubiquitin-binding domains

Like other post-translational modifications, ubiquitination can be recognised through specific domains defined as Ub-binding Domains (UBDs), which are able to directly interact with monoubiquitin and/or polyubiquitin chains [11]. Different structures of UBDs have been described so far: Ub-Interacting Motifs (UIMs), Ub-Associated (UBA) domains, Coupling of Ub Conjugation to Endoplasmic Reticulum Degradation (CUEs) domains, Polyubiquitin-Associated Zinc Fingers (PAZ) motifs and Npl4 Zinc Finger (NZF) motifs, as well as Gram-like Ub-binding in Eap45 (GLUE) and Vps27/HRS/STAM (VHS) domains. These UBDs are relatively small (20-150 aa) and recognise the hydrophobic patch (I44), thereby binding non-covalently to Ub. UBDs can display affinity for specific Ub linkages, depending on their structural conformation and distances between multiple hydrophobic patches. Their ability to selectively bind different Ub modifications reflects the variety of functions covered by this post-translational modification within the cell [10, 11].

1.2 The innate immune system

"Immunity" comprises all of the defence mechanisms that are necessary for an organism to preserve host-microbial homeostasis, and to prevent infection and colonisation by pathogens. The human immune system is composed of two subsystems, which are characterised by different timing and reaction specificities: the innate immune system and the adaptive immune system [12]. The innate immune system is the older component of immunity, and its primary role is to prevent infection. This is achieved through anatomical and physiological barriers, together with cellular components. The innate immune system is comprised of different cell types (basophils, eosinophils, neutrophils, Langerhans cells, mast cells, monocytes/macrophages, dendritic cells and Natural Killer (NK) cells) that are able to sense invariant structures present in invading pathogens through a vast collection of specific receptors defined as PRRs [13]. While the innate immune response is not considered to be specific, the adaptive immune response is tailored against specific pathogens. This targeted response is achieved through T and B lymphocytic cells, and it requires the *de novo* generation of specific immune receptors via somatic recombination [12].

1.2.1 Pattern Recognition Receptors

PRRs are the main receptors used by innate immune cells to recognise and bind highly conserved molecular motifs in pathogen regions, define as Microbe-associated Molecular Patterns (MAMPs) or Pathogen-associated Molecular Patterns (PAMPs). In addition to these cell-extrinsic structures, PRRs can sense a group of endogenous molecules described as Danger-associated Molecular Patterns (DAMPs). Activation of PRRs via these different ligands triggers an inflammatory response, mainly mediated by the Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells (NF- κ B) transcription factor (TF), together with Activator protein 1 (AP1) and the Interferon Response Factors (IRFs). As a result, cytokines, chemokines, antimicrobial peptides and inflammatory mediators are produced [12, 14].

Four main families of PRRs have been described so far: Toll-like Receptors (TLRs) and C-type Lectin Receptors (CLRs) are transmembrane proteins, while Retinoic Acid-inducible Gene (RIG)-I-like Receptors (RLRs) and Nucleotide-binding Oligomerisation Domain (NOD)-like Receptors (NLRs) are cytoplasmic proteins. Additionally, a further set of cytosolic DNA-binding proteins has been recently reported, involved in viral and microbial DNA sensing (Figure 1.3) [15, 16].

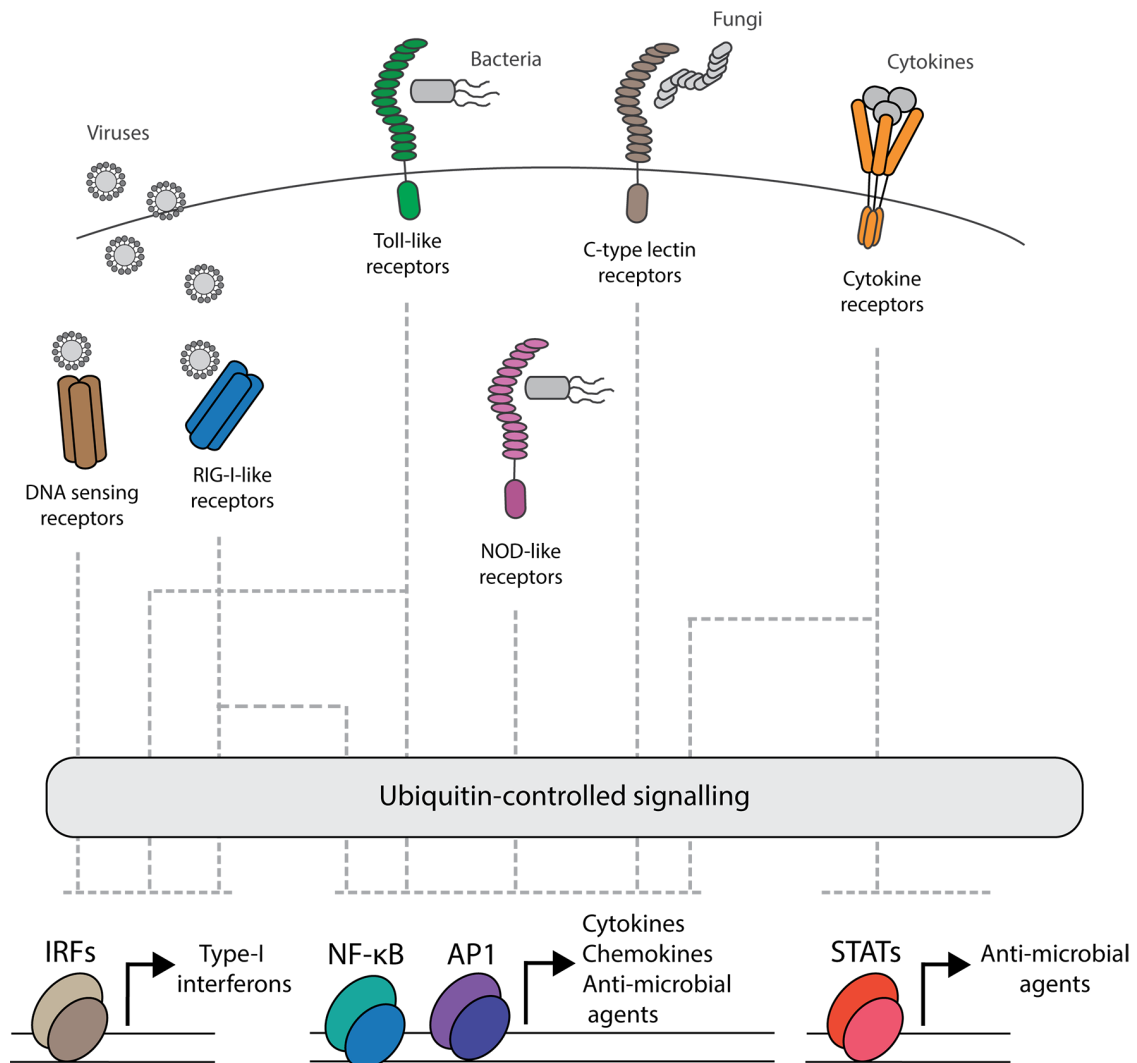


Figure 1.3: Pattern recognition signalling. Schematic representation of each of the major types of PRRs, their respective ligands and the main groups of TFs activated upon receptor stimulation. Importantly, all of these signalling events are reliant on Ub-mediated signalling events. Cytokine receptors can also activate an inflammatory response via NF- κ B and Signal Transducer and Activator of Transcription (STAT) TFs.

1.2.2 NOD2 receptor signalling

The NLR family members NOD1 and NOD2 recognise conserved bacterial cell wall components named Peptidoglycans (PNGs). NOD1 senses γ -D-glutamyl-*meso*-diaminopimelic acid (iE-DAP), whereas NOD2 recognises muramyl dipeptide (MDP), and these PNG structures can be present in both gram-positive and gram-negative bacteria [17]. Structurally, NOD1 and NOD2 receptors are very similar. They possess one or two Caspase Activation and Recruitment Domains (CARDs), respectively, followed by a single oligomerisation NOD domain and a multiple Leucine-rich Repeats (LRRs) domain that is important for ligand sensing (Figure 1.4) [17].

In response to MDP-mediated activation, NOD2 oligomerises and recruits the adaptor Receptor-interacting Serine/Threonine-protein Kinase 2 (RIPK2 (RICK/CARDIAK)) via its CARD domain. Following recruitment, RIPK2 is polyubiquitinated with Lys63- and Met1-linked Ub chains, generated by various E3 ligases engaged via receptor activation, including cellular Inhibitor of Apoptosis 1/2 (cIAP1/2), the X-linked Inhibitor of Apoptosis Protein (XIAP) and the Linear Ub Chain Assembly Complex (LUBAC) [18–20]. Lys63-linked Ub chains recruit the Transforming Growth Factor (TGF) β -activated Kinase 1 (TAK1)-TAK1-binding Protein (TAB) complex, while Met1-linked Ub chains promote recruitment of the NF- κ B Essential Modulator (NEMO), a subunit of the I κ B Kinase (IKK) complex. Recruitment and activation of TAK1-TAB is crucial for IKK complex phosphorylation. This step leads to the ubiquitination and degradation of the NF- κ B Inhibitory Factor (I κ B α), and subsequent nuclear translocation of the NF- κ B TF. Additionally, The TAK1-TAB complex activates the three major families of Mitogen-Activated Protein Kinases (MAPKs): p38, JNK, and ERK (Figure 1.4) [8, 20, 21].

Ubiquitination is crucial for proper NOD2 signalling and the subsequent inflammatory response. In addition to E3 ligases generating polyubiquitin chains, DUBs are also recruited at the receptor complex to counterbalance and eventually inhibit downstream signalling. Three Ub hydrolysing enzymes have been described as acting at the NOD2 receptor complex, presenting different linkage specificities: A20,

CYLD and OTULIN. In particular, A20 cleaves Lys11-, Lys48- and Lys63-linked Ub chains, CYLD preferentially hydrolyses Lys63- and Met1-linked Ub chains, and OTULIN is selective for Met1-linked Ub chains [8, 22–24]. The IKK complex and MAPKs are common mediators of inflammation following PRR activation. The IKK complex specifically induces the activation and nuclear translocation of NF- κ B TF (a heterodimeric complex containing the subunits p50 (NF- κ B1) and p65 (RelA)), while MAPKs activate the AP1 family of TFs (c-Fos, c-Jun, Activating Transcription Factor (ATF) and Jun Dimerization Protein (JDP)). Hence, the NF- κ B and AP1 TFs are responsible for mounting the NOD2-mediated inflammatory response, mainly by inducing the transcription of pro-survival and pro-inflammatory genes (Figure 1.4) [16, 17, 21].

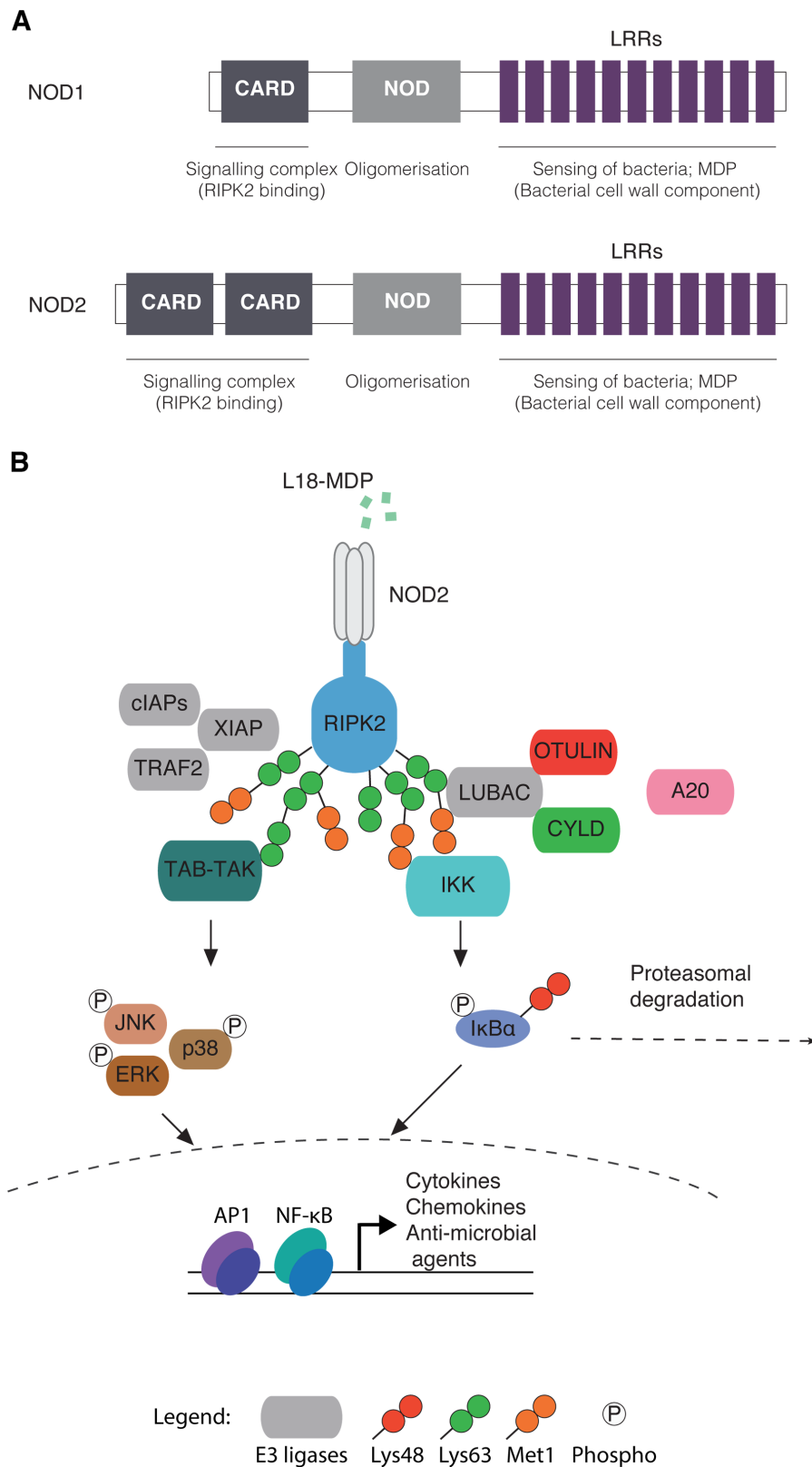


Figure 1.4: NOD2 signalling pathway. (A) the domain structures of NOD1 and NOD2 highlights their similarity, with NOD2 containing an extra CARD domain. (B) Schematic representation of the NOD2 signalling pathway and downstream transcriptional responses.

1.3 The deubiquitinase CYLD

1.3.1 Structure and function of CYLD

The deubiquitinase CYLD was initially identified as the gene responsible for a heritable skin tumour syndrome named cylindromatosis. This condition is characterised by the development of multiple cylindromas, benign skin tumours arising from the hair follicles, usually appearing on the head and neck. *CYLD* thereby functions as a bona fide tumour suppressor gene. Indeed, cylindromas present loss of heterozygosity, and mutations in this gene mainly affect the USP domain, which is responsible for CYLD's catalytic activity. Interestingly, despite their rapid growth, cylindromas rarely become malignant [25, 26].

The *CYLD* gene is located on chromosome 16q and encodes for a 956-aa protein, though a smaller isoform lacking exon seven is also produced via alternative splicing. CYLD is expressed in all human tissues (the smaller isoform is expressed in all tissues apart from the kidney) and it contains three Cytoskeleton-Associated Protein Glycine-rich (CAP-Gly) domains, a Proline-rich (PR) region and a USP domain (also called the Ub Carboxyl-terminal Hydrolase (UCH) domain). The first two CAP-Gly domains are located in the N-terminal part of CYLD, and are important for tubulin and/or microtubule interaction, cell growth and cell division [26, 27]. The PR region is located between the second and third CAP-Gly domains, and it constitutes an SH3 structure mediating protein protein interactions. This area contains a consensus binding sequence that is specific for the E3 ligase TRAF2, mediating CYLD binding and therefore the deubiquitination of this target [28, 29]. The third CAP-Gly domain follow the PR region, and is involved in binding NEMO, which is the γ subunit of the $\text{I}\kappa\text{B}$ kinase complex that is responsible for NF- κB activation [28, 30]. At the C-terminus, the CYLD USP domain, presenting the catalytic triad Cys601, His871 and Asp889, allows the hydrolysis of Lys63- and Met1-linked Ub chains. Cys601 single-aa substitution completely abrogates CYLD deubiquitinase activity. A Zinc-finger-like B-box Motif (B-box) is also present within the USP domain

of CYLD and is important for both CYLD homodimerisation and cytoplasmic localisation, but dispensable for CYLD catalytic activity (Figure 1.5) [31–33].

The majority of CYLD germline mutations associated with cylindromas development are located between exon 9 and 20, the region encoding for the USP domain [34]. Germline mutations are likely to produce inactive or truncated form of the CYLD protein and they include frameshift mutations (44%), nonsense mutations (25%), splice-sites mutations (11%), missense mutations (11%) and a small fraction of germline deletions (2.7%) [25, 26, 34]. In addition to the USP catalytic domain, the PR region located between exon 7 and exon 8, important for TRAF2 binding, has been reported as an hotspot mutation site (Figure 1.5) [34].

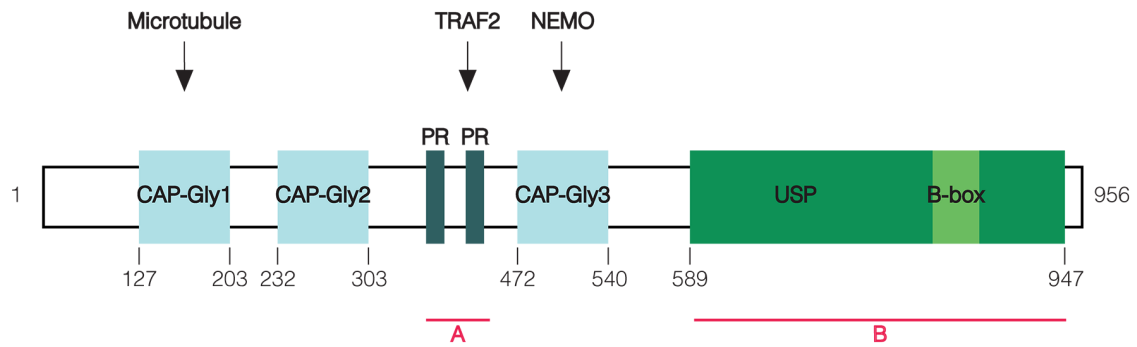


Figure 1.5: Schematic representation of CYLD’s domain structure. Domain structure of *CYLD*, showing three CAP-Gly domains, the PR region, and the USP domain (with the embedded B-box). The binding areas for tubulin and/or microtubules association, TRAF2 and NEMO are annotated. A and B regions highlighted in red contain hotspot mutation sites.

1.3.2 CYLD in immunity and pathogen infection

The NF- κ B signalling pathway was the first CYLD-regulated pathway to be described. Specifically, CYLD deubiquitinating activity was reported to inhibit NF- κ B activation downstream the TNF Receptor (TNFR), TLR2, TLR4 and NOD2 signalling pathways [33, 35–41]. CYLD recruitment to these innate immune receptor complexes has been reported to occur through direct interaction with different adaptors, such as NEMO, TRAF2 and p62. In addition, recent data from our group and others have uncovered a new adaptor protein named Spermatogenesis

Associated Protein 2 (SPATA2), linking CYLD to the LUBAC complex. SPATA2-mediated CYLD interaction with LUBAC seems to be essential for the recruitment of CYLD to the TNFR and NOD2 receptor complexes [35, 36, 42, 43].

Different phenotypes have been described for *CYLD* Knockout (KO) mice. In line with the pro-inflammatory role of NF- κ B signalling, *CYLD* KO mice have been shown to present inflammatory infiltrates in several tissues such as the liver, spleen and lung [44, 45]. Moreover, T cells from *CYLD* KO mice have been described as being hypersensitive to T Cell Receptor (TCR) stimulation, due to enhanced activation of NF- κ B signalling, leading to spontaneous lymphocyte infiltration in the liver and colonic inflammation [46]. CYLD is also important for NK T cell development and activation [47]. Additionally, *CYLD* KO mice present an increased number of B cells, as a compensatory mechanism due to the reduction of mature T cells [48].

Conversely, some *CYLD* KO strains have no overt phenotype, but display uncontrolled NF- κ B and MAPKs signalling with subsequent increased antimicrobial response when challenged with pathogens. Accordingly, *CYLD* KO mice are protected from *Streptococcus pneumoniae* infection and subsequent acute lung injury. Moreover, *CYLD* KO mice are protected from severe listeriosis following *Listeria monocytogenes* infection through enhanced NF- κ B signalling, leading to Reactive Oxygen Species (ROS) and Interleukin (IL)-6 production. In contrast, CYLD has been reported to promote the Interferon (IFN)- β -dependent antiviral response following Vesicular Stomatitis Virus (VSV) infection [49–52].

1.3.3 CYLD as a tumour suppressor

Despite its defined role as a tumour suppressor in humans, *CYLD* KO mice do not recapitulate the cylindromatosis phenotype. Indeed, KO of *CYLD* in mice does not lead to spontaneous tumour development and progression. However, these mice are susceptible to colitis-associated tumorigenesis following Dextran Sulfate Sodium (DSS) treatment, or the development of papillomas after a single dose of 7,12-Dimethylbenzanthracene (DMBA) followed by 12-O-tetra-decanoylphorbol-13

acetate (TPA) treatment [44, 53]. In contrast, hepatocytes-specific inactivation of *CYLD* (exon nine deletion, which determines a deubiquitinase-deficient form of CYLD) promotes spontaneous apoptosis of these cells via JNK activation, subsequent inflammation and late-onset hepatocellular carcinoma [54]. Similarly, mice carrying *CYLD* exon nine deletion in epithelial cells develop hair defects, sebaceous adenoma or basaloid tumours if challenged with TPA [55].

In conclusion, in *CYLD* KO mice, the tumour suppressive function of this deubiquitinating enzyme seems to be exploited only in an inflammation-driven context, such as DSS or TPA treatment, while conditional catalytically inactive CYLD expression promotes the spontaneous development of hepatocellular carcinoma and skin tumours. In addition to NF- κ B and MAPK signalling, CYLD regulates other important signalling pathways involved in tumour development and progression, such as Wnt, TGF β 1, and Notch [56–58].

1.4 Macrophages

1.4.1 Macrophage development and tissue residency

Macrophages are multifunctional and evolutionarily conserved phagocytic cells present in vertebrates [59, 60]. Despite being initially discovered by Eli Metchnikoff as part of the innate immune system, these cells have subsequently been described as being important in vertebrate tissue homeostasis and development. Accordingly, the functions of important tissues and organs, such as the brain, heart and bones, rely on these cells. Considering their plurifunctionality, defects in macrophage functions are associated with several chronic pro-inflammatory diseases including type 2 diabetes, atherosclerosis, obesity and cancer, in addition to other pathological conditions such as fibrosis and osteoporosis [60, 61]. Macrophages are present throughout all tissues and they can be divided in two major populations based on their origin: tissue-resident macrophages and monocyte-derived macrophages.

Tissue-resident macrophages originate from yolk sac, foetal liver, and bone marrow Haematopoietic Stem Cells (HSCs) during development. This population acquires different phenotypes and physiological functions based on the tissue of residence. This functional specialisation is mediated by tissue-specific signals, able to drive temporary or permanent transcriptional programmes in these cells. Several populations of tissue-resident macrophages have been described so far: lung alveolar macrophages, spleen red-pulp macrophages, osteoblasts/osteoclasts, epidermal Langerhans cells, liver Kupffer cells, brain microglia, and cardiac, peritoneal, pancreatic and kidney macrophages [59, 60].

Monocyte-derived macrophages originate from circulating monocytes, and their function is to replenish the population of tissue-resident macrophages both in steady-state conditions and in response to inflammation. Monocytes can infiltrate tissues and differentiate into macrophages, which can then acquire tissue-specific properties. This homeostatic process is enhanced in response to several challenging conditions, such as injury, infection or inflammation. In addition to monocyte-driven replenishment, it was recently shown that macrophages have a self-renewal capacity,

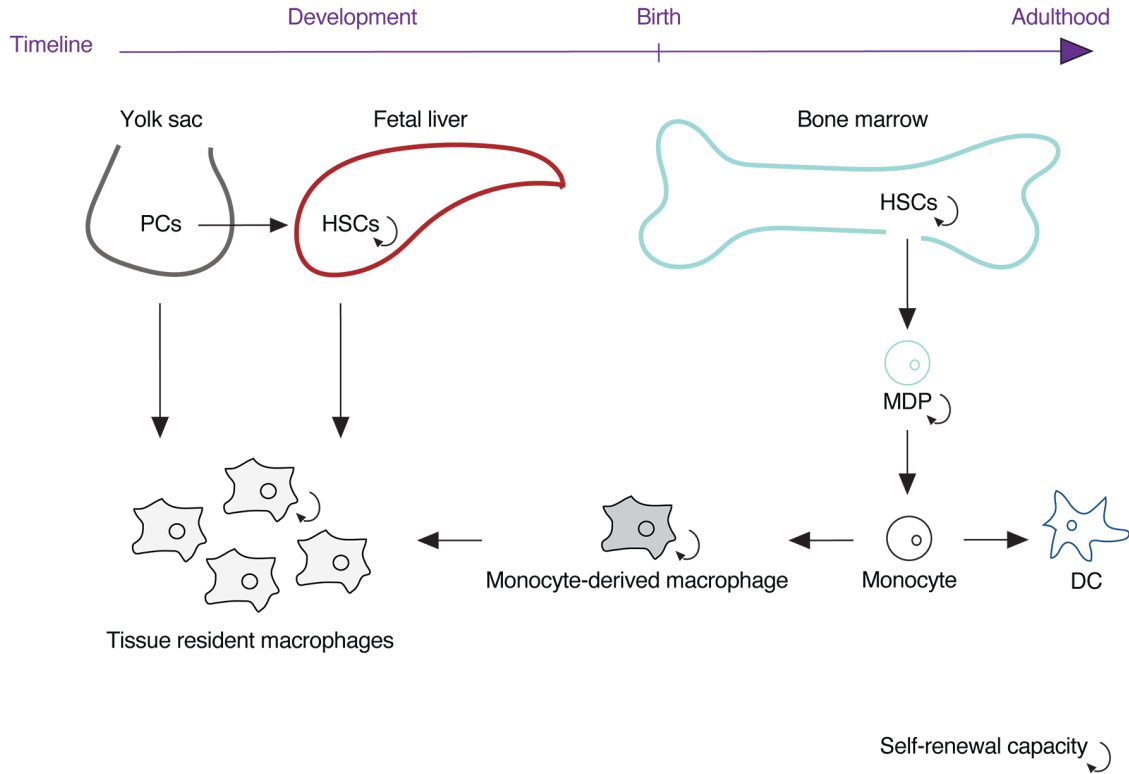


Figure 1.6: Macrophage development and maintenance. Schematic representation of tissue-resident macrophages development and maintenance. Tissue-resident macrophages originate from the yolk sac, foetal liver and bone marrow, and have a self-renewal capacity. Monocyte-derived macrophages replenish the pool of tissue-resident macrophages. Precursor Cells (PCs), Macrophage Dendritic cells Progenitor (MDP), Dendritic Cell (DC).

despite the fact that, following differentiation from proliferative progenitors or monocytes, the cell cycle is suspended. This arrest can be overcome to maintain the macrophage population during development, homeostasis or under challenging conditions. Interestingly, this self-renewal capacity does not lead to a loss of differentiated properties or transformation [60, 62]. Macrophage self-renewal is triggered via Macrophage Colony-stimulating Factor Receptor (M-CSFR) by M-CSF and IL-34, an alternative ligand for this receptor, or through Granulocyte-macrophage Colony-Stimulating Factor Receptor (GM-CSFR) via GM-CSF. Furthermore, it has been recently shown that during T helper-2 (Th2) response, IL-4 also drives macrophage proliferation (Figure 1.6) [62, 63].

1.4.2 Macrophages in innate immunity

Macrophages, together with neutrophils, monocytes, dendritic cells and mast cells, are professional phagocytic cells. In addition to engulfing and processing foreign materials, macrophages have the ability to distinguish among *self* and *non-self* components through the expression of PRRs, which are crucial for their immunological activity [64, 65].

In addition to the origin-related classification reported above, macrophages have been further categorised based on phenotype and gene expression profiles following cytokine or microbial stimulation: *Classically activated macrophages*, orchestrate the defence response against bacteria, protozoa and viruses, have pro-inflammatory activity and are important for anti-tumour immunity. *Alternatively activated macrophages* are involved in wound-healing and resolution of inflammation, and therefore present anti-inflammatory properties. *Tumor Associated Macrophages* (TAMs) are phenotypically shaped by the tumour microenvironment and actively suppress antitumour immunity. *Myeloid-Derived Suppressor Cells* (MDSCs) are immature myeloid precursor cells that are characterised by their ability to suppress T cell response.

Macrophages are constantly exposed to various and simultaneous stimuli within tissues. Despite their strictly defined characterisation, based on gene expression and phenotype, they are extremely plastic cells. Therefore, they can quickly adapt their phenotype in response to immunoregulatory stimuli, invading pathogens, foreign substances, metabolites and dead cells [64, 65]. Furthermore, it is important to consider that studies focussed on describing macrophage phenotypes used primary macrophages and cell lines, which were *in vitro* exposed to single or combinatory ligands. In a tissue context, the number of stimuli is increased, and it has been shown that macrophages do not respond to cytokines or microbial agonist challenges to the same extent. This suggests an important role for the microenvironment in shaping macrophage responses to stimuli, and is in line with the notion that, at steady-state, these cells preserve tissue integrity, presenting an anti-inflammatory phenotype described as *tolerance* [64–66].

Classically activated macrophages

PRR expression is important in macrophages in order to sense danger signals, which can include microbial components such as Lipopolysaccharide (LPS) and exogenous DNA/RNA molecules, or endogenous molecules such as alarmins, released at high levels during sterile injury [65]. Accordingly, danger signals are divided in two main groups based on origin: PAMPs deriving from microorganisms (hence *non-self*) and DAMPs (cell-derived thus *self*) [67].

Danger signals released by tissue injury or infection activate macrophages toward a pro-inflammatory phenotype, which is characterised by secretion of pro-inflammatory mediators including Reactive Nitrogen Species (RNS), ROS, and pro-inflammatory cytokines. RNS and ROS act directly on the microorganism causing nitrosative stress, while cytokines participate in the activation of antimicrobial mechanisms and influence the adaptive immune response. As an example, IL-12 secretion by phagocytic cells triggers T helper-1 (Th-1) cells to produce IFN- γ , which can then induce antimicrobial properties in macrophages (Figure 1.7) [64, 65].

Functional and molecular studies on activated macrophages have been performed using primary macrophages and cell lines stimulated with either IFN- γ or IFN- γ together with TLRs ligands, such as LPS or TNF- α . In particular, the combination of IFN- γ and LPS stimulation mimics the encounter with a gram-negative bacteria in a Th-1 response context, and it is widely accepted and referred to as *classical* activation or M1 polarisation. Conversely, macrophage stimulation with IFN- γ alone, triggering antimicrobial features but not a PRRs-mediated NF- κ B response, is defined as *priming* [68–70].

The initial observation of *Mackanness* in 1960 on classically activated macrophages was their enhanced antimicrobial activity against *Listeria monocytogenes* and *Bacillus Calmette-Guerin*, upon secondary exposure to these pathogens [71–73]. More recently, *Mills et al.* reported that macrophages exposed to Th-1 or directly stimulated with LPS and IFN- γ produce ROS [68]. Nowadays, the activated macrophages phenotype has been extensively characterised. These cells have increased antimicrobial and antitumour features, and they secrete a variety of

pro-inflammatory cytokines including $\text{TNF-}\alpha$, $\text{IFN-}\gamma$, IL-12, IL-6, IL-1 β , CCL2 and IL-23. Moreover, they present a higher capacity to present antigens and induce T cell proliferation [74, 75]. Despite their crucial role in host-defence, uncontrolled or excessive activation of macrophages has been shown to lead to chronic inflammatory and autoimmune syndromes, highlighting the importance of regulating this process within the tissues (Figure 1.7) [74, 75].

Downstream signalling responsible for activation

Several receptors have been recognised to be involved in macrophage activation, and they can be grouped in two distinct classes based on their downstream signalling. There are receptors activating NF- κ B and AP1, next to receptors activating the STAT family of TFs. Among the receptors responsible for NF- κ B activation via the IKK complex and AP1 via Jun kinases, TLR3, TLR4, RIG-1 and cyclic GMP-AMP synthase (cGAS) can trigger IRF3 activation, and therefore transcription of the $\text{IFN-}\gamma$ gene (*IFNG*). Others, including TNFR and IL-1R, do not activate IRF3. Among the STATs activating receptors, $\text{IFN-}\gamma$ Receptor (IFNGR) triggers STAT1 and STAT3 activation forming homo or heterodimers, while the $\text{IFN-}\alpha$ and $\text{IFN-}\beta$ Receptor (IFNAR) activates STAT1-STAT2-IRF9 trimers and STAT1 homodimers [65].

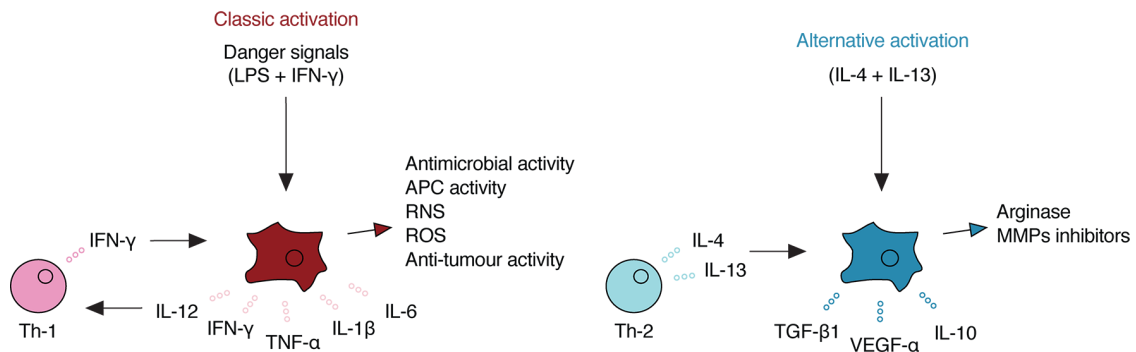


Figure 1.7: Schematic representation of macrophage activation. Schematic representation of *classically activated* and *alternatively activated* macrophages with individual properties. Antigen Presenting Cells (APC), Matrix metalloproteinases (MMPs).

Alternatively activated macrophages

Similarly to activation, there are immunoregulatory stimuli capable of inducing anti-inflammatory features in macrophages that are important for resolving inflammation and promoting wound-healing. This wound-healing phenotype is usually described as *alternative activation*, and it is characterised by a reduced ability to produce toxic oxygen and nitrogen radicals, lower secretion of pro-inflammatory mediators and a failure to present antigens to T cells [74]. Alternatively activated macrophages present diminished antimicrobial activity and counteract primed macrophages, repairing damaged tissue, restoring homeostasis and promoting an anti-inflammatory environment. The stimuli responsible for inducing this phenotype are the signature cytokines produced during a Th-2 response: IL-4 and IL-13. In addition to adaptive immune cells, basophils and mast cells can also produce IL-4 and drive *alternative activation* in macrophages, resulting in the upregulation of arginase and production of Vascular Endothelial Growth Factor- α (VEGF- α), IL-10 and TGF β 1.

As part of the wound-healing process, the enzyme arginase directly contributes to extracellular matrix production and VEGF- α to blood vessel development, while TGF β 1 enhances the expression of metalloproteinases inhibitors and promotes the differentiation of fibroblasts into myofibroblasts synthesising collagen [64, 74, 76]. Furthermore, as previously discussed, IL-4 stimulation directly drives macrophage proliferation (Figure 1.7) [62].

1.4.3 Macrophage antimicrobial mechanisms

Macrophages recognise invading pathogens mainly through PRRs. Extracellularly, TLRs 1/2, 2/6, 4, 5 and 9 sense bacterial lipopeptides, LPS, flagellin, and DNA sugar phosphate backbones, respectively. Moreover, other surface receptors such as C-type lectins and mannose receptors bind bacteria and initiate phagocytosis. Intracellularly, NOD1, NOD2, Galectin 3 and cGAS recognise l-Ala- γ -d-Glu-meso-diaminopimelic Acid (DAP), MDP, β -galactosides and intracellular DNA respectively. Following pathogen sensing, macrophages can activate several antimicrobial mechanisms and, in parallel, trigger the inflammatory response [77].

Phagocytosis and efferocytosis

Phagocytosis is defined as the internalisation of particles over 0.5 microns in size (contrary to endocytosis, which involves targets under 0.5 microns) and it is a crucial mechanism for antimicrobial defence. Following pathogen sensing and binding, surface PRRs can trigger actin polymerisation creating a phagocytic cup, and subsequently a phagosome. After internalisation, the phagosome vacuole acquires microbicidal and degradative features through maturation. During maturation, the phagosome's protein and lipid composition is highly dynamic, and is dependent on Golgi-derived transport vesicles and endosomes fusing with it [77–79].

Phagosome maturation can be divided in three main steps: The *early phagosome* represents the closure of the phagocytic cup, including the pathogen. During this process, the calcium-dependent phosphatase calcineurin is activated to prevent fusion with lysosomes. The pH in this compartment is almost neutral (6/6.5), since it lacks the v-ATPases proton pumps. The *late phagosome* is characterised by Golgi-derived vesicles and endosomes fusion, which results in the acquisition of lysosomal enzymes and Lysosome-associated Membrane Proteins (LAMP1 and 2). Lysosomal enzymes include cathepsins B, L and D, phospholipases and peptidases. The late phagosome presents a lower pH (5) due to the v-ATPases proton pumps, which is necessary for the activity of the delivered enzymes. The *phagolysosome* represents the complete fusion of the late phagosome and lysosome, with a low pH (4/4.5) and high hydrolysing activity. These conditions are adverse for the majority of engulfed pathogens, which get degraded in this compartment [77–79].

IFN γ treatment of murine macrophages increases *Mycobacterium avium* clearance through enhanced phagosome maturation and fusion with the lysosomal compartment [78, 80]. Moreover, IFN γ priming selectively upregulates cathepsins B and L in macrophages [81].

Efferocytosis can be described as the engulfment of dead cells, dying cells and cellular debris via phagocytosis. Pathogen infection can cause three different types of cell death - apoptosis, pyroptosis or necroptosis - depending on the infecting microorganism. Apoptosis is a programmed and caspase 3, 6 and 7-dependent

form of cell death. Apoptotic cells do not release cytoplasmic contents and they are cleared via efferocytosis. Pyroptosis is triggered by inflammasome activation, and is mainly driven by caspase 1 and 11 together with the pore-forming protein gasdermin. This form of cell death is characterised by membrane disruption and the release of cytoplasmic contents with pro-inflammatory outcomes. Necroptosis is also pro-inflammatory programmed cell death, similar to pyroptosis, but is induced via RIPK3 activating the pore-forming protein Mixed Lineage Kinase Domain-like (MLKL). Evolutionarily, these different types of programmed cell death represent mechanisms to restrict pathogen replication and spread, activating the innate/adaptive immune system. Efferocytosis of pathogen-infected cells and debris is therefore an important mechanism to clear the tissues from infection, and this is mainly performed by macrophages and neutrophils (Figure 1.8) [77, 78, 82].

Production of radicals and amino acid deprivation

A well-recognised activated macrophage strategy to eliminate intracellular pathogens is nitrosative stress. Transcription and activation of Nitric Oxide Synthase (NOS) or NADPH oxidase 2 (NOX2) enzymes allows the production of RNS and ROS respectively. Such radical production affects the stability of microbial proteins and lipids, and generates microbial DNA damage. IFN γ treatment in macrophages directly induces NOS transcription and recruitment of NOX2 to the phagosome.

Amino acid deprivation within the phagosome is important to limit intracellular pathogen growth. This function is carried out by the Indoleamine 2,3 Dioxygenases (IDO1 and IDO2), which deplete tryptophan through its conversion into N-formylkynurenine. *IDO1* and *IDO2* transcription is also induced following IFN γ stimulation of macrophages (Figure 1.8) [77, 79, 83].

Antimicrobial peptides and proteins

Pathogen growth or integrity within the phagosome can be also compromised through antimicrobial peptides and proteins. Several classes of antimicrobial effectors have been described, including defensins, lysozymes, lipases and proteases. Defensins are small proteins able to bind negatively charged molecules on gram-positive

and gram-negative bacterial surfaces, inducing membrane permeabilisation and disruption. Lysozymes, lipases and proteases can hydrolyse carbohydrates, lipids and proteins, respectively. These microbial effectors are part of the IFN γ - and NF- κ B-mediated immune responses, and are delivered within the phagosome during maturation (Figure 1.8) [78, 79].

Macroautophagy and xenophagy

Following activation or priming, macrophages upregulate the macroautophagy machinery, including the lipid-binding proteins MAP1LC3A, MAP1LC3B and MAP1LC3C, the Unc-51 Like Autophagy Activating Kinase 1 and 2 (ULK1 and ULK2), and Autophagy-related (ATG) proteins [67, 77]. Autophagy is canonically used to degrade and recycle senescent cellular organelles and compartments, via targeting of these ubiquitinated components to the autophagosome. Similarly to the phagosome, the autophagosome undergoes maturation and fusion with lysosomes; therefore, this pathway can be exploited to eliminate intracellular pathogens. Senescent organelles undergoing recycling, cellular starvation and nutrient deprivation trigger autophagy via Adenosine Monophosphate Kinase (AMPK) and the Mammalian Target Of Rapamycin (mTOR). In addition, IFN γ , TLR2, TLR4 and vitamin D have been described as directly activating autophagy and macroautophagy mechanisms [67, 77]. While macroautophagy and autophagy are aimed to degrade *self* cellular components, xenophagy is defined as the elimination of *non-self* material via selective autophagy [84]. Similarly to senescent organelles, intracellular bacteria are decorated with ubiquitin chains following detection by PRRs. Opsonization of targeted bacteria involves Lys63- and Met1-linked ubiquitin chains conjugation, and it results in xenophagy adaptors binding and initiation of the autophagosome maturation [67, 84]. Four xenophagy adaptors have been described so far involved in intracellular pathogen clearance: p62, TANK-binding Kinase 1 (TBK1), Nuclear Domain Protein 10 (NDP52) and Optineurin (OPTN). These adaptors bind concomitantly ubiquitinated bacteria, using UBDs, and LC3 family members, promoting xenophagy and degradation of targeted

pathogens [67, 84]. In particular, OPTN has been shown to sense Lys63- and Met1-linked ubiquitin chains present on *Salmonella typhimurium* and subsequently bind LC3 family members, bridging the pathogen to the autophagosome [85, 86]. Accordingly, OPTN dysfunction in mice is associated with increased sensitivity to *Salmonella typhimurium* infection [87].

Transient metals deprivation/accumulation

Transient metals within the cytoplasm and the microenvironment are determinant for host-pathogen interaction. Several metals, such as iron, zinc, manganese and copper, are essential for the metabolic processes and proliferation of microbes. At the same time, these metals are important for the host-microbial response. Indeed, the formation of RNS and ROS is catalysed by these elements. Moreover, their availability affects immune cell proliferation. IFN γ priming of macrophages directly modulates iron levels. This is achieved by inhibiting the expression of the transferrin receptor, which is involved in extracellular iron uptake. To further reduce iron availability in the cytoplasm and phagosomes, IFN γ primed macrophages block the translation of the iron-storing protein ferritin and activate the iron-exporter protein ferroportin. In contrast to iron, copper and zinc uptake is increased in primed and activated macrophages. Copper and zinc catalyse the formation of toxic radicals, therefore they are crucial for antimicrobial properties. Furthermore, the enzyme Natural Resistance-associated Macrophage Protein 1 (NRAMP1), induced by IFN γ treatment, also actively transfers manganese and iron from the phagosome to the cytosol, limiting nutrients for bacterial growth (Figure 1.8) [77, 79].

Bacterial evasion mechanisms

Despite the efficiency of antimicrobial mechanisms, pathogens have developed strategies to survive, such as resistance to antimicrobial factors, interference with phagosome initiation/maturation, the prevention of RNS and ROS formation, and low pH quenching. For the purposes of this thesis, I will focus only on mechanisms related to *Listeria monocytogenes* and *Salmonella typhimurium*.

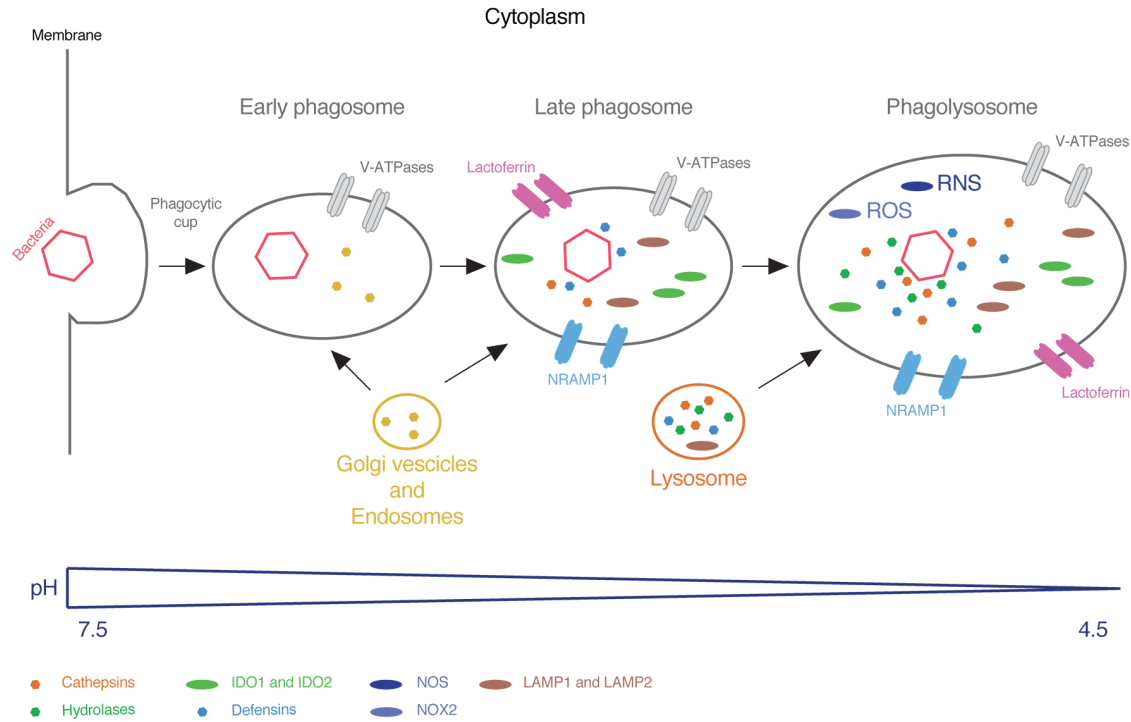


Figure 1.8: Phagocytosis. Schematic representation of phagosome maturation and the antimicrobial activities acquired during this process.

The gram-positive bacterium *Listeria monocytogenes* is engulfed by both professional phagocytic and epithelial cells via scavenger receptors (active internalisation) or internalins (InlA and InlB) (passive internalisation), respectively [88]. During the formation of the early phagosome, *Listeria monocytogenes* can actively create pores in the phagosome membrane through the expression of phospholipases and Listeriolysin-O (LLO), inhibiting its maturation and fusion with endosomes and lysosomes. Moreover, these pores can be used by the bacteria to reach the cytosol where it replicates quicker, with a generation time of about 30 minutes. In addition to cytoplasmic replication, *Listeria monocytogenes* can also replicate within the impaired vacuoles, which are then called Spacious *Listeria*-containing Phagosomes (SLAPs). Replication in SLAPs involves a generation time of around 8 hours. It does not cause the destruction of the host cell, and primarily occurs in macrophages [78, 88].

The formation of SLAPs requires medium-low expression levels of the LLO enzyme, sufficient to impair phagosome acidification avoiding disruption of the

phagosome membrane [88].

Listeria monocytogenes infection occurs through ingestion and commence in the intestine where this pathogen gain access to lymph and blood. This initial phase of infection is crucial to allow *Listeria monocytogenes* dissemination and migration to the liver and the spleen, which are the target organs [88]. In particular, Kupffer cells which are the liver resident macrophages are crucial to control *Listeria monocytogenes* infection and colonisation [78, 88]. Several PRRs are engaged in *Listeria monocytogenes* recognition, such as TLR1, 2, 6, 8, RIG1 and NLRs. The NOD2 receptor signalling pathway has been shown to be required for *Listeria monocytogenes* clearance *in vivo* (Figure 1.9) [89].

Gram-negative *Salmonella typhimurium* is able to induce its own phagocytosis through the expression of SipA and SipC effectors, encoded by the *Salmonella Pathogenicity Island 1* (SPI1), and modulating the actin cytoskeletal reorganisation [90, 91]. Following internalisation, the generation and maturation of Salmonella-containing Vacuoles (SCVs) is achieved by the expression of genes encoded by the *Salmonella Pathogenicity Island 2* (SPI2) [90, 91]. *Salmonella typhimurium* survives and proliferates within phagocytic cells inside SCVs for up to 24 hours. The primary role of SCVs is to protect the bacteria from antimicrobial effectors. In addition, *Salmonella typhimurium* produces a protein called Salmonella Pathogenicity Island C (SPIC) that can directly interfere with SCV-lysosome fusion [92, 93].

Salmonella typhimurium infection occurs through ingestion and lead to acute intestinal inflammation. Following colonisation and crossing of the primary cellular barrier of the gut, composed by intestinal epithelial cells, *Salmonella typhimurium* invades the lamina propria [94]. In the lamina propria *Salmonella typhimurium* is sensed by phagocytic cells (macrophages and dendritic cells) resident in the gut-associated lymphoid tissues [90, 91, 94]. *Salmonella typhimurium* infection can be sensed by macrophages via several PRRs, such as TLR1, 2 and 4 located on the outer membrane. Differently, intracellular colonisation of this gram-negative bacterium can be sensed through TLR3, 7, 8, 9, 11 and NLRs (Figure 1.9) [94].

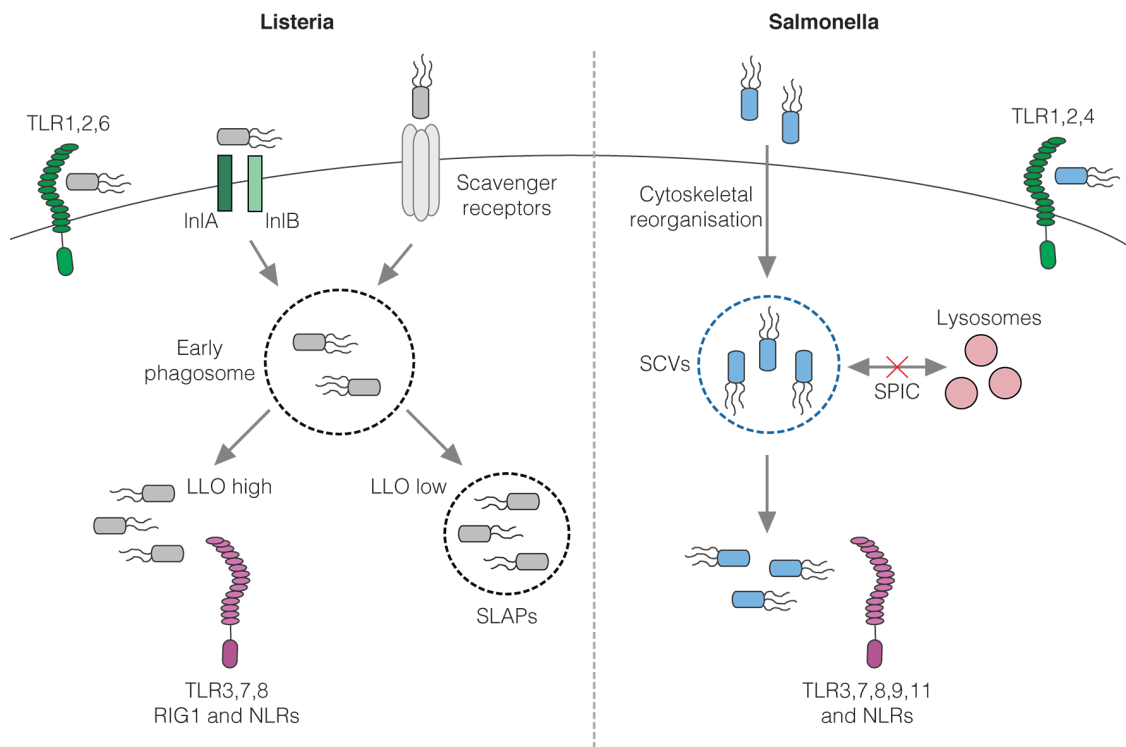


Figure 1.9: Pathogens infection and recognition. Schematic representation of *Listeria monocytogenes* (left) and *Salmonella typhimurium* (right) infection of phagocytic and non-phagocytic cells. Detection of these pathogens occurs through membrane-bound or intracellular innate immune receptors mainly activating NF- κ B and IRFs transcription factors.

1.5 IFN γ signalling

IFN γ is an important cytokine that mediates inflammation and immunity. It is produced by all innate and adaptive immune cells [95]. However, in an inflammatory context, the main producers of this cytokine are macrophages, NK cells, Th-1 and CD8-positive cytotoxic T lymphocytes. In particular, innate and innate-like immune cells' production of IFN γ during infection occurs at early stages, ahead of the antigen-specific adaptive response. Production of this cytokine is induced following the activation of PRRs, or via IL-12 and IL-18 [95, 96]. In addition to the immune compartment, IFN γ production has also been described in mucosal epithelial cells following bacterial infection [97, 98].

IFN γ binds and signals through the IFNGR, which is composed of two subunits (IFNGR1 and IFNGR2) and expressed in all cell types. Following IFN γ binding, the heterodimer IFNGR1-IFNGR2 activates the receptor-associated Janus Kinases (JAK1 and JAK2). These two tyrosine kinases initially phosphorylate the cytoplasmic tails of the receptor, generating a docking site for STAT TFs, which are in turn phosphorylated by JAK1 and JAK2 [66, 95]. JAK1 and JAK2 tyrosine-phosphorylate the STAT1 and STAT3 TFs, allowing their dimerisation and translocation to the nucleus, binding Gamma Activation Sites (GAS) and inducing specific gene transcription. Subsequently, these TFs are dephosphorylated by nuclear phosphatases, inducing their nuclear export and the termination of their transcription (Figure 1.10) [66, 79, 95].

1.5.1 JAK-STAT axis

The mammalian JAK family is composed of four members (JAK1, JAK2, JAK3 and TYK2) and they all mediate hormone- or cytokine-receptor signalling. Mammalian JAKs have four domains: the FERM and the SH2-like domains mediate interaction with the upstream receptor, while the pseudokinase and kinase domains regulate tyrosine phosphorylation [66]. JAKs can directly activate members of the STAT TFs family, which has seven components in mammals: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6. Despite their different roles, all the STAT family

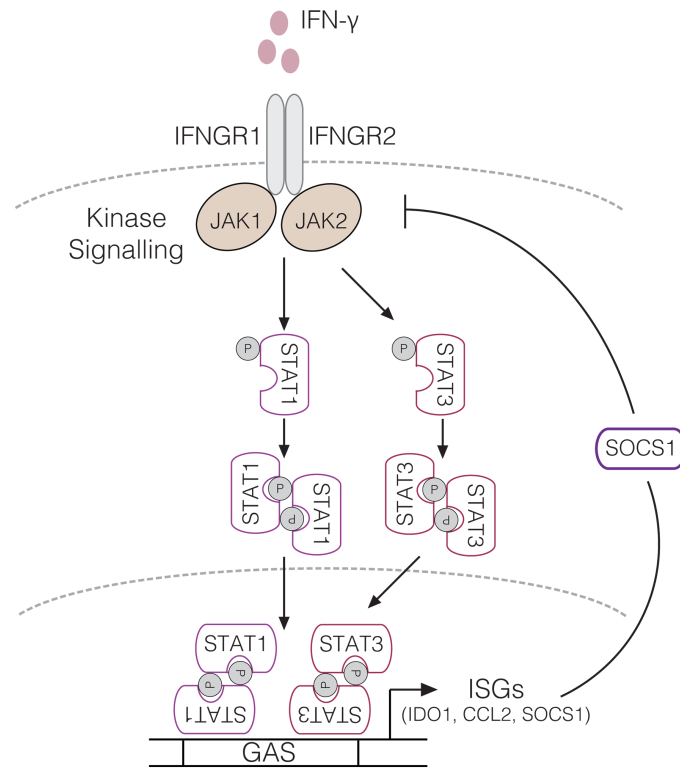


Figure 1.10: IFN γ signalling. Schematic representation of the IFN γ signalling pathway. Gamma Activation Sites (GAS), Interferon Stimulated Genes (ISGs).

members have an N-terminal and a coiled-coil domain involved in protein protein interactions. The coiled-coil domain also contains a Nuclear Localisation Sequence (NLS). The DNA-binding and *linker* domains are important for transcription and affinity for different DNA-regulatory elements. An SH2 domain, similar to that of JAK proteins, is involved in interaction with the receptor complex. Lastly, the trans-activation and C-terminal domains contain the tyrosine residues necessary for activation (Figure 1.11) [66].

STATs have been described to bind largely within enhancers or DNA regulatory elements in promoter areas. Moreover, they can directly cooperate with histone acetyltransferases such as p300 or methyltransferases including Enhancer of Zeste Homolog 2 (EZH2) to remodel the chromatin state. An interesting example is the IFN γ priming of macrophages, where STAT1-mediated recruitment of CREB-binding Protein (CBP)/p300 promotes accessible chromatin in favour of an enhanced and prolonged TLR-mediated transcriptional response [99].

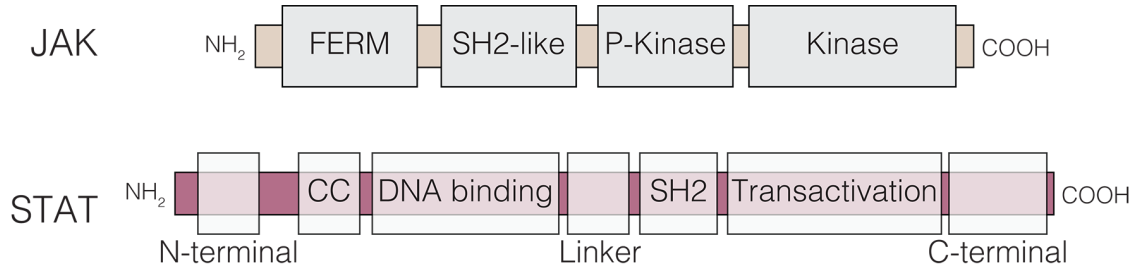


Figure 1.11: JAK and STAT structures. Schematic representation of JAK and STAT protein structures. JAK: FERM, SH2-like, pseudokinase and kinase domains. STAT: N-terminal, Coiled-Coil (CC), DNA-binding, linker, SH2, trans-activation and C-terminal domains.

STAT TFs can cooperate with each other following activation to orchestrate downstream transcriptional responses. Furthermore, they can coordinate and interact with other TFs, either cross-activated or *de novo* synthesised, such as IRFs. The selective binding of STAT family members is determined by the STAT-specific binding motifs, designed on the palindromic consensus sequence TTC(N2-4)GAA; however, in some conditions this specificity is lost, and STATs can bind each other's motifs. In addition, Genome-wide Chromatin Immunoprecipitation and Sequencing (ChIP-Seq) analysis has revealed that many STAT binding sites do not contain STAT sequences, suggesting possible degeneration in their recognition of DNA motifs or alternative recruiting strategies, such as direct binding with other TFs [66, 95].

The JAK-STAT axis is controlled at several levels in terms of activation, fine-tuning and suppression. As mentioned above, tyrosine phosphorylation is the canonical post-translational modification that defines STAT dimerisation and activation. Additionally, serine phosphorylation is important in fine-tuning STAT transcriptional outputs. As an example, STAT1 and STAT3 share the same reported C-terminal site of serine phosphorylation (S727), and mutation of this site results in gene-specific reduction of the IFN γ mediated transcriptional response [100, 101]. TLR signalling also feeds into the IFN γ transcriptional response. STAT1 S727 phosphorylation can be triggered by p38 MAPK via TLRs agonists such as LPS and CpG DNA [102].

Inhibition of STAT signalling occurs through dephosphorylation of activating residues or through the induction of specific repressors, such as members of the

Suppressors of Cytokine Signalling (SOCS) family (SOCS1, SOCS2 and SOCS3). SOCS family members present the SH2 domain and directly bind JAKs at the receptor complex, blocking STAT phosphorylation (Figure 1.10) [66].

1.5.2 STAT1- and STAT3-mediated transcriptional response

Macrophages are the major physiological targets of IFN γ . Indeed, this cytokine was initially described as *macrophages activating factor* [95]. The majority of IFN γ -related studies have been carried out in primary macrophages or cell line models, and its effect has been shown to be a combination of transcriptional, epigenetic and metabolic mechanisms, which last several days post-treatment [95].

IFNGR complex engagement mainly activates STAT1, but also STAT3 to a lesser extent. Following IFN γ stimulation, STAT1 signalling, similarly to STAT3, peaks from 10 to 60 minutes, and ends around 6 hours later. An initial wave of Interferon-stimulated Genes (ISGs) can be identified peaking 1 hour post-treatment, while a second wave starts around 2 hours and escalating for up to 12 hours following stimulation. This second wave, sustained beyond the initial JAK-STAT engagement, relies on cooperation among STATs and *de novo*-transcribed TFs [95, 103]. An example is IRF1 or the IRF1/IRF8 complex, which are both induced following IFN γ stimulation and important in the context of infection [104]. Furthermore, STAT1 directly cooperates with NF- κ B to initiate the transcription of specific genes, such as *NOS* [105].

Since STAT1 is the major TF activated following IFN γ stimulation, STAT1-deficient mice present impaired antiviral and antimicrobial responses when challenged with *mouse hepatitis virus* (MHV) and *Listeria monocytogenes*, respectively [106, 107]. STAT3 activation by the IFN γ receptor complex can occur through JAK or via the SRC tyrosine kinase. Furthermore, STAT3 activation is increased in the absence of STAT1. These two TFs bind very similar but not identical DNA-binding motifs, and they transcriptionally activate both different and overlapping set of genes. A subset of STAT1-dependent genes, including *SOCS3* and *CEBPB*, can become STAT3 targets in the absence of STAT1. In addition, a subset of ISGs,

including *CCL2*, are directly targeted by STAT3 and are unaffected by the absence of STAT1. In conclusion, both the STAT1 and STAT3 pathways are important to the IFN γ transcriptional network, and their induction is dependent on STAT levels and their availability within the cell [103, 108–110]. In addition, several kinases, including AKT, Mitogen-Activated Protein Kinases (MAPKs) and Protein Kinase C (PKC), are activated following IFN γ treatment and are involved in STAT1 serine phosphorylation [108, 109, 111].

STAT1 and STAT3 ISGs mainly encode immune effectors and mediators including cytokines, phagocytic receptors, antigen-presenting molecules, and antibacterial and antiviral factors, that contribute to the primed macrophage phenotype. Furthermore, this transcriptional network switches macrophage *tolerance*, facilitating NF- κ B mediated TLR transcriptional programmes [95]. Therefore, IFN γ signalling is crucial to orchestrate innate and adaptive immunity in an infection-related context. Accordingly, depletion of the *IFNG* gene impairs the clearance of gram-negative *Bordetella pertussis* in mice. Cytosolic replication of gram-negative *Shigella flexneri* in Mouse Embryonic Fibroblasts (MEFs) is inhibited by 24 hours pre-treatment with IFN γ . Moreover, IFN γ -driven autophagy is important for *Burkholderia cenocepacia* and *Mycobacterium tuberculosis* clearance in macrophages [112–115].

1.6 Aims of the study

The aims of this study were threefold:

1 Generation of Lys63- and Met1-specific ubiquitin binders.

1a/ Generate Lys63- and Met1-specific Ub binders with localised cytoplasmic or nuclear activity.

1b/ Investigate the direct role of Lys-63-linked Ub chains within the NOD2 signalling pathway.

2 Investigate CYLD in macrophage differentiation and activation.

2a/ Characterise the mechanisms of CYLD regulation in macrophages during differentiation and in response to activating stimuli.

2b/ Identify the role of CYLD within the IFN γ signalling pathway.

3 Investigate the importance of CYLD during bacterial infection.

3a/ Characterise CYLD-depleted macrophages.

3b/ Evaluate the role of CYLD in intracellular bacterial growth.

3c/ Identify important components underlying CYLD-enhanced intracellular bacterial clearance.

2

Materials

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2.1 Materials

2.1.1 Chemicals, reagents, and consumables

Table 2.1: Reagents used in this study

Reagent	Supplier	Code
2-mercaptoethanol	Gibco	31350-010
4,6-diamidin-2-fenilindolo (DAPI)	Molecular probes	D1306
45 μ m filter (low protein binding)	Sartorius stedim	16555-K
Acetic Acid	Fisher	A/0360/PB17
Acetone	Fisher	A/0600/17
Acrylamide	Severn Biotech	20-210-10
Agarose	Web Scientific	AGR-500
Ammonium peroxide sulphate (APS)	-	-

Continuation of Table 2.1

Reagent	Supplier	Code
Ampicillin	Sigma-Aldrich	A9518
Anchored oligoes	Sigma	T ¹⁸ VN
Anti-c-MYC magnetic beads	Thermo	88842
Anti-V5 agarose	Sigma	A7345
Blotting paper	GE healthcare	3030-917
Bovine serum albumin (BSA)	Sigma	A9647
Brefeldin A	BioLegend	420601
Bromophenol blue	Fisher	BP510-500
Complete protease inhibitor cocktail	Roche	04 693 124 001
Dimethyl sulphoxide (DMSO)	Sigma	D2650
Dithiothreitol (DTT)	Sigma	43819
DMEM media	Gibco	31966-021
dNTPs	NEB	N0446S
ECL developing solution	Amersham	RPN2209
ECL enhancer solution	Amersham	RPN2235
Ethanol	Merck	32221
Ethylendiaminetetraacetic acid (EDTA)	Fluorochem	239931
Fixation buffer	Biolegend	420801
Foetal bovine serum (FBS)	Labtech	FCS-SA
Formaldehyde 16 % (w/v)	Thermo	28908
FLAG peptide	Sigma	F3290
Fludarabine	Selleckchem	S1491
Fugene 6	Promega	E2692
Fugene HD	Promega	E2312
Gel Filtration Markers Kit	Sigma	MWGF1000
Glutathione Sepharose 4 Fast Flow	GE healthcare	17-5132-02
GlutaMAX	Gibco	35050-038
Glycerol	Honeywell	G7757
Glycine	Fisher	G/0800/60
Gentamicin	Sigma	G1522
Hydrochloric acid (HCl)	Fisher	H/1200/PB15
Hygromycin B	Invitrogen	10687010
IGEPAL CA-630 (NP-40)	Sigma-Aldrich	I3021
Isopropanol	Merck	24137
L18-Muramyl dipeptide (L18-MDP)	Invivogen	tlrl-lmdp
Latex beads fluorescent	Sigma-Aldrich	L4530
Lipofectamine RNAiMAX	Invitrogen	100014472
Lipopolysaccharide LPS-EK Ultrapure from E.coli	Invivogen	EK12
McCoy's 5A	Gibco	36600-021
MES running buffer	Invitrogen	NP0002-02
Methanol	Honeywell	32213
Micro BioSpin columns	BioRad	7326204

Continuation of Table 2.1

Reagent	Supplier	Code
Microscopy cover glasses	Marienfeld	0112520
Microscopy slides	Thermo	AGAA00008
Milk powder	Marvel	3023034
Manganese Chloride (MnCl ₂)	NEB	B1761S
Monensin	BioLegend	420701
MOPS running buffer	Invitrogen	NP0001-02
N-Ethylmaleimide (NEM)	Sigma	E1271
Necrostatin - 1s	Biovision	2263
NheI-HF	NEB	R3131S
Nitrocellulose membrane	GE Healthcare	10600002
NuPAGE 4-12% Bis-Tris midi gels	Invitrogen	WG1403BX10
OptiMEM	Gibco	31985
Oxozeaeonol	Tocris	3604
PBS (10x)	Fisher	BP399-20
PBS (Sterile)	Gibco	14190-094
Penicillin/Streptomycin (P/S)	Gibco	15070
Pentadecamers	IDT	169190224
peqGREEN DNA dye	Peqlab	37-5010
Phorbol 12-myristate 13-acetate	Sigma	P8139
PhosSTOP inhibitors	Roche	04 906 837 001
Phusion polymerase	NEB	M0530L
Phusion polymerase reaction buffer (5x)	NEB	B0518S
PMP buffer	NEB	B0761S
Polyethylene glycol (PEG)	Aldrich	P2139
Ponceau stain	Sigma	P7170
Potassium chloride (KCl)	Sigma	P9541
ProLong Gold Antifade Mountant	Thermo	P36930
Protein A/G PLUS beads	Santa cruz	sc-2003
Proteinase K	Axxora	MBI-19900
Puromycin	Invivogen	ant-pr
PVDF membrane	GE healthcare	10600023
RevertAID reverse transcriptase	Thermo	EP0441
Recombinant human GM-CSF	Peprotech	300-03
Recombinant human IL-6	Peprotech	200-06
Recombinant human IL-10	Peprotech	200-10
Recombinant human IFN- γ	Peprotech	300-02
Recombinant murine IFN- γ	Peprotech	315-05
RiboLock	Thermo	EO0381
RPMI medium	Gibco	42401-018
RT buffer (5x)	Thermo	LT-02241
S.O.C. medium	Invitrogen	15544-034
SalI-HF	NEB	S3138S
Saponin	Fluka	47036

Continuation of Table 2.1

Reagent	Supplier	Code
SeeBlue protein standard	Invitrogen	LC5925
Sodium azide	Sigma	S2002
Sodium chloride (NaCl)	SLS	CHE3316
Sodium dodecyl sulphate (SDS)	Fisher	1281-1680
Sodium phosphate dibasic heptahydrate (Na ₂ HPO ₄)	Sigma-Aldrich	S9390
Sodium phosphate monobasic monohydrate (NaH ₂ PO ₄)	Sigma-Aldrich	S9638
Sodium pyruvate	Gibco	11360-039
STAT3 inhibitor BP1-102	Selleckchem	S7769
Streptavidin magnetic beads	Thermo	88817
T4 DNA ligase	NEB	M0202L
T4 ligation buffer (10x)	NEB	B0202S
Tetramethylethylenediamine (TEMED)	Amresco	0761
TNF (recombinant human)	Peprtech	300-01A
Tris base	Fisher	BP152-1
Triton X-100	Sigma	T9284
Trypsin-EDTA	Gibco	25300-054
Tween-20	Fisher	BP337-500
X-ray film	Fujifilm	47410 19289

2.1.2 Buffers and solutions

Table 2.2: Buffers used in this study

Buffer	Composition
1 % agarose gel	1 % w/v agarose powder in 1 x TAE buffer (boiled until homogenous) plus 2-3 μ l/50 ml peqGREEN
Blocking solution	5 % Milk powder in PBS-Tween
Laemmli sample buffer	50 mM Tris (pH 6.8), 10 % Glycerol, 100 mM DTT, 2 % SDS, 0.1 % Bromophenol blue
Perm/wash buffer	2 % FBS, 0.1 % saponin, 0.1 % NaN ₃ in PBS
Phosphate buffered saline (PBS)	1.47 mM KH ₂ PO ₄ , 2.68 mM KCl, 136.9 mM NaCl, 7.97 mM Na ₂ HPO ₄
PMP buffer (no salt)	50 mM HEPES, 2 mM DTT, 0.01 % Brij 35, pH 7.5
RIPA lysis buffer	20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 % NP-40, 1 % Sodium deoxycholate, 1 mM β -glycerophosphate supplemented with 5 mM NEM, 1 mM DTT, cOmplete protease inhibitor cocktail and phosSTOP

Continuation of Table 2.2

Reagent	Supplier
SDS running buffer	192 mM Glycine, 25 mM Tris base, 0.1 % SDS
TAE buffer	40 mM Tris base, 1 mM EDTA-Na, 20 mM acetic acid, pH 8.5
TBS + 0.5 % NP-40 lysis buffer	TBS and 0.5 % NP-40 supplemented with 5 mM NEM, 1 mM DTT, Complete protease inhibitor cocktail and phosSTOP
Transfer buffer	25 mM Tris, 192 mM glycine, 10 % ethanol
Tris-buffered saline	50 mM Tris-Cl (pH 7.5), 150 mM NaCl
TUBE lysis buffer	20 mM Na ₂ HPO ₄ , 20 mM NaH ₂ PO ₄ , 1 % NP-40, 2 mM EDTA supplemented with 5 mM NEM, 1 mM DTT, cOmplete protease inhibitor cocktail and phosSTOP

2.1.3 Commercial kits

Table 2.3: Commercial kits used in this study

Kit	Supplier	Code
MycoAlert mycoplasma detection kit	Lonza	LT07-318
Nucleospin gel and PCR clean-up	Machery-Nagel	740609.250
Nucleospin plasmid	Machery-Nagel	740588.250
Purelink HiPure Midiprep kit	Invitrogen	K210014
Qiaprep spin miniprep kit	Qiagen	27106
Quick-gDNA Blood prep Microprep	Zymo research	D3071
RNase-free DNase set	Qiagen	79254
RNeasy Mini kit	Qiagen	74106
SYBR Select master mix	Applied Biosystems	4472908
ROS Detection Cell-Based Assay Kit (DCFDA)	abcam	ab113851

2.1.4 Antibodies

Table 2.4: Antibodies used in this study

Antibody	Host species	Supplier / Code
anti-Actin	Mouse	Millipore / MAB1501
anti-CYLD	Mouse	Santa cruz / sc- 74435
anti-CYLD	Rabbit	Cell signalling / 8462
anti-HOIP/RNF31	Rabbit	Aviva / ARP43241
anti-HOIP/RNF31	Sheep	ReD / AF8039
anti-I κ B α	Rabbit	Cell signalling / 9242
anti-IKK γ /NEMO	Rabbit	Santa cruz / sc-8330

Continuation of Table 2.4

Antibody	Host species	Supplier / Code
anti-CXCL-8 (APC conjugated)	Rabbit	BioLegend / 511410
anti-p38 MAPK	Rabbit	Cell signalling / 9212
anti-phospho-p38 MAPK	Rabbit	Cell signalling / 4511
anti-p65	Rabbit	Cell signalling / 8242
anti-phospho p65	Rabbit	Cell signalling / 3033
anti-RIPK2	Rabbit	Santa cruz / sc-22763
anti-Ubiquitin (P4D1)	Mouse	Cell signalling / 3936
anti-STAT1 (p91)	Rabbit	Santa cruz / E23 sc-346
anti-phospho-STAT1 (Y701)	Goat	Santa cruz / sc-7988
anti-phospho-STAT1 (Y727)	Goat	Santa cruz / sc-16570
anti-STAT3	Rabbit	Santa Cruz / sc-482
anti-STAT3	Mouse	Cell Signalling / 9139S
anti-phospho-STAT3 (Y705)	Rabbit	Cell signalling / D3A7 9145
anti-phospho-STAT3 (S727)	Rabbit	Cell signalling / 9134S
anti-OPTN	Rabbit	Abcam / ab23666
anti-NOD2	Rabbit	Sigma / PRS2513
anti-NOD2	Mouse	Millipore / (2D9) 04-145
anti-TetR	Rabbit	Novus Biological NBP1-78424
anti-mouse-HRP	Goat	Dako / P0447
anti-rabbit-HRP	Goat	Bio-Rad / 1706515
anti-rat-HRP	Goat	ThermoFisher / 31470
anti-sheep-HRP	Donkey	ReD systems / HAF016
anti-mouse Alexa Fluor 594	Goat	Thermo
anti-rabbit Alexa Fluor 546	Goat	Thermo
anti-rabbit Alexa Fluor 488	Goat	Thermo

2.1.5 Plasmids

Table 2.5: Plasmids used in this study

Insert	Backbone	Source
GFP	pEGFP-C1	Gyrd-Hansen lab
GFP-K63-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-M1-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-NZF-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-NLS	pEGFP-C1	Gyrd-Hansen lab
GFP-NLS-K63-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-NLS-M1-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-NES	pEGFP-C1	Gyrd-Hansen lab
GFP-NES-K63-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-NES-M1-SUB	pEGFP-C1	Gyrd-Hansen lab
GFP-NLS	pDestination Tet	Gyrd-Hansen lab

Continuation of Table 2.5

Insert	Backbone	Source
GFP-NLS-K63-SUB	pDestination Tet	Gyrd-Hansen lab
GFP-NLS-M1-SUB	pDestination Tet	Gyrd-Hansen lab
GFP-NES	pDestination Tet	Gyrd-Hansen lab
GFP-NES-K63-SUB	pDestination Tet	Gyrd-Hansen lab
GFP-NES-M1-SUB	pDestination Tet	Gyrd-Hansen lab
FLAG-CYLD	CMV2	Gyrd-Hansen lab
FLAG-CYLD ^{C601A}	CMV2	Gyrd-Hansen lab
FLAG-CYLD	pBABE	Gyrd-Hansen lab
FLAG-CYLD ^{C601A}	pBABE	Gyrd-Hansen lab
ShCTR Puro	pTRIP	Gyrd-Hansen lab
ShCYLD Puro	pTRIP	Gyrd-Hansen lab
ShCTR-GFP	pTRIP	Gyrd-Hansen lab
ShCYLD-GFP	pTRIP	Gyrd-Hansen lab
pLVX-Tet-On Advanced	pLVX	Clontech
pLVX-Tight-Puro	pLVX	Clontech
pDONR 221	pDONR	Invitrogen

2.1.6 Oligos

Table 2.6: qRT-PCR oligos used in this study

Name	Sequence
hHPRT	F - 5'-AGCCAGACTTTGTTGGATTTG-3' R - 5'-TTTACTGGCGATGTCAATAGG-3'
hTNF	F - 5'-TGCTGCAGGACTTGAGAAGA-3' R - 5'-GAGGAAGGCCTAAGGTCCAC-3'
hCXCL8	F - 5'-TCTGGCAACCCTAGTCTGCT-3' R - 5'-AAACCAAGGCACAGTGGAAC-3'
hGABARAPL1	F - 5'-AGGGTCCCCGTGATTGTAGA-3' R - 5'-TAAGGCGTCCTCAGGTCTCA-3'
hGABARAP	F - 5'-CAGAACCCCAGCAATCCCTT-3' R - 5'-GGGAGCAACACAAGGCAGAAA-3'
hGABARAP	F - 5'-CAGAACCCCAGCAATCCCTT-3' R - 5'-GGGAGCAACACAAGGCAGAAA-3'
hMAP1LC3C	F - 5'-TACATGACCTACGCCTCCCA-3' R - 5'-GACACGTCTGTCAGAGCACA-3'
hMAP1LC3B	F - 5'-ACTGCAGAGCCCGTTTCTTT-3' R - 5'-CCTGTGCCATTTACTGCCAC-3'
hRIPK2	F - 5'-CTGGATCTCAAAGGGCTGCA-3' R - 5'-TTGCTCTGGATCCACTGCTG-3'
hNOD2	F - 5'-CATCCTGGAAGTCTGGCTCC-3' R - 5'-ACATCCTCCCGGAGACTTCA-3'
hOPTN	F - 5'-AAGGCTCTGGCTTCCAAACA-3' R - 5'-ATCTGAGCCCTGAGGATGGT-3'
hIRF1	F - 5'-GAAGGCCAACTTTCGCTGTG-3' R - 5'-CTCCTTCTACCCTCCCTCCC-3'
hIL-1 β	F - 5'-CCAGCTACGAATCTCCGACC-3' R - 5'-GGGAAAGAAGGTGCTCAGGT-3'
hIDO1	F - 5'-GCACCAGAGGAGCAGACTAC-3' R - 5'-TGGATTTGGCAGAGCAAAGC-3'
hMRC1	F - 5'-GATTTTAACGGGTGCTGGCC-3' R - 5'-CCTGGGCTTGACTGACTGTT-3'
hCCR7	F - 5'-TGCCTGTGTCAAGATGAGGT-3' R - 5'-AAGTTCCGCACGTCCTTCTT-3'
hMAF	F - 5'-AGCTGGTGACCATGTCTGTG-3' R - 5'-CTTGAGGTGGTCGACTTGCT-3'
hIRF2	F - 5'-TCTCCCAGGGCTACTCACTC-3' R - 5'-ACTGCAATGTGCTAGTGCT-3'
hIRF3	F - 5'-AGGGCCTTGGTAGAAATGGC-3' R - 5'-AATCCATGCCCTCCACCAAG-3'
hIRF9	F - 5'-GCTCCAGCCATACTCCACAG-3' R - 5'-GGCTCTACACCAGGGACAGA-3'

Continuation of Table 2.6primers

Name	Sequence
hSOCS1	F - 5'-GGTCCCCCTGGTTGTTGTAG-3' R - 5'-ACCCCTGGTTTGTGCAAAGA-3'
mRIPK2	F - 5'-CTGGAAACCACAGCTGGGAT-3' R - 5'-TACAGCCAAGGAACACGAGG-3'
mOPTN	F - 5'-ATTCACTCCTGCCCCAAGTG-3' R - 5'-ACCAAGAGCTTTGGGGACTG-3'
mNOD2	F - 5'-ACTTTTCCCGAGGAGTCGTG-3' R - 5'-TTTAGAGGCAGAAACGCGGT-3'
mCCL2	F - 5'-CAGCCAGATGCAGTTAACGC-3' R - 5'-GCTGCTGGTGATCCTCTTGT-3'
m18S	F - 5'-GTAACCCGTTGAACCCCAT-3' R - 5'-CCATCCAATCGGTAGTAGCG-3'

2.1.7 Cell lines

Parental cell lines

Cell line	Cell type	Source
THP-1	Human monocytic leukemic cell line	Gyrd-Hansen lab
U937	Human monocytic lymphoma cell line	Gyrd-Hansen lab
Cyld ^{-/-} wt MEF	Mouse embryonic fibroblasts	Maurer lab
HCT-116	Human colonic cell line	Gyrd-Hansen lab
HEK293T	Human embryonic kidney cells	Gyrd-Hansen lab
U2OS/NOD2	Osteosarcoma cells	Gyrd-Hansen lab

Table 2.7: Parental cell lines used in this study

Genetically modified cell lines

Table 2.8: Self-generated modified cell lines

Cell line	Cell type
THP-1-ShCTR	THP-1
THP-1-ShCYLD	THP-1
THP-1-ShCTR-GFP	THP-1
THP-1-ShCYLD-GFP	THP-1
THP-1-ShCTR-GFP-ShOPTN	THP-1
THP-1-ShCYLD-GFP-ShOPTN	THP-1
THP-1 ^{GFP-NES}	THP-1
THP-1 ^{GFP-NES-K63}	THP-1
THP-1 ^{GFP-NES-M1}	THP-1
U937-ShCTR	U937

Continuation of Table 2.8primers

Cell line	Cell type
U937-ShCYLD	U937
U2OS ^{CYLD-WT}	U2OS
U2OS ^{CYLD-KO}	U2OS
U2OS ^{CYLD-C601A}	U2OS
MEF ^{CYLD-KO}	Cyld ^{-/-} wt MEF
MEF ^{CYLD-WT}	Cyld ^{-/-} wt MEF
MEF ^{CYLD-C601A}	Cyld ^{-/-} wt MEF
HCT-116-HOIP ^{-/-}	HCT-116

3

Methods

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3.1 Cell biology

3.1.1 Cell culture

Cultivation of cell lines was performed under sterile conditions at 37 °C and 5 % CO₂. THP-1 and U937 cells were grown in suspension using Roswell Park Memorial Institute (RPMI) medium supplemented with 10 % v/v foetal calf serum (FCS), 60 µg/ml penicillin/100 µg/ml streptomycin (P/S), 50 µM 2-mercaptoethanol, 1 mM Sodium pyruvate and GlutaMAX. Cells were counted every two days and kept at a density between 500,000 and 1,000,000 cells/ml adding fresh media. Once every two weeks cells were passaged into a new flask. U2OS/NOD2 cells, HEK 293 cells, mouse embryonic fibroblasts (MEFs), and all genetically modified variants of these lines were grown as a monolayer using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % v/v foetal calf serum (FCS) and 60 µg/ml penicillin/100 µg/ml streptomycin (P/S). Cells were passaged every 2-4 days to maintain them at a density not exceeding 95 % confluency in the flask (T 75 or T 175). HCT-116 cells and genetically modified variants of this line were grown as a monolayer using McCoy's 5A (Modified) Medium, (GlutaMAX) supplemented with 10 % v/v foetal calf serum (FCS) and 60 µg/ml penicillin/100 µg/ml streptomycin (P/S). Cells were passaged every 2-4 days to maintain them at a density not exceeding 95 % confluency in the flask (T 75 or T 175). Splitting of cells consisted of discarding existing media, washing with phosphate-buffered saline (PBS) and adding Trypsin/EDTA solution (4 ml/T 175 or 15 cm plate, 2 ml/T 75 or 10 cm plate). Cells with trypsin solution were incubated for up to 10 minutes in the 37 °C incubator to allow detachment from the plate followed by re-suspension in DMEM medium for experimental use or re-plating at a ratio between 1:6 and 1:20. Once per month, all cell lines were re-plated into new flasks.

3.1.2 Transient transfection of cells

Transfection of DNA with FuGENE 6/HD reagents

FuGENE 6 (Promega) was used for transfection of U2OS/NOD2 cell lines whereas FuGENE HD (Promega) was used for the transfection of HEK293 cells. For both FuGENE 6 and FuGENE HD, the transfection reagent to DNA ratio was kept consistent at 3:1 for the number of cells transfected (see table 3.1).

Plate	DNA (μg)	FuGENE 6/HD (μl)	OptiMEM (μl)
12 well	0.2	0.6	2 x 50
6 well	1	3	2 x 100
10 cm	3.5	10	2 x 500

Table 3.1: Volumes/concentrations of FuGENE 6/HD transfection mix

For transfection, cells were plated to allow for around 60 % confluency on the day of transfection. The transfection mix was prepared through the separate dilution of DNA and FuGENE 6/HD to optiMEM and each were thoroughly mixed. After incubation for 5 minutes, the two solutions were mixed and incubated for a further 10 minutes at room temperature before dropwise addition to the cells. Fresh media was added after 24 hours. 48 hours after initial transfection cells were stimulated/harvested for experimental use.

3.1.3 Macrophages differentiation and activation *in vitro*

THP-1 and U937 human monocytic cells were differentiated into macrophages *in vitro* following 72 hours incubation with Phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and 24 hours rest in fresh media. PMA concentration and exposure time were optimised based on cellular morphology, observed via microscopy, and expression levels of differentiation markers, measured through qRT-PCR. To obtain activated macrophages, following 72 hours incubation with PMA, fresh media was added containing LPS (50 ng/ml) and IFN- γ (20 ng/ml) for 2 hours and then replaced with fresh media for 22 hours. Macrophage activation was monitored via cellular morphology and expression levels of macrophage activation markers through qRT-PCR (Figure 5.1).

Following 72 hours of PMA treatment cells were always washed twice with fresh media to remove possible residual PMA. Similarly, following 2 hours of LPS, IFN- γ , IL-6 or IL-10 stimulation, cells were washed twice with fresh media and kept in fresh media for subsequent time points. *De facto*, constant exposure to LPS, IFN- γ , IL-6 or IL-10 in all experiments is never longer than 2 hours.

Production of lentivirus

Lentiviral particles were used to induce the stable expression of non-targeting and CYLD-specific ShRNA in THP-1 and U937 cells. Moreover, lentivirus were used to stably express the Tet-repressor construct and NES or NLS variants of linkage-specific ubiquitin binders (GFP, GFP-K63-SUB, GFP-M1-SUB) in THP-1 cells.

Production of viral particles was performed in the packaging cell line HEK-293T. These cells were plated into 10 cm dishes containing 10 ml of media and, at around 60 % confluency, transfected with the psPAX packaging vector, the pMD.G envelope vector and the desired lentiviral vector as detailed in table (see table 3.2).

Reagent	Volume / Amount
psPAX packaging vector	6 μ g
pMD.G envelope vector	1.5 μ g
Lentiviral vector	4.5 μ g
FuGENE HD	36 μ l
OptiMEM	1.5 ml

Table 3.2: Volumes / quantities used for the generation of lentivirus in HEK293T cells

24 hours after transfection, 10 ml of fresh media were carefully added to the cells. Cells were then incubated for 72 hours in order to allow for viral particles production. Subsequently, the media was harvested and debris removed through centrifugation 4000 x g for 15 minutes. 8.5 ml of media were mixed with 0.5 ml 3 M NaCl and 1 ml PEG-8000 to allow viral particles precipitation through agitation during overnight (O/N) incubation at 4 °C. Following this, the mix was centrifuged at 3500 x g for 15 minutes at 4 °C to pellet the virus. The supernatant was carefully removed and the viral pellet was resuspended in 100 μ l of sterile PBS before snap freezing with liquid nitrogen (LN₂).

Transduction of cells

50,000 cells were plated into a 12 well plate containing 1 ml of media. Infection was performed adding 10 $\mu\text{g}/\text{ml}$ of polybrene and four different dilutions of the concentrated virus stock (2 to 5 μl). Spin infection was performed at 1000 x g for 90 minutes and after O/N incubation fresh media was added the next day. Selection was started 72 hours post-infection using puromycin (1 $\mu\text{g}/\text{ml}$) or blasticidin S (10 $\mu\text{g}/\text{ml}$) and continued until control cells had all died.

3.1.4 Stable knockdown of genes by ShRNA

ShRNA-mediated gene silencing was performed through lentiviral transduction of the construct containing the desired ShRNA. In particular, suspension cell lines, such as THP-1 and U937, were incubated with 2 to 5 $\mu\text{l}/\text{ml}$ of concentrated virus and 10 $\mu\text{g}/\text{ml}$ of polybrene for 24 hours, including an initial round of spin infection performed at 1000 x g for 90 minutes. Following 24 hours incubation with the virus, the media was replaced with fresh media for 48 hours. Subsequently, selection of successfully transduced cells was performed with Puromycin (1 $\mu\text{g}/\text{ml}$) until control cells had all died.

All the stable knockdowns have been initially performed with 5 different ShRNA as described above. Knockdown efficiency was then tested via western blotting and the ShRNA presenting the most efficient knockdown was used for further experiments.

ShCTR- and ShCYLD-GFP THP-1 cells were generated as described above. In lack of a selection marker, following transduction ShCTR and ShCYLD infected cells were sorted based on GFP expression using the Sh800 Sony Sorter. The 10 % of cells with highest expression of GFP was sorted for both samples (ShCTR and ShCYLD) and then expanded. Knockdown efficiency was measured via western blot analysis.

3.1.5 Generation of THP-1 with doxy-inducible expression of ubiquitin binders

Expression of NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB was performed through lentiviral transduction, using the Lenti Tet-On Advanced Inducible Expression System from Clontech. The constructs were cloned into the inducible expression system using Gateway cloning according to the manufacturer's protocol. In particular, NLS or NES variants of ubiquitin binders and GFP were initially amplified via attb-flanked PCR in order to be inserted in a pDONR vector through Gateway cloning using the enzyme BP clonase. Subsequently, the pDONR vectors containing the ubiquitin binders were incubated with the pLVX-Tight-Puro destination vector, together with the enzyme LR clonase. With this second Gateway step ubiquitin binders were cloned within the pLVX-Tight-Puro vector. This vector presents a cytomegalovirus (CMV) promoter with Tet-responsive elements, allowing transcription only in the co-presence of Tet protein and doxycycline. Lentiviral particles containing the pLVX-Tight-Puro vectors with the ubiquitin binders and GFP were generated as described above. In parallel, a lentiviral vector containing the Tet protein (pLVX-Tet-On Advanced) was generated in the same way. THP-1 monocytic cells were initially transduced with pLVX-Tet-On Advanced lentivirus and selected with blasticidin (10 $\mu\text{g}/\text{ml}$). Following selection, single clones were grown and expression of the Tet protein was evaluated via western blotting. The clone with the highest expression of the Tet protein (THP-1^{Tet}) was used for generation of inducible cell lines. (THP-1^{Tet}) were subsequently transduced with pLVX-Tight-Puro vectors containing NLS or NES variants of ubiquitin binders and selected with puromycin (1 $\mu\text{g}/\text{ml}$). Inducible expression of NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB was monitored via flow cytometry following addition of increasing concentration of doxycycline 50 to 500 $\mu\text{g}/\text{ml}$. 500 $\mu\text{g}/\text{ml}$ was observed to be the best concentration to induce a strong expression of the constructs between 24 and 48 hours post treatment.

3.1.6 Generation of ShCTR- and ShCYLD-THP-1 with expression of mCherry-EGFP-LC3

Lentiviral particles containing the mCherry-EGFP-LC3 construct were generated as reported above. ShCTR- and ShCYLD-THP-1 cells were transduced with 5 μ l/ml of concentrated virus and 10 μ g/ml of polybrene for 24 hours, including an initial round of spin infection performed at 1000 x g for 90 minutes. Following 24 hours incubation with the virus, the media was replaced with fresh media for 48 hours. Subsequently, due to the impossibility of using puromycin as a selection marker (ShCTR- and ShCYLD-THP-1 cells were previously selected with puromycin) infected cells were sorted based on GFP expression using the Sh800 Sony Sorter. The 10 % of cells with highest expression of GFP was sorted for both samples (ShCTR and ShCYLD) and then expanded.

3.1.7 Bright-field microscopy

Bright-field microscopy of live cells was performed using the Olympus CKX41 microscope endowed with camera and linked to the Image J/Fiji software Micro Manager.

3.1.8 Confocal microscopy staining

Confocal microscopy imaging, using the Zeiss LSM 710, was used to monitor cellular localisation of NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB. Moreover, this technique was used to evaluate RelA nuclear translocation following NOD2 receptor activation. 100,000 to 300,000 cells were plated in a 12 well plate with 1 ml of media on 13 mm microscopy cover glasses with thickness grade 1.5, previously washed with 70 % ethanol and rinsed with media. Following seeding, cells can be treated, transduced or transfected according to the experiment. Subsequently, cells were washed with PBS and fixed in 800 μ l of 4 % formaldehyde (FA) pre-diluted in PBS for 15 minutes at room temperature. Cells were washed with PBS and permeabilized with 500 μ l of 0.1 % Triton-X in PBS for 10 minutes. Cells were washed with PBS and blocked with 500 μ l of 5 % BSA/0.01 % Triton-X in PBS for 1 hour at room temperature. Primary antibody staining was performed on an even

parafilm layer with 100 μ l of primary antibodies diluted 1:500 in 1 % BSA/0.01 % Triton-X in PBS O/N at 4 °C. Secondary antibody staining was performed diluting the secondary conjugated antibodies 1:1000 in PBS and using 1 ml of this solution for each microscopy cover glass, incubating for 1 hour at room temperature. Cells were washed twice with PBS and 4,6-diamidin-2-fenilindolo (DAPI) staining was performed in 1 ml of 1 μ g/ml DAPI in PBS solution for 10 minutes. Cells were then washed with PBS and mounted on glass slides using 8 μ l of ProLong Gold mounting media and left O/N at room temperature to dry.

In case of imaging for endogenous GFP- or mCherry-labelled constructs, following fixation in FA the microscopy cover glasses can be stained with DAPI and mounted directly on the glass slides.

3.1.9 Intracellular cytokine staining

Flow cytometry was used for the detection of intracellular cytokines. Cells were cultured in 12 well plates and grown to 90 % confluency. Cells were stimulated for 5-6 hours with desired ligand in presence of 5 μ g/ml brefeldin A and 2 μ M monensin protein transport inhibitors. Non stimulated cells were only incubated with 5 μ g/ml brefeldin A and 2 μ M monensin for 5-6 hours. Subsequently, cells were washed once with PBS, dissociated with Trypsin/EDTA solution and collected by centrifugation of 300 x g for 5 minutes. Cells were then fixed with 150 μ l of IC fixation buffer (Biolegend) O/N at 4 °C, washed with PBS, and permeabilised with perm/wash buffer. After a further centrifugation of 600 x g for 3 minutes at 4 °C, cells were incubated for 1 hour at RT with perm/wash buffer containing anti-CXCL-8/APC (Biolegend: 100 x), or anti-IL-1 β /Alexa 647 (Biolegend: 100 x), or anti-IFN- γ /FITC (Biolegend: 100 x), or anti-TNF- α /PE-Cyanine7 (Biolegend: 100 x) antibodies. Following incubation, cells were washed once with perm/wash buffer and resuspended in PBS for analysis by flow cytometry (FACS Canto Flow cytometer, BD Biosciences, San Jose, CA). Analysis of the data was performed with FlowJo software (TreeStar, Ashland, OR), and statistical analysis by two-way ANOVA in Prism 6 (GraphPad Software Inc).

3.1.10 RNA isolation, cDNA synthesis, and qRT-PCR

Cells were plated into 12 well plates and, at around 90 % confluency, stimulated with selected ligand for desired time points (IFN- γ , LPS, IL-6 and IL-10 stimulation have been always performed for a maximum of 2 hours and then fresh media was added for further time points. Diversely, L18-MDP stimulation was performed constantly for indicated time without adding fresh media). Lysis of cells and extraction of RNA was performed using an RNeasy Mini kit (Qiagen) and on-column Dnase digestion was performed with the RNase-free Dnase Set (Qiagen) according to the manufacturer's protocol. Following extraction, total RNA was reverse transcribed using RevertAidTM reverse transcriptase (Thermofisher) and a mixture of anchored oligo (dT)20 primers and random pentadecamers alongside RiboLock (Thermofisher) as per table 3.3. As indicated, 3.5 μ l of mix 1 was added to 10 μ l of extracted RNA and incubated at 65 °C for 5 minutes. Samples were immediately put on ice and 6.5 μ l of mix 2 was added followed by heating to 25 °C for 10 minutes, 42 °C for 60 minutes, and 70 °C for 10 minutes. Resulting cDNA was diluted and qRT-PCR was performed using the SYBR Select Master Mix (Applied Biosystems), primer pairs for genes of interest (plus HPRT or 18S as internal control) as detailed in table 2.6, and RNase free water as per table 3.3.

Mix (per sample)	Reagent	Volume
cDNA mix 1 (3.5 μ l)	Pentadecamers (10 μ M)	1 μ l
	Anchored oligo dT primes (100 μ M)	0.5 μ l
	dNTPs (10 mM)	2 μ l
cDNA mix 2 (6.5 μ l)	RT buffer (5 x)	4 μ l
	RiboLock	0.5 μ l
	RevertAid TM reverse trascriptase	0.5 μ l
	RNase-free H ₂ O	1.5 μ l
qRT-PCR mix	cDNA	1 μ l
	SYBERGreen mix	5 μ l
	Primer pairs (Forward and Reverse - 20 μ M)	1 μ l
	RNase-free water	3 μ l

Table 3.3: Volumes/quantities of reagents used for cDNA synthesis and qRT-PCR

The qRT-PCR mix was pipetted into 384 well plates and run as per table 3.4 on a Lightcycler[®] 480 II qRT-PCR machine (Roche). The resulting cycle

values (Ct values) were analysed by calculating the deltaCt and deltadeltaCt values through normalisation to the HPRT in human samples or 18S in mouse control, and subsequently to the parental, WT or unstimulated condition (referred as "Fold induction"). In case of monocyte-to-macrophage differentiation the resulting cycle values (Ct values) were analysed by calculating the deltaCt and deltadeltaCt values through normalisation to the HPRT control and subsequently to the control monocytic state prior PMA-mediated differentiation in order to be aware of differences in transcriptional induction during differentiation (referred as "Fold over Monocytes"). Graphpad Prism 6 was used for plotting the relative fold changes as well as performing statistical tests. For qRT-PCR data, Two-way ANOVA test was used to determine significance where sample number was sufficient, otherwise One-way ANOVA test or unpaired T-test were used.

Name	Temp.	Duration	Ramp rate	Cycles	Acquisition
Warming	50 °C	2 min	4.4 °C/sec	1	None
Melting	95 °C	2 min	4.4 °C/sec	1	None
Amplification	95 °C	15 sec	4.4 °C/sec	40	-
	60 °C	1 min	2.2 °C/sec		Quant
Melting curve	95 °C	1 min	4.4 °C/sec	1	-
	55 °C	30 sec	2.2 °C/sec		-
	95 °C	-	0.11 °C/sec		Continuous (5/1 °C)

Table 3.4: Details of the qRT-PCR programme used on the Lightcycler 480

3.1.11 Bacterial infection

Growth conditions

Salmonella typhimurium was grown on ampicillin plates. The day prior infection, a single colony of *Salmonella typhimurium* was incubated in 3 ml of LB media containing ampicillin (100 µg/ml) O/N at 37 °C shaking. The morning of the infection, 100 µl of *Salmonella typhimurium* were diluted in 3 ml of fresh LB without ampicillin for around 2.5 hours in order to have the bacteria in exponential phase.

Listeria monocytogenes was grown on brain heart infusion (BHF) plates. The day prior infection, a single colony was incubated in 3 ml of BHF media O/N

at 37 °C shaking. The morning of the infection 500 μ l of *Listeria monocytogenes* were diluted in 4.5 ml of fresh BHF media for around 2 hours in order to have the bacteria in exponential phase.

Concentration of bacteria in a liquid culture is defining the exponential phase of growth and it can be evaluated through the Optical Density (OD) measured at 600 nanometers (OD600). Usually, an exponential phase bacteria present on OD600 of 0.5 to 0.6. Optical density readings were performed with the Jenway 6300 visible spectrophotometer. The number of bacteria per ml in exponential phase can be obtained multiplying the OD600 $\times 10^9$. All infection experiments were performed using a multiplicity of infection (MOI) of 10, calculated according to the number of cells seeded in every experiment.

Colony forming units assay

For *Salmonella typhimurium* or *Listeria monocytogenes* infections, cells were plated in a 12 well plate (200,000 U2OS cells and 500,000 to 1,000,000 THP-1 or U937 cells) with 1 ml of media. Prior infection cells were washed and rested in fresh media without P/S for 2.5 hours. Cells were incubated with exponential phase *Salmonella typhimurium* or *Listeria monocytogenes* for 30 minutes including an initial 5 minutes of spin infection at 2000 x g. Subsequently, media with bacteria was aspirated and fresh media with gentamicin (50 μ g/ml) was added for 10 minutes, in order to kill remaining extracellular bacteria. At this stage, time 0 samples were collected washing them twice with PBS and lysing them with 200 μ l of 0.5 % Triton-X in PBS. Time point 3 and time point 24 were rested in fresh media with gentamicin (25 μ g/ml) for the remaining time and then harvested in the same way. Lysates were diluted 10, 100 and 1000 times in 0.5 % Triton-X in PBS and plated as a 10 μ l drop on ampicillin plates for *Salmonella typhimurium* or BHF plates for *Listeria monocytogenes*. Ampicillin plates were then incubated O/N at 28 °C, while BHF plates were incubated O/N at 37 °C. Colony count was performed the day after on 10x or 100x dilutions and normalised to time point zero (referred as CFUs ratio).

3.1.12 Phagocytic assay

Cells were incubated for 30 minutes with 10 μ l of yellow-green fluorescent latex beads carboxylate-modified polystyrene 2 μ m at 37 °C 5 % CO₂. Subsequently, cells were washed twice with PBS and harvested adding Trypsin/EDTA solution. Cells with trypsin solution were incubated in the 37 °C incubator to allow detachment from the plate and re-suspended in fresh medium. Cells were collected by centrifugation at 300 x g for 5 minutes, resuspended in 400 μ l of PBS and analysed by flow cytometry (FACS Canto Flow cytometer, BD Biosciences, San Jose, CA). Analysis of the data was performed with FlowJo software (TreeStar, Ashland, OR), and statistical analysis by two-way ANOVA in Prism 6 (GraphPad Software Inc).

3.1.13 Lysosomal activity assay

Cells were incubated for 30 minutes with 10 μ g/ml of DQ-Red-BSA at 37 °C 5 % CO₂. Subsequently, cells were washed twice with PBS and harvested adding Trypsin/EDTA solution. Cells with trypsin solution were incubated in the 37 °C incubator to allow detachment from the plate and re-suspended in fresh medium. Cells were collected by centrifugation at 300 x g for 5 minutes, resuspended in 400 μ l of PBS and analysed by flow cytometry (FACS Canto Flow cytometer, BD Biosciences, San Jose, CA). Analysis of the data was performed with FlowJo software (TreeStar, Ashland, OR), and statistical analysis by two-way ANOVA in Prism 6 (GraphPad Software Inc).

3.1.14 Autophagic flux assay

Cells presenting stable expression of the mCherry-EGFP-LC3 construct were washed with PBS and harvested adding Trypsin/EDTA solution. Cells with trypsin solution were incubated in the 37 °C incubator to allow detachment from the plate and re-suspended in fresh medium. Cells were collected by centrifugation at 300 x g for 5 minutes, resuspended in 400 μ l of PBS and analysed by flow cytometry (FACS Canto Flow cytometer, BD Biosciences, San Jose, CA). The autophagic flux was defined as the ratio between the median fluorescence of mCherry and the median

fluorescence of EGFP in the whole population of cells for each sample [116, 117]. Analysis of the data was performed with FlowJo software (TreeStar, Ashland, OR), and statistical analysis by two-way ANOVA in Prism 6 (GraphPad Software Inc).

3.1.15 Cellular Reactive Oxygen Species Detection Assay

Adherent cells: After, differentiation and/or treatment cells have been harvested with 200 μ l of trypsin and subsequently 300 μ l of fresh media. While the cells were in suspension in a 1.5 ml tube, DCFDA (20 mM) was added to have a final working concentration of 20 μ M. Cells were then incubated for 30 minutes at 37 °C 5 % CO₂ for 30 minutes and collected by centrifugation at 300 x g for 5 minutes, resuspended in 400 μ l of PBS and analysed by flow cytometry (FACS Canto Flow cytometer, BD Biosciences, San Jose, CA). Analysis of the data was performed with FlowJo software (TreeStar, Ashland, OR), and statistical analysis by two-way ANOVA in Prism 6 (GraphPad Software Inc).

3.2 Protein biochemistry

3.2.1 Western blotting

Samples prepared for western blotting were heated with Laemmli's sample buffer (LSB) (to a final 1 x concentration) to 95 °C for 5 minutes to ensure complete denaturing. Two electrophoresis systems were used: the BioRad mini system and the Invitrogen Novex system. For the BioRad system, self-cast 10 % Bis-Tris gels were placed into the tank with 1 x running buffer and samples were loaded into the wells with one well of SeeBlue pre-stained protein ladder (Invitrogen) to indicate the molecular weight (MW) of identified proteins. The gel tank was topped up with buffer and run at a constant 140 V until the dye front had run off the gel. For the Invitrogen system, samples were loaded into 4-12 % gradient gels (Invitrogen) with 1 x MOPS or 1 x MES buffer with one well of SeeBlue pre-stained protein ladder (Invitrogen). After loading, gels were run at a constant 190 V until the dye front had run off the gel.

Gels were equilibrated in 1 x transfer buffer for 10 minutes followed by transfer using a semi-dry system (OWL HEP1, Thermo) to transfer protein to nitrocellulose or PVDF membrane (GE Healthcare). A constant current of 100 mA/gel (BioRad) or 150 mA/gel (Invitrogen), with a maximum voltage of 24 V, was applied for a duration of 1 hour. Protein transfer efficiency was checked through the addition of Ponceau stain to the membrane for 5 minutes, after which the gel was washed 2 x with dH₂O. Nonspecific interactions were blocked through the addition of 5 % w/v milk in PBS-Tween (PBST) for 1 hour at RT, followed by 1 x with PBST and incubation O/N at 4 °C with the primary antibody diluted in 1 % Bovine serum albumin (BSA) in PBST.

After incubation with the primary antibody, membranes were washed 2 x with PBST and incubated with the appropriate secondary antibody diluted in PBST for 1 hour at RT. After 3-6 x 5 minutes washes with PBST the membranes were incubated with ECL solution for 1 minute for developing. For low abundance or difficult to detect proteins, 10 % enhancer solution was added to the ECL solution to increase signal.

3.3 Molecular biology

3.3.1 Plasmid preparation

For small scale plasmid prep (mini-prep), TOP10 competent *E.coli* were incubated with the plasmid of interest on ice for 5 minutes. Heat shock was performed for 1 minute at 42 °C before being placed back on ice. 500 μ l of S.O.C media was added to each transformation, mixed, and 250 μ l were pipetted on to agar plates containing ampicillin at a concentration of 100 μ g/ml. After allowing the mix to dry into the plate at RT, the plate was placed into a 37 °C incubator O/N to allow for bacterial growth. The following day, 5-10 colonies were carefully picked from the plates and cultured O/N at 37 °C in 4 ml of L-broth containing 100 μ g/ml ampicillin (this is also a starter prep for midis). Following this, 1 ml of the culture was taken for purification of plasmid DNA with a Qiagen spin miniprep kit or Machery-Nagel nucelospin plasmid kit as per manufacturer's protocol.

For large scale plasmid production (midi-prep), the Machery-Nagel Purelink HiPure midiprep kit was used. Samples were prepared for extraction firstly through the addition of 150 μ l of a starter prep (as detailed above) to 100 ml of L-broth containing ampicillin at a concentration of 100 μ g/ml and subsequent O/N incubation at 37 °C, shaking. Following incubation, all 100 ml of culture were used for midi-prep as per manufacturer’s protocol. For both mini- and midi-preps, the extracted plasmid was eluted in RNase and DNase-free dH₂O.

3.3.2 Cloning and mutagenesis

Gateway cloning of NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB

For the creation of the pLVX-Tight-Puro vector containing NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB, the construct sequences were amplified from respectively pEGFP-C1 plasmids using PCR primers flanked by 25 bp attb sequences (attb1: ACAAGTTTGTACAAAAAAGCAGGCT attb2: ACCACTTTGTACAAGAAAGCTGGGT), followed by 18-25 bp of template. The PCR performed for templates amplification is detailed in table 3.5 and table 3.6. The resulting PCR products were run on a 1 % agarose gel with 2.5 μ l of peqGREEN (per 50 μ l of agarose gel mix) at 90 V for 20 minutes and the resulting bands were excised and purified using a Machery-Nagel nucleospin gel and PCR clean-up kit as per manufacturer’s protocol.

Component	Volume (μ l)
5 x Phusion buffer	10
dNTPs (10 mM)	1
Forward primer (attb1) (10 μ M)	1
Reverse primer (attb2) (10 μ M)	1
Template (100 ng/ μ l)	1
Phusion DNA polymerase	0.5
dH ₂ O	up to 20

Table 3.5: Reaction mix for attb-PCR products

Step	Temperature (°C)	Time (mm:ss)
Denaturing	98	01:00
Amplification (25 cycles)	98	00:15
	60	00:30
	72	00:40
Final extension	72	05:00

Table 3.6: Details of the PCR cycles for attb-PCR amplification

PCR products were measured by Nanodrop and diluted to 100 ng/ μ l and used for BP recombination reactions with pDONR vectors also diluted to 100 ng/ μ l . BP recombination reactions were performed as per table 3.7 at 25 °C O/N.

Component	Volume/Amount
5 x BP reaction buffer	4 μ l
dH ₂ O	7 μ l
attb-PCR product	2 μ l
pDONR	3 μ l
BP clonase	4 μ l

Table 3.7: BP clonase recombination reaction

Following BP recombination reaction the products were transformed into TOP10 *E.coli* and resulting colonies were picked and grown for mini-prep as previously described (pDONR vector present kanamycin resistance). pDONR vectors containing NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB were confirmed by sequencing and diluted to 100 ng/ μ l for LR recombination reactions with the destination vector pLVX-Tight-Puro also diluted to 100 ng/ μ l. LR recombination reactions were performed as per table 3.8 at 25 °C O/N followed by 30 minutes treatment with 20 μ g/ μ l Proteinase K at 37 °C.

Component	Volume/Amount
5 x LR reaction buffer	4 μ l
dH ₂ O	7 μ l
pDONR	3 μ l
pLVX-Tight-Puro	2 μ l
LR clonase	4 μ l

Table 3.8: LR clonase recombination reaction

Following LR recombination reaction the products were transformed into TOP10 *E.coli* and resulting colonies were picked and grown for mini-prep as previously described (pLVX-Tight-Puro vector present ampicillin resistance). pLVX-Tight-Puro vectors containing NLS or NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB were confirmed by sequencing and diluted to 100 ng/ μ l for lentivirus generation.

3.4 Statistical analyses

Statistics

In all experiments, the standard error of the mean (SEM) was used to indicate the error bars of replicates, as multiple technical replicates were used in each experiment to generate a mean value. This sampling distribution was calculated for each experiment and the SEM refers to the change in the mean over each set of biological replicates (for each experiment $n \geq 3$).

Statistical analyses shown in this thesis were calculated by: Two-way ANOVA (analysis of variance) test. Two-way ANOVA test calculates the mean differences between groups with two independent variables (in this study, the cell type) on one dependent variable (stimulation). One-way ANOVA (analysis of variance) test. One-Way ANOVA test calculates differences between the means of independent groups (in this study stimulated and non stimulated cells). Unpaired T-test. Unpaired T-test compares two independent groups with the null hypothesis that population means in two independent groups are equal.

RNA-Microarray analysis

RNA-Microarray analysis and detection of differentially expressed genes between different experimental conditions was performed using Limma Bioconductor package [118]. Differentially expressed genes were selected based on adjusted $P < 0.05$. Gene ontology (GO) analysis was performed using ToppFun (ToppGene Suite) [119]. GSEA was performed using javaGSEA v2.2.2 software (<http://software.broadinstitute>

.org/gsea/downloads.jsp) with 1000 permutations and default parameters using selected gene sets from the MSigDB (www.broadinstitute.org/gsea/msigdb) database. All RNA-Microarray analysis have been performed by Jagat Chauhan.

RNA-Sequencing analysis

RNA-Sequencing quality control of raw sequencing reads was performed using FastQC, (www.bioinformatics.babraham.ac.uk/projects/fastqc/). Low quality bases and polyA were trimmed using cutadapt. All trimmed reads were aligned to the hg19 genomic reference using STAR software [120] with the parameters “-outSAMtype BAM Unsorted SortedByCoordinate -quantMode TranscriptomeSAM GeneCounts”. The reads count were normalised with The Trimmed Mean of M-values Normalisation Method (TMM) and tested for differential expression using the limma voom method [118]. Genes with $P < 0.05$ and adjusted $P < 0.05$ were considered significantly differentially expressed. Gene ontology (GO) analysis was performed using ToppFun tool [119]. We conducted GSEA using selected gene sets from the MSigDB (www.broadinstitute.org/gsea/msigdb) database with 1000 permutations and default parameters. All RNA-Sequencing analysis have been performed by Jagat Chauhan.

4

Ubiquitin-binding domains: a tool to investigate receptor signalling pathways

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4.1 Introduction

Following NOD2 receptor activation, Lys63- and Met1-linked Ub chains are conjugated to the RIPK2 adaptor molecule. Met1-linked Ub chains are required for NOD2 downstream signalling [17, 22, 23]. The requirement for Lys63-linked Ub chains is inferred by the fact that several E3 ligases, conjugating Lys63-linked Ub chains, are recruited to the NOD2 receptor complex following activation, such as XIAP, TRAF2, cIAP1/2 and PELI3 [121–126]. In addition, published and ongoing works in Gyrd-Hansen laboratory demonstrated that in the absence of the deubiquitinating enzyme CYLD (preferentially cleaving Lys63 and Met1 chains) or OTULIN (specific for Met1 chains) the NF- κ B-dependent pro-inflammatory signalling following L18-MDP stimulation is increased [22, 23]. These data enforce the importance of Lys63- and Met1-linked Ub chains within the NOD2 receptor system; however, direct evidence for the role of Lys63 Ub chains in the NOD2 signalling pathway was still lacking, and it will be addressed in this chapter.

4.2 NOD2 signalling individually requires Lys63- and Met1-linked ubiquitin chains

4.2.1 Generation of cytoplasmic and nuclear ubiquitin-binding constructs

A GFP-coupled tandem UIM domain from Receptor-associated Protein 80 (RAP80) was obtained from Niels Mailand's laboratory (GFP-K63-SUB) in order to address the direct function of Lys63-linked Ub chains within the NOD2 receptor signalling pathway [127]. A GFP-coupled Ubiquitin-binding in ABIN and NEMO (UBAN) domain from NEMO (GFP-M1-SUB) was developed in Gyrd-Hansen lab in order to investigate the role of Met1-linked Ub chains [22]. Following ectopic expression in U2OS/NOD2 cells (henceforth referred to as U2OS cells), the cellular distribution of the two constructs was dissimilar, the GFP-K63-SUB was mainly localised in the nuclear portion, while the GFP-M1-SUB was mainly localised in the cytoplasm. In order to directly compare these constructs, and identify if the Ub linkages

4. Ubiquitin-binding domains: a tool to investigate receptor signalling pathways⁶⁶

were acting at a cytoplasmic or nuclear level, I generated variants of the binders that accumulate either in the nucleus or cytoplasm. To do this, I cloned two I κ B α Nuclear Export Sequences (NESs) downstream the GFP, GFP-K63-SUB and GFP-M1-SUB to generate GFP-NES, GFP-NES-K63-SUB and GFP-NES-M1-SUB respectively. Similarly, I cloned the Simian Virus 40 (SV40) Nuclear Localisation Sequence (NLS) downstream the GFP (GFP-NLS) and the GFP-M1-SUB (GFP-NLS-M1-SUB), while two SV40 NLS were cloned downstream the GFP-K63-SUB (GFP-NLS-K63-SUB) (Figure 4.1).

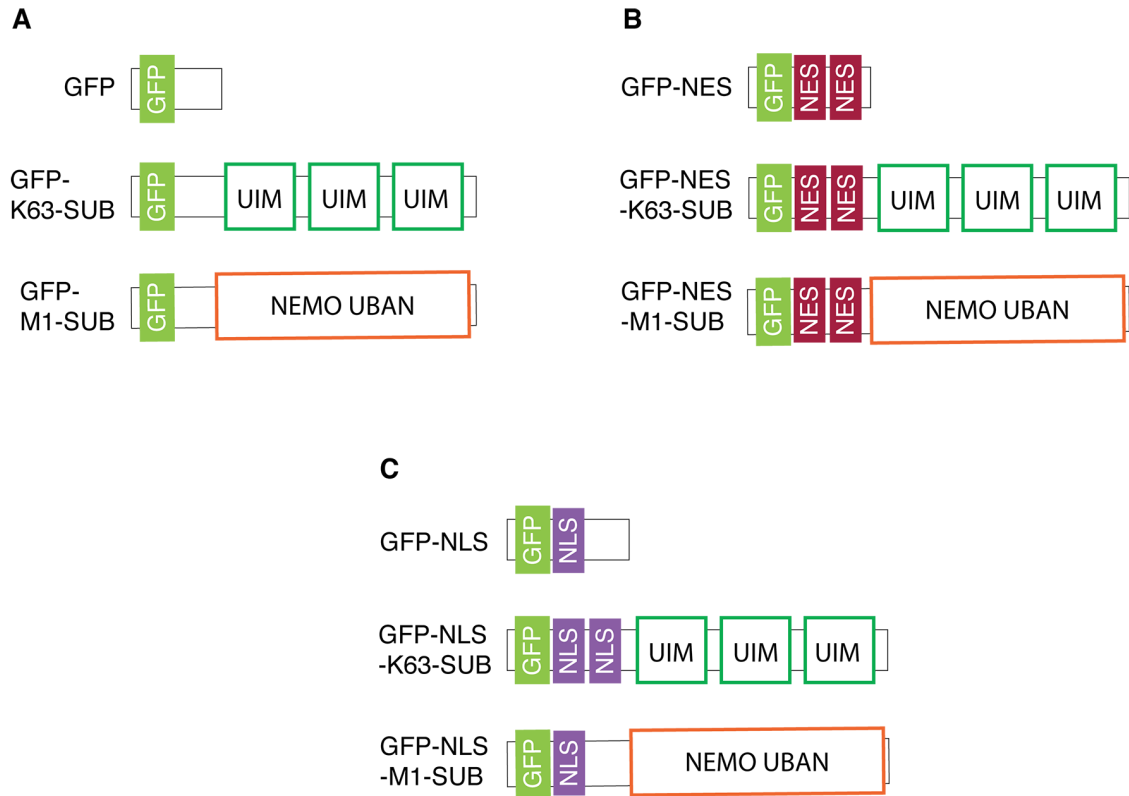


Figure 4.1: GFP-coupled ubiquitin-binding constructs. (A) Schematic representation of GFP, GFP-K63-SUB generated from RAP80 UIM domain [128] and GFP-M1-SUB generated from NEMO UBAN domain [22] (B) Schematic representation of NES variants of GFP, GFP-K63-SUB and GFP-M1-SUB constructs (C) Schematic representation of NLS variants of GFP, GFP-K63-SUB and GFP-M1-SUB constructs.

The cytoplasmic or nuclear-specific distribution of GFP-labelled constructs was evaluated via immunofluorescence in U2OS cells. Expression levels of GFP, GFP-K63-SUB or GFP-M1-SUB constructs were monitored in all the experiments

4. Ubiquitin-binding domains: a tool to investigate receptor signalling pathways⁶⁷

via flow cytometry or microscopy, and no significant differences were observed among the three constructs (Figure 4.2).

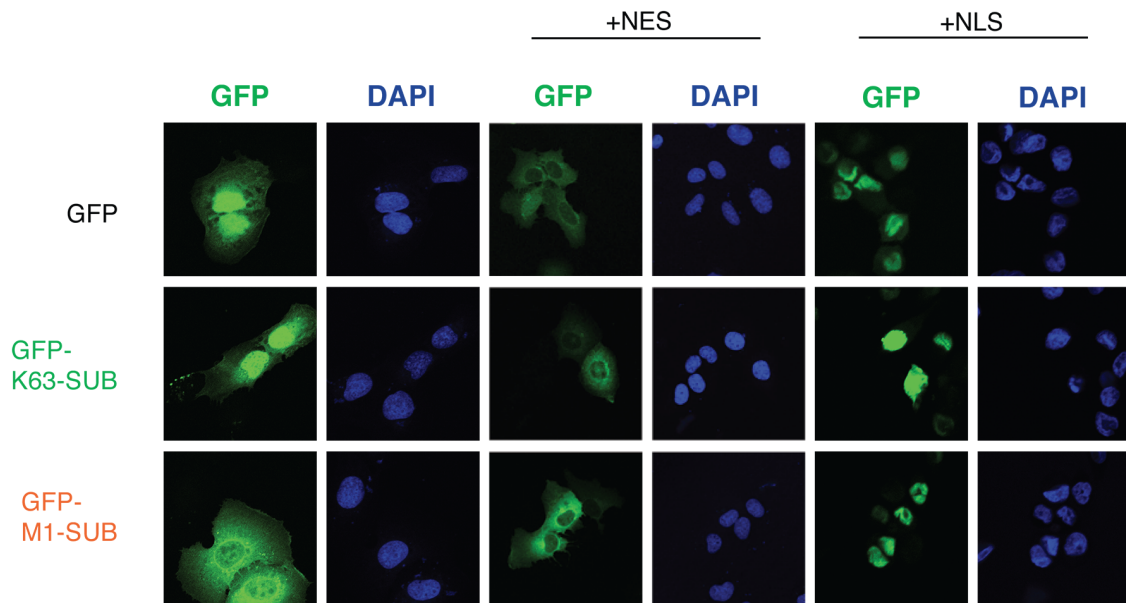


Figure 4.2: Nuclear and cytoplasmic variants of ubiquitin binders. Confocal microscopy analysis of cellular distribution for GFP, GFP-K63-SUB and GFP-M1-SUB constructs and respective NES or NLS variants in U2OS cells transfected for 48 hours.

4.2.2 GFP-K63-SUB and GFP-M1-SUB expression impairs RelA nuclear translocation following NOD2 stimulation

To assess the individual role of Lys63 and Met1-linked Ub chains within the NOD2 signalling pathway, GFP, GFP-K63-SUB or GFP-M1-SUB were transiently transfected in U2OS cells. Following stimulation with L18-MDP, RelA nuclear translocation was assessed via immunofluorescence and quantified using the Fiji software. RelA nuclear translocation was shown to be reduced in cells transfected with GFP-K63-SUB and GFP-M1-SUB but not in GFP only or not successfully transfected cells (Figure 4.3).

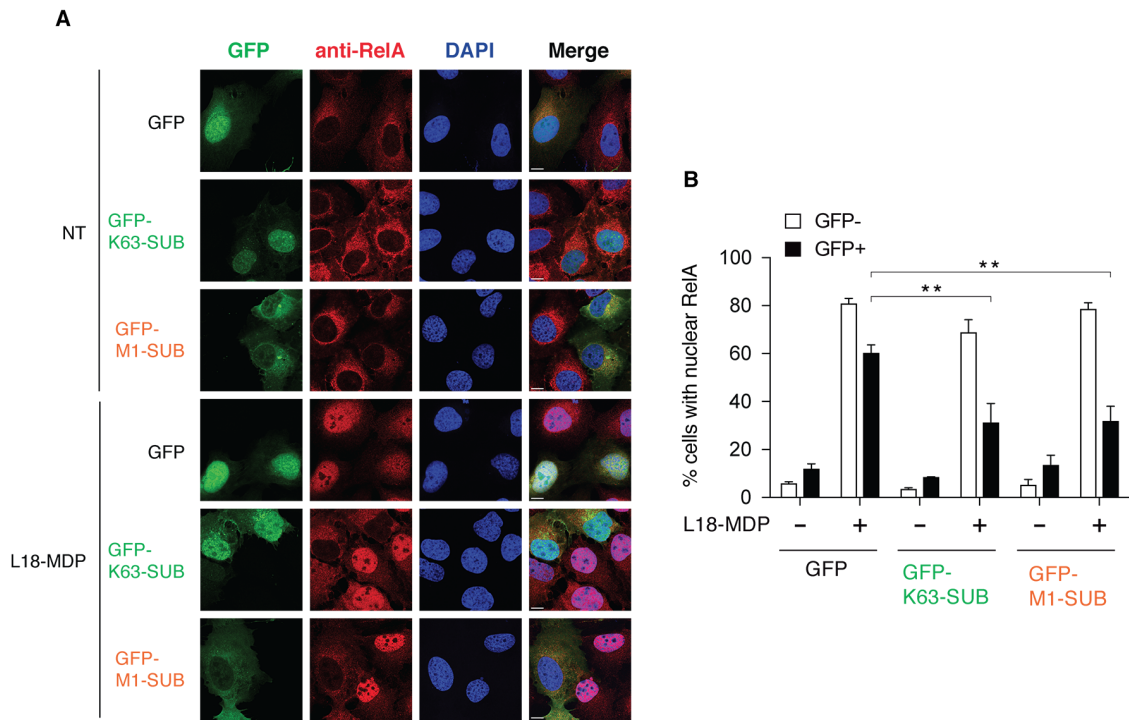


Figure 4.3: RelA nuclear translocation relies on Lys63 and Met1 Ub linkages. (A) Nuclear translocation analysis of RelA (red) assessed through immunofluorescence in response to L18-MDP (1 μ g/ml, 1 hr) or no treatment (NT) in U2OS cells following 48 hrs transfection with GFP, GFP-K63-SUB or GFP-M1-SUB (scale bar, 10 μ m) (B) Quantification of (A) using Fiji software analysis. Triplicates. Two Way ANOVA statistical test.

4.2.3 GFP-K63-SUB and GFP-M1-SUB expression impairs CXCL-8 production following NOD2 stimulation

To investigate if impaired RelA nuclear translocation resulted in reduced transcription and translation of pro-inflammatory mediators, U2OS cells were transfected with GFP, GFP-K63-SUB or GFP-M1-SUB and the production of CXCL-8, following NOD2 stimulation, was assessed via intracellular cytokine staining and flow cytometry. The production of CXCL-8 was decreased in GFP-K63-SUB and GFP-M1-SUB, but not in GFP only expressing cells. Interestingly, GFP-K63-SUB or GFP-M1-SUB expression did not completely abolish CXCL-8 production following NOD2 receptor stimulation. I speculated this is a consequence of heterogeneous expression of the linkage-specific Ub binders in U2OS cells. Therefore, to investigate a concentration dependent effect of GFP-K63-SUB and GFP-M1-SUB, GFP positive

4. Ubiquitin-binding domains: a tool to investigate receptor signalling pathways ⁶⁹

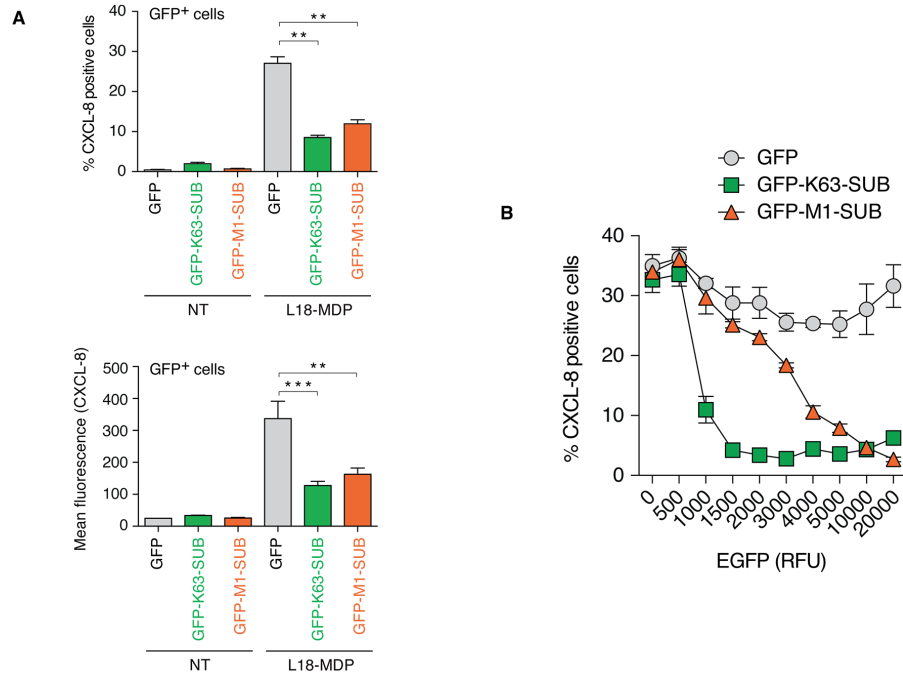


Figure 4.4: CXCL-8 production relies on Lys63 and Met1 ubiquitin linkages. (A) CXCL-8 production measured as % of CXCL-8 positive cells or as mean fluorescence via flow cytometry in U2OS cells following 48 hrs transfection with GFP, GFP-K63-SUB or GFP-M1-SUB and L18-MDP stimulation (200 ng/ml, 6 hrs) (B) Gating of GFP positive cells in (A) based on RFU values. The values on the x axis indicate the minimal and maximal RFU values for each fraction. Triplicates. Two Way ANOVA statistical test.

cells were gated based on relative fluorescence units (RFUs). Ten different gates were generated, from RFU = 0 to RFU = 20000, and the percentage of CXCL-8 positive cells was calculated for each fraction. The higher the expression of the Ub chain-specific binding constructs, the lower was the percentage of CXCL-8 positive cells observed following L18-MDP stimulation, while no differences were observed in GFP only transfected cells (Figure 4.4 and 9.1).

In order to evaluate if GFP-K63-SUB and GFP-M1-SUB were acting at the cytoplasmic or nuclear level, I transfected U2OS cells with GFP-NLS, GFP-NLS-K63-SUB or GFP-NLS-M1-SUB and measured CXCL-8 production following L18-MDP treatment. Expression of NLS variants of these constructs did not affect the production of CXCL-8 following NOD2 receptor stimulation. This is in line with the notion that NOD2 is an intracellular cytoplasmic receptor (Figure 4.5 and 9.1).

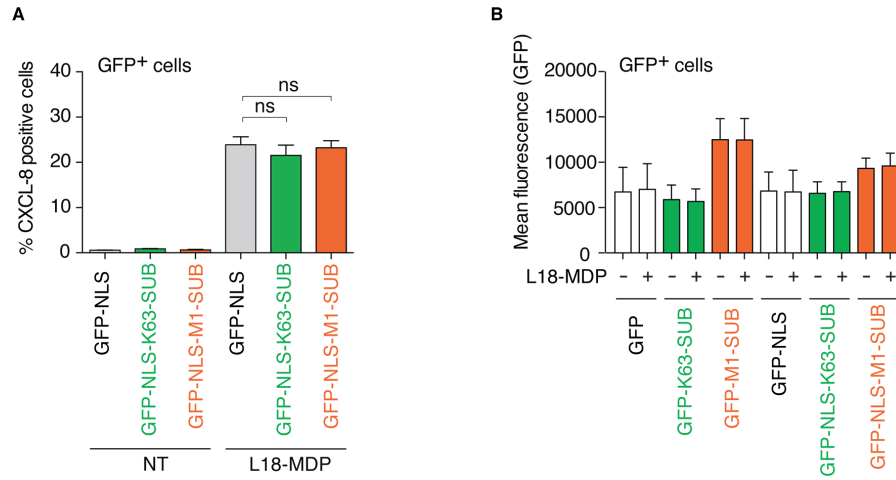


Figure 4.5: Nuclear ubiquitin binders do not affect CXCL-8 production. (A) CXCL-8 production measured as % of CXCL-8 positive cells via flow cytometry in U2OS cells following 48 hrs transfection with GFP-NLS, GFP-NLS-K63-SUB or GFP-NLS-M1-SUB and L18-MDP stimulation (200 ng/ml, 6 hrs) (B) Expression levels of GFP, GFP-K63-SUB or GFP-M1-SUB constructs with and without NLS in U2OS cells 48 hrs post transfection. Triplicates. Two Way ANOVA statistical test.

4.2.4 The LUBAC catalytic subunit HOIP is required for effective NOD2 signalling

HOIL-1 Interacting Protein (HOIP) is the catalytic subunit of the LUBAC complex, the only known E3 ligase generating Met1-linked Ub chains. To test the role of Met1-linked Ub chains in NOD2 signalling, I used *HOIP* KO HCT-116 cells previously generated in Gyrd-Hansen lab, and stimulated them with L18-MDP. Following stimulation, I evaluated NF- κ B activation through CXCL-8 transcript production via qRT-PCR. *HOIP* KO cells displayed a marked reduction in CXCL-8 mRNA levels following L18-MDP stimulation (Figure 4.6). Consistent with the previous experiments, performed using the GFP-M1-SUB construct, deletion of HOIP completely prevents NOD2-mediated expression of CXCL-8.

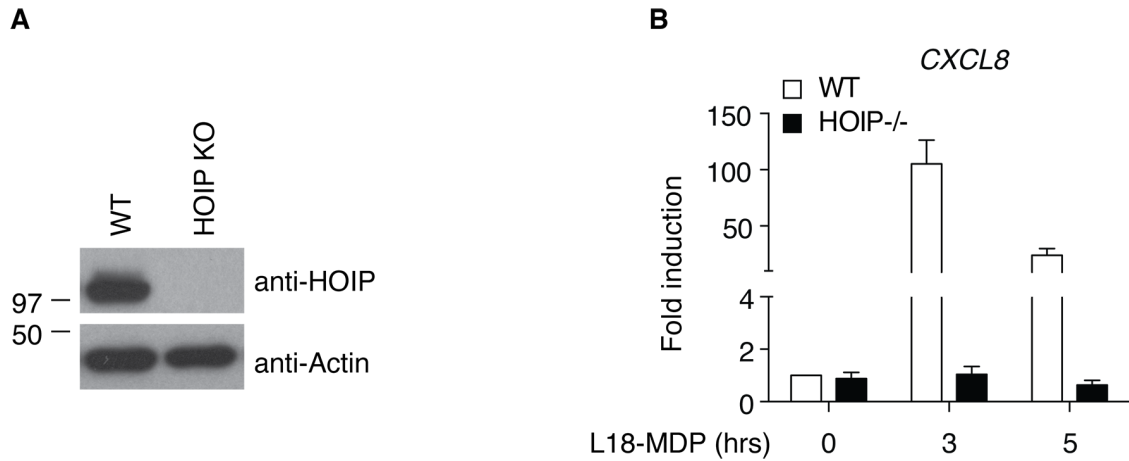


Figure 4.6: HOIP is required for CXCL-8 production. (A) Western blot (WB) showing HOIP levels in HCT-116 cells and HCT-116 CRISPR/Cas *HOIP* KO cells. (B) qRT-PCR for CXCL-8 transcript levels following L18-MDP (200 ng/ml) stimulation for indicated times and normalised to untreated control cells. Triplicates.

4.2.5 Lys29- and Lys33-linked ubiquitin chains are not involved in NOD2 signalling pathway

I generated a further GFP-labelled linkage-selective Ub binder, using the Npl4 Zinc Finger 1 (NZF1) domain of the deubiquitinating enzyme TRABID (GFP-NZF-SUB) with specificity for Lys29- and Lys33-linked Ub chains [129]. To test a possible involvement of Lys29- and Lys33-linked Ub chains within the NOD2 receptor signalling pathway, GFP and GFP-NZF-SUB were transfected in U2OS cells and CXCL-8 production was assessed via intracellular cytokine staining and flow cytometry following NOD2 receptor stimulation. The production of CXCL-8 was not affected by the expression of GFP-NZF-SUB, suggesting no function for these two Ub linkages within the NOD2 receptor system (Figure 4.7).

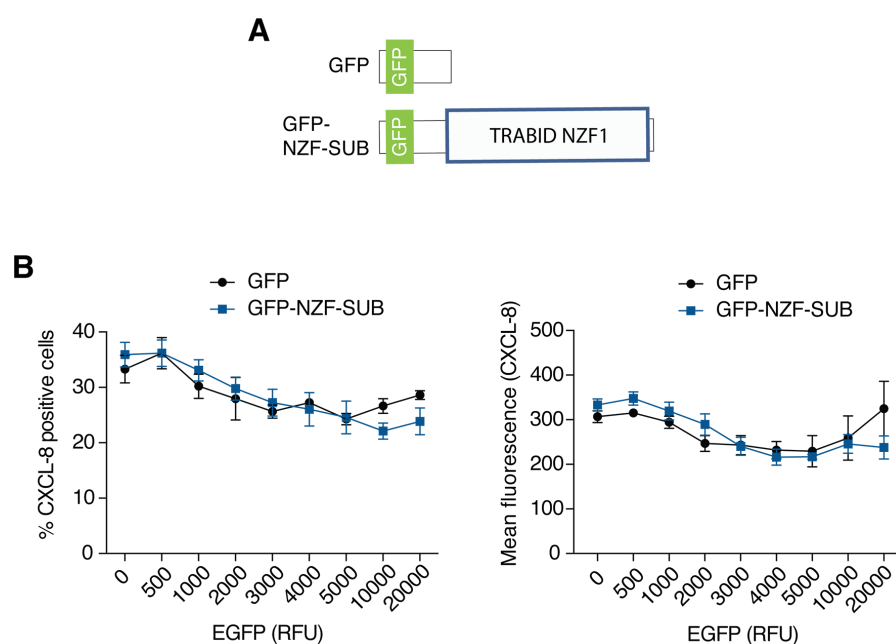


Figure 4.7: Lys29- and Lys33-specific ubiquitin-binding domain. (A) Schematic representation of GFP and GFP-NZF-SUB generated from TRABID NZF1 domain [129]. (B) CXCL-8 production measured as % of CXCL-8 positive cells or mean fluorescent via flow cytometry in U2OS cells following 48 hrs transfection with GFP or GFP-NZF-SUB and L18-MDP stimulation (200 ng/ml, 6 hrs). GFP positive cells were gated based on RFUs as in figure 4.4, the values on the x axis indicate the minimal and maximal RFU values for each fraction. Triplicates.

4.3 Discussion

The NOD2 receptor is responsible for sensing and clearance of intracellular bacteria, and it acts mainly through NF- κ B and MAPK activation [17, 130]. Met1-linked Ub chains are indispensable for NOD2 signalling pathway and effective transcriptional response [22]. In this chapter I provided direct evidence for Lys63-linked Ub chains activity at the NOD2 receptor complex. Interfering with Lys63- or Met1-linked Ub chains via ectopic expression of specific Ub-binding domains abrogated RelA nuclear translocation and production of the pro-inflammatory mediator CXCL-8, following NOD2 stimulation with the ligand L18-MDP.

Importantly, Lys63- and Met1-linked Ub chains activity in this system is restricted to the cytoplasmic compartment, in line with the notion that the NOD2 receptor complex assembles in the cytosol following homotypic oligomerization triggered by the ligand MDP. *De facto*, nuclear-confined ectopic expression of Lys63- or Met1-specific Ub-binding domains does not impair NOD2 receptor signalling, and production of pro-inflammatory mediators.

Here I showed a direct approach to investigate the activity of specific Ub linkages within immune receptor signalling pathways, taking advantage of GFP-labelled Ub-binding constructs. These domains can directly bind specific Ub linkages, blocking their scaffolding functions and recognition by other proteins. In principle, the same approach could be used to investigate the role of specific Ub linkages in other cytoplasmic or nuclear cellular processes. Moreover, these data collectively show for the first time the indispensable function of Lys63-linked Ub chains in controlling NOD2 receptor complex activation and downstream signalling.

5

CYLD is an IFN- γ target gene

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5.1 Introduction

CYLD negatively regulates NF- κ B and MAPK pathways, controlling inflammation and cell death in epithelial and immune cells [8, 35, 40, 45, 54, 131]. A20 is a deubiquitinating enzyme negatively regulating the NF- κ B pathway. This enzyme is transcriptionally induced following NF- κ B-activating stimuli, such as TNF- α and lipopolysaccharide (LPS), in order to disassemble these inflammatory receptor signalling complexes [132, 133]. Similarly to A20, *Jono et al.* reported CYLD to be transcriptionally induced by a variety of NF- κ B stimuli in epithelial cells, including TNF- α , IL-1 β and *Haemophilus influenzae* (NTHi) [134]. Despite the important role of CYLD in controlling inflammation and immune responses, the regulation of this deubiquitinating enzyme in immune cells has not yet been investigated. Monocytes and monocyte-derived macrophages are key cellular components of our innate immune system and they orchestrate inflammation in response to pathogens. I hypothesised a transcriptional regulation for the *CYLD* gene in this cell type, dependent on cell state and immunoregulatory stimuli.

The data presented in this chapter indicate *CYLD* as an IFN- γ responsive gene. Furthermore, I identify CYLD induction to be dependent on STAT1 and STAT3 TF activation. Lastly, I show that STAT3-specific signalling pathways, such as IL-6 or IL-10, do not induce CYLD transcription as previously reported [135].

5.2 An *in vitro* model to study human monocyte differentiation and macrophage activation

5.2.1 THP-1 and U937 monocytes differentiation and activation

To investigate CYLD transcriptional regulation in human macrophages, I used THP-1 and U937 human (pro-)monocytic cells. THP-1 and U937 are immortalised cell lines, derived from an acute monocytic leukemia patient and a histiocytic lymphoma patient, respectively, and can be differentiated into macrophages by phorbol-12-myristate-13-acetate (PMA) treatment [136–141]. THP-1 and U937

cells are widely used as an *in vitro* model to recapitulate human monocyte-to-macrophage differentiation. Furthermore, they have been described to respond similarly to primary macrophages following pathogen infection or immunoregulatory stimuli such as TNF- α and LPS [136–141].

I optimised a monocyte-to-macrophage differentiation protocol for THP-1 and U937 cells, treating these cells with PMA for 72 hours, followed by 24 hours of rest in fresh media. Activated monocyte-derived macrophages were generated with 72 hours PMA treatment, followed by stimulation with LPS + IFN- γ for 2 hours and 22 hours of rest in fresh media (Figure 5.1).

To evaluate THP-1 monocyte-to-macrophages differentiation and activation, RNA-microarray analysis was performed on THP-1 monocytic cells, THP-1-derived macrophages, and THP-1-derived macrophages treated with LPS + IFN- γ . RNA-microarray analysis displayed a distinctive gene expression profile for each condition. Gene Ontology (GO) analysis of differentially expressed genes comparing THP-1 monocytes and THP1-derived macrophages showed an upregulation of immune and defense response genes, in line with the function of these cells in the innate immune context. Downregulated genes included cell cycle and mitotic-related genes, due to the fact that these cells stop dividing following differentiation into macrophages. In agreement with the notion that LPS + IFN- γ stimulation induces an activated pro-inflammatory state in macrophages [136], GO analysis of differentially expressed

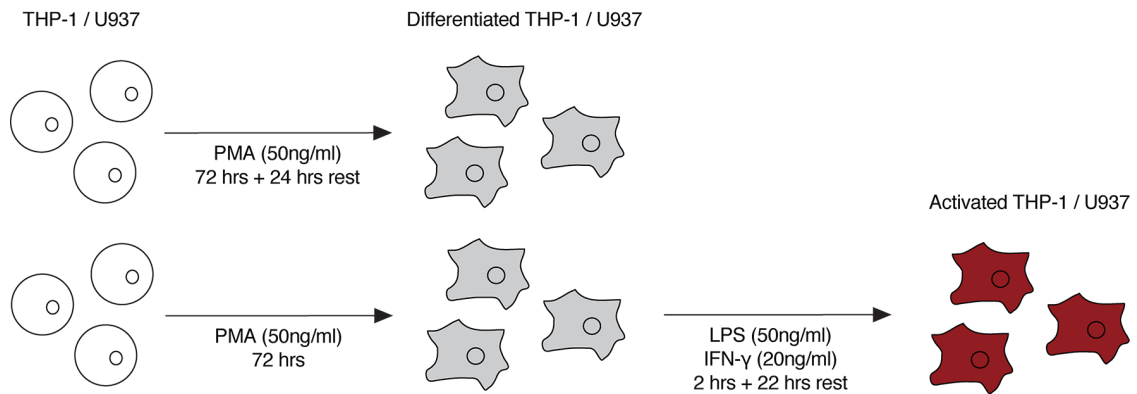


Figure 5.1: THP-1/U937 cells differentiation and activation. Schematic representation of the differentiation and activation protocols for THP-1 and U937 human monocytic cells.

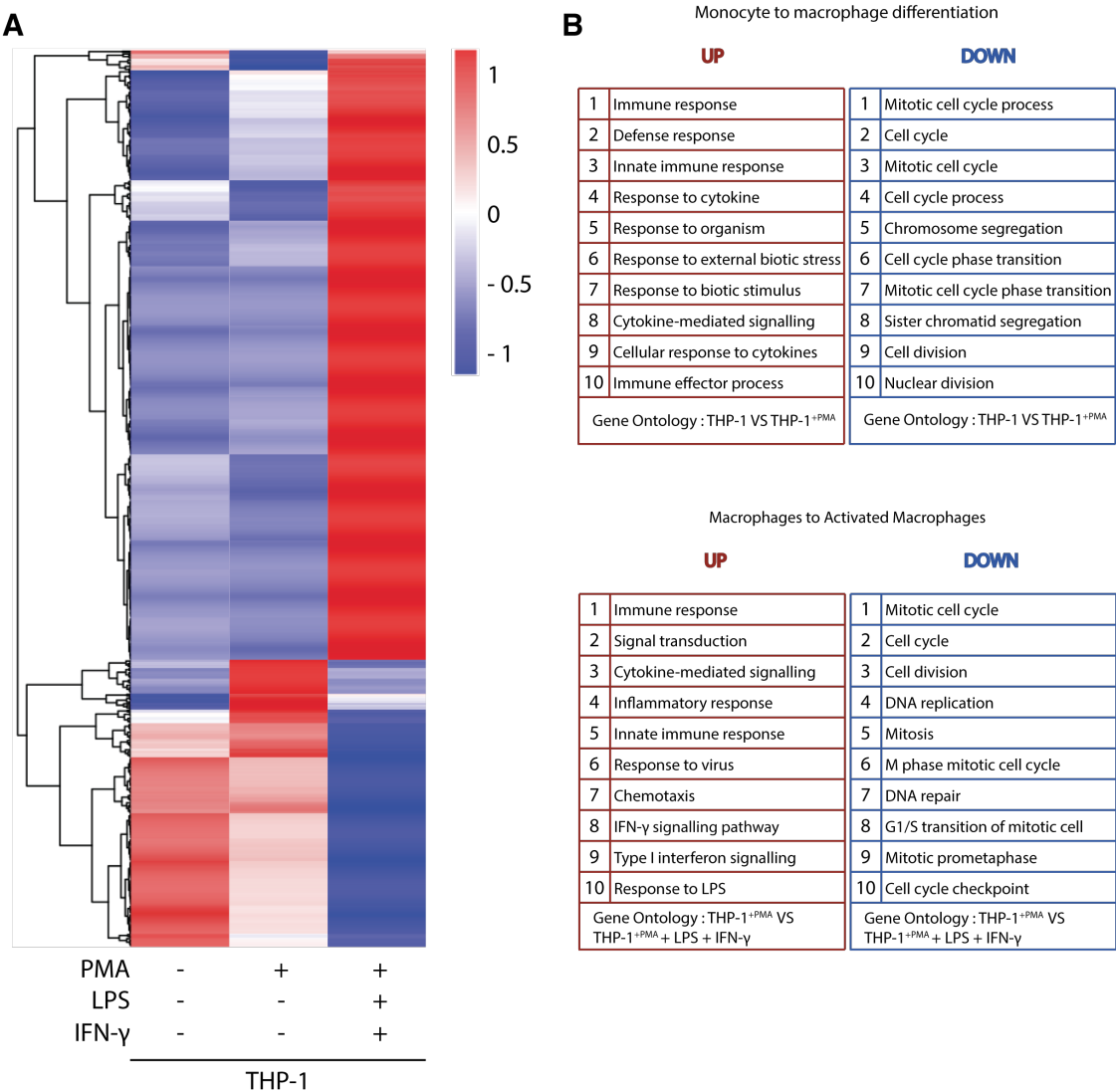


Figure 5.2: THP-1 human monocytic cells differentiation and activation. (A) Heat map from RNA-microarray analysis for gene expression profiling in THP-1 monocytes, THP-1-derived macrophages and THP-1-derived macrophages activated with LPS (50 ng/ml) + IFN- γ (20 ng/ml) treated for 2 hrs + 22 hrs rest (B) GO analysis of differentially up or downregulated genes comparing THP-1 monocytes with THP-1-derived macrophages, or comparing THP-1-derived macrophages with THP-1-derived macrophages activated with LPS + IFN- γ from figure (A). RNA-microarray. Triplicates.

genes comparing untreated and LPS + IFN- γ treated THP-1-derived macrophages showed an enrichment for immune response, inflammatory response, and specifically IFN- γ - or LPS-mediated response genes (Figure 5.2).

Validation of the RNA-microarray results was performed via qRT-PCR, including previously reported up (*IDO1*, *CCL2* and *IL-1 β*) or downregulated (Mannose Receptor C-type 1 *MRC1*) genes in activated THP-1-derived macrophages [141].

RNA-microarray analysis showed a mild transcriptional induction of *CYLD* as part of the monocyte-to-macrophage differentiation process, but a significant induction following LPS + IFN- γ stimulation. Increased *CYLD* mRNA levels following differentiation and activation with LPS + IFN- γ were confirmed by qRT-PCR (Figure 5.3).

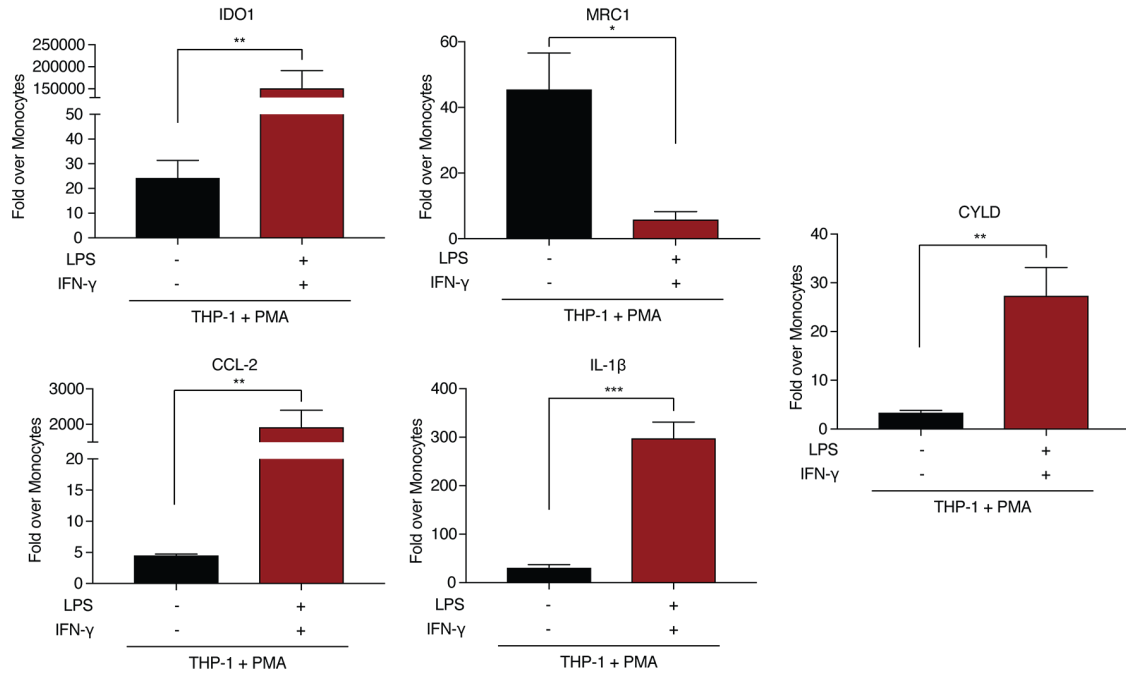


Figure 5.3: *CYLD* mRNA levels are increased in activated macrophages. qRT-PCR for *CYLD*, *IDO1*, *MRC1*, *CCL2* and *IL-1 β* in THP-1-derived macrophages untreated and LPS (50 ng/ml) + IFN- γ (20 ng/ml) treated for 2 hrs + 22 hrs rest, normalised to untreated monocytic state. Triplicates. One Way ANOVA statistics.

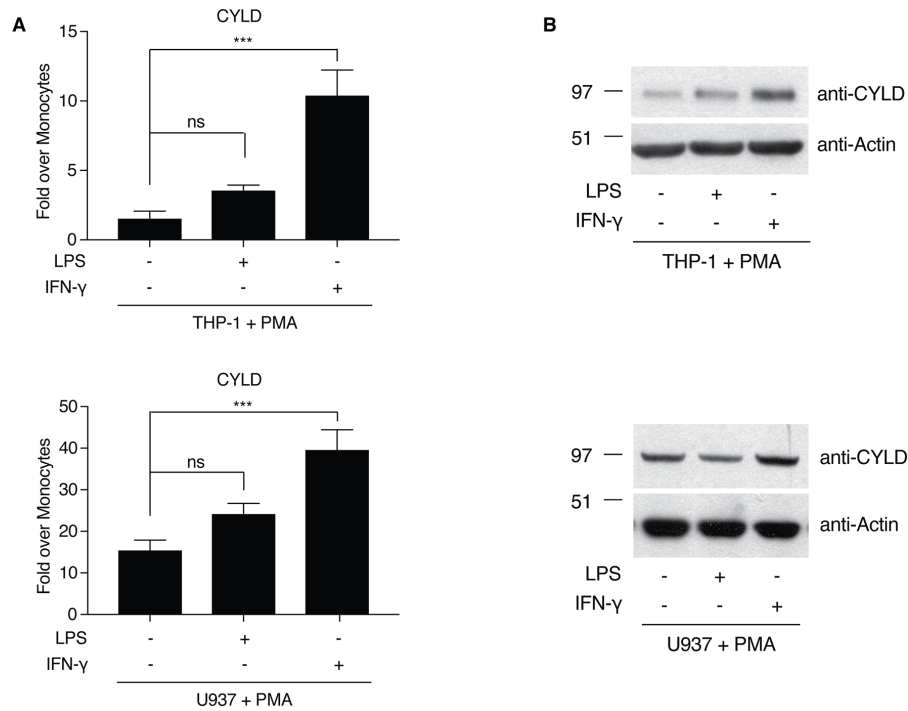


Figure 5.4: *CYLD* is induced via *IFN- γ* in activated macrophages. (A) qRT-PCR for *CYLD* in untreated, LPS (50 ng/ml) or *IFN- γ* (20 ng/ml) treated THP-1- or U937-derived macrophages. LPS or *IFN- γ* treatment was carried for 2 hrs + 22 hrs rest and the fold induction was normalised to the untreated monocytic state (B) Protein quantification via WB of (A). Triplicates. One Way ANOVA statistics.

5.3 *CYLD* is transcriptionally activated following *IFN- γ* stimulation in human macrophages

5.3.1 *CYLD* transcription in activated macrophages is induced via *IFN- γ*

In line with the reported finding of *CYLD* being an $\text{NF-}\kappa\text{B}$ target gene, I investigated if *CYLD* transcriptional induction in activated macrophages was due to LPS or *IFN- γ* stimulation. To address this, I stimulated THP-1-derived macrophages with LPS or *IFN- γ* independently and evaluated *CYLD* mRNA levels via qRT-PCR and protein levels through western blotting (WB). *CYLD* transcript and protein levels were mildly induced by LPS stimulation, whilst *IFN- γ* treated cells showed a significant upregulation of *CYLD*. Similar results were observed in U937-derived macrophages following LPS or *IFN- γ* stimulation (Figure 5.4).

CYLD transcriptional activation and protein accumulation in response to *IFN- γ*

were further characterised for various time points post-treatment through qRT-PCR and WB in THP-1- or U937-derived macrophages (Figure 5.5). Transcriptional induction of CYLD following IFN- γ stimulation is slow, it starts between 1 and 2 hours and it accumulates up to 24 hours post-treatment. This dynamic is different from IFN- γ early response genes such as *IRF1*, where transcription starts around 30 minutes post-stimulation. CYLD transcriptional dynamic suggests the necessity of *de novo*-synthesised TFs, such as IRFs which are known to cooperate with STATs for delayed gene transcription [95]. WB analysis showed that CYLD protein accumulates around 8 hours and it increases up to 24 hours. Increased CYLD protein levels in response to IFN- γ were also observed by WB analysis in Peripheral Blood Mononuclear cells (PBMCs) from healthy donors differentiated into macrophages (Figure 5.5). In addition, IFN- γ -dependent CYLD upregulation was tested in HCT-116 epithelial cells, where qRT-PCR and WB analysis showed an IFN- γ -dependent increase in CYLD mRNA and protein levels (Figure 9.2).

5.3.2 Increased CYLD mRNA and protein levels rely on STAT1 and STAT3 activation

In response to IFNGR activation both STAT1 and STAT3 TFs are activated through phosphorylation. Therefore, I investigated their engagement in CYLD transcriptional activation. As these TFs are partially redundant in mediating the IFN- γ downstream transcriptional response, I tried to dissect STAT1 and STAT3 transcriptional activities using specific inhibitors: fludarabine (causing depletion of STAT1 and STAT1 Y701 phosphorylated form) and BP1-102 (blocking STAT3 phosphorylation Y705) [108, 142–144].

Inhibition of STAT1 and STAT3 activation following IFN- γ stimulation in THP-1-derived macrophages was assessed through WB by reduction in Y701-phosphorylated STAT1 and Y705-phosphorylated STAT3. WB analysis showed a combinatory inhibition of STAT1 and STAT3 phosphorylation in the presence of both fludarabine or BP1-102. As both inhibitors separately inhibited STAT1 and STAT3, it was not possible to separate their activity by these means (Figure 5.6).

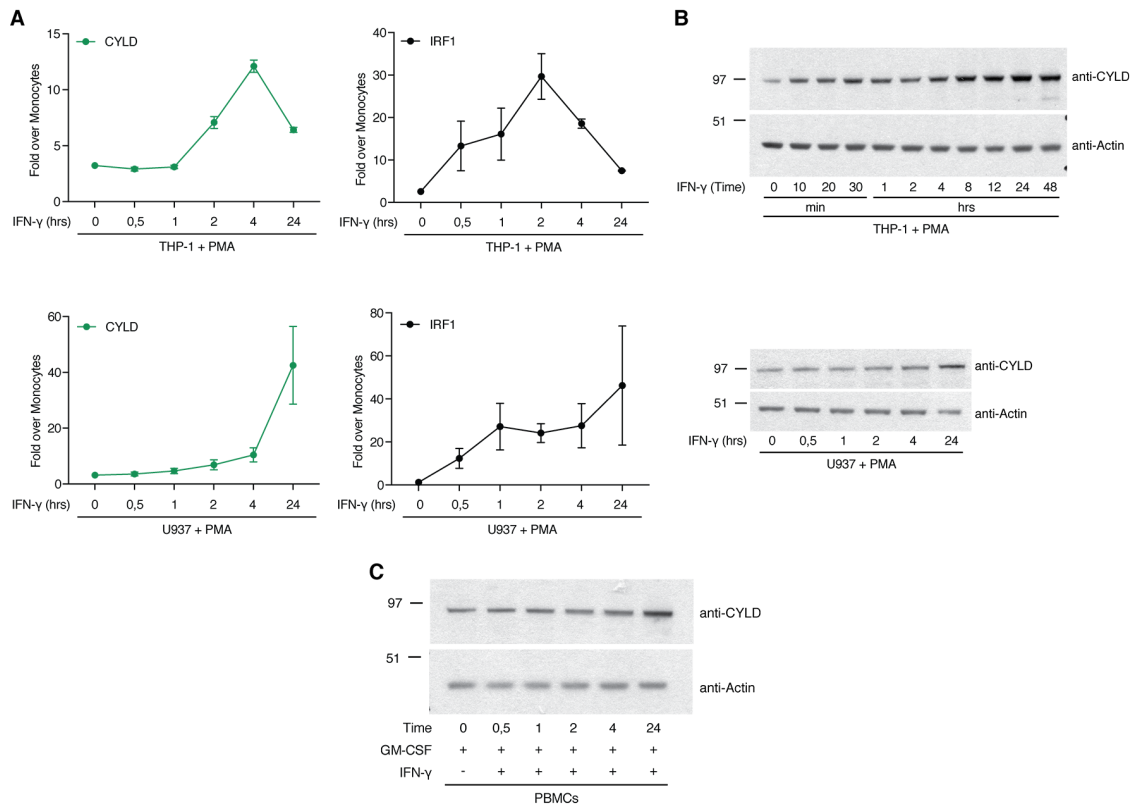


Figure 5.5: CYLD is a late IFN- γ responsive gene. (A) qRT-PCR for CYLD and IRF1 in THP-1- or U937-derived macrophages treated with IFN- γ (20 ng/ml) for indicated times and normalised to the untreated monocytic state (B) WB for CYLD in THP-1- or U937-derived macrophages treated with IFN- γ (20 ng/ml) for indicated times (C) WB for CYLD in GM-CSF-differentiated PBMCs treated with IFN- γ (20 ng/ml) for indicated times. Triplicates.

Nonetheless, treatment with these inhibitors completely blocked IFN- γ induced CYLD expression, showing a requirement for STAT1 and STAT3, and confirming the IFN- γ signalling role in this induction (Figure 5.6).

5.3.3 STAT3-specific signalling pathways do not activate CYLD induction

In order to investigate a possible individual role for STAT3 in CYLD induction I tested whether STAT3-specific stimuli, such as IL-6 or IL-10, were increasing CYLD mRNA and protein levels. THP-1-derived macrophages were stimulated with IL-6 or IL-10 for various time points and CYLD transcript levels were measured via qRT-PCR while protein levels through WB. qRT-PCR and WB analysis displayed no differences in CYLD levels in response to IL-6 or IL-10

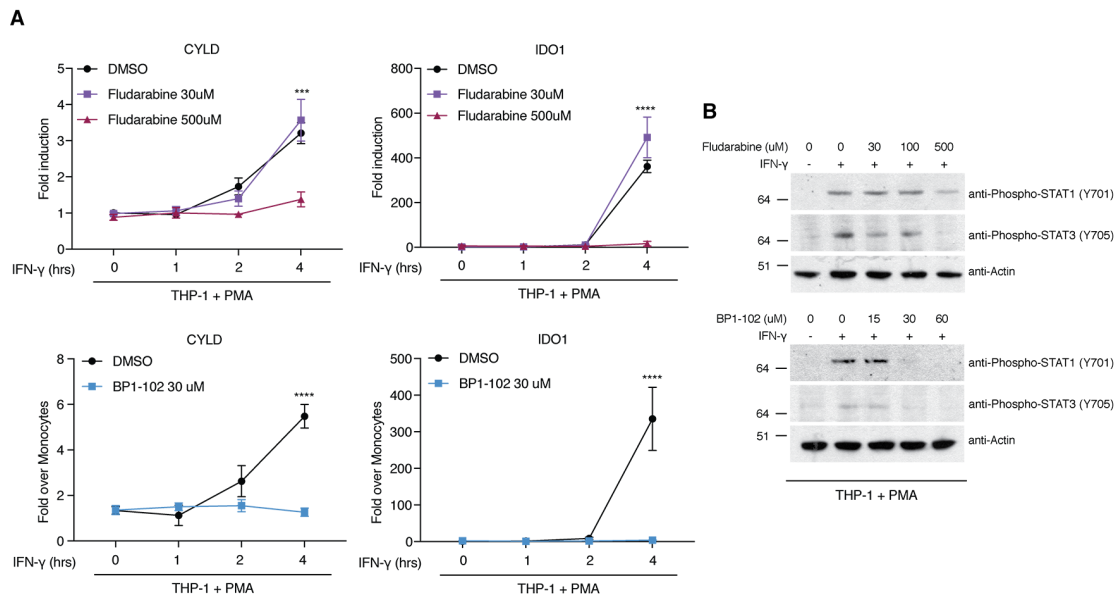


Figure 5.6: IFN- γ induced STATs are required for *CYLD* transcription. (A) qRT-PCR for *CYLD* and *IDO1* in THP-1-derived macrophages incubated with fludarabine (30 μ M or 500 μ M, 1 hr) or BP1-102 (30 μ M, 1hr) and treated with IFN- γ (20 ng/ml) for indicated times and normalised to the DMSO-treated control cells. (B) WB for Phospho-STAT1 (Y701) and Phospho-STAT3 (Y705) in THP-1-derived macrophages incubated with fludarabine (30 μ M, 100 μ M, or 500 μ M, 1 hr) or BP1-102 (15 μ M, 30 μ M and 60 μ M, 1hr) and treated with IFN- γ (20 ng/ml, 30 mins). Triplicates. Two Way ANOVA statistics.

stimulation. These data suggest that STAT3 activation alone is not sufficient to drive *CYLD* induction (Figure 5.7).

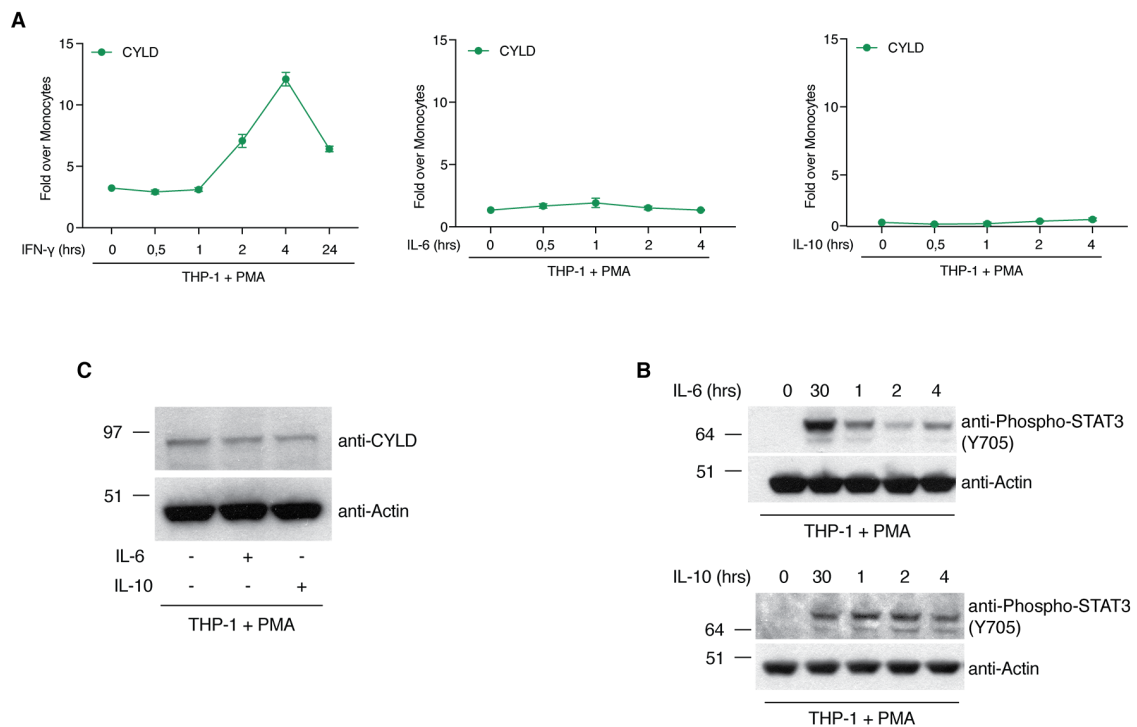


Figure 5.7: STAT3-specific stimuli do not induce *CYLD*. (A) qRT-PCR for *CYLD* in THP-1-derived macrophages treated with IFN- γ (20 ng/ml) or IL-6 (10 ng/ml) or IL-10 (10 ng/ml) for indicated times and normalised to the untreated monocytic state (B) WB for Phospho-STAT3 (Y705) in THP-1-derived macrophages treated with IL-6 (10 ng/ml) or IL-10 (10 ng/ml) for indicated times (C) WB for *CYLD* in THP-1-derived macrophages treated with IL-6 (10 ng/ml) or IL-10 (10 ng/ml) for 2 hrs + 22 hrs rest. Triplicates.

5.4 Discussion

CYLD was initially observed to be transcriptionally induced by a variety of NF- κ B-activating stimuli in epithelial cells [134]. Despite its important activity in regulating innate immune pathways, CYLD transcriptional regulation in this context was still not clear. In this chapter I identified CYLD to be induced in activated human macrophages, suggesting a possible role for this enzyme in this specific cell state. Increased CYLD mRNA and protein levels in activated macrophages could appear counterproductive for the pro-inflammatory function of these cells. However, an increased CYLD activity could be important to regulate an excessive and sustained NF- κ B-mediated inflammatory response.

In addition, I showed that NF- κ B activation through LPS stimulation only mildly induces *CYLD* gene transcription. Instead, I identified IFN- γ as the predominant stimulus for CYLD induction in human macrophages and epithelial cells. IFN- γ -dependent CYLD upregulation was confirmed by interfering with the IFN- γ downstream TFs (STAT1 and STAT3) using two different STATs inhibitors. CYLD transcriptional dynamic suggests that this gene is part of the second wave of IFN- γ regulated genes. CYLD mRNA accumulation resembles the transcriptional dynamics of known IFN- γ late response genes, such as *IDO1* and *CCL2*. These data suggest the involvement of *de novo*-synthesised TFs in CYLD induction, such as IRF1, IRF2 and IRF9, which are known to cooperate with STAT1 and STAT3 in orchestrating the IFN- γ secondary transcriptional response [66, 95].

Lastly, I investigated CYLD transcriptional regulation in STAT3-specific immunoregulatory stimuli, namely IL-6 and IL-10. No induction of CYLD was observed following IL-6 or IL-10 stimulation, suggesting that STAT3 activation alone is not sufficient to drive CYLD transcription, and enforcing the IFN- γ stimulus-specificity in this regulation.

6

CYLD regulates IFN- γ response in human macrophages

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6.1 Introduction

IFN- γ signalling primes macrophages and regulates several biological functions, including immune surveillance, pathogen clearance and antiviral response [95, 142]. I previously showed that *CYLD* is an IFN- γ target gene. In light of the important function of NF- κ B and IFN- γ signalling pathways in macrophages, in this chapter I will explore the consequences of *CYLD* depletion during differentiation and activation of human THP-1 monocytic cells. In addition, I will investigate the role of *CYLD* within the IFN- γ signalling pathway in THP-1-derived macrophages. The data presented indicate *CYLD* as a negative regulator of the IFN- γ signalling pathway, showing that its deubiquitinase activity is required for this regulation. In line with this, Lys63- and Met1-linked Ub chains are involved in the IFN- γ -mediated transcriptional response. Furthermore, *CYLD*-depleted THP-1-derived macrophages present an over-production of proinflammatory cytokines, including IFN- γ , as previously reported for murine macrophages [45, 51].

6.2 *CYLD*-depleted macrophages

6.2.1 *CYLD*-deficient macrophages present morphological features of activated macrophages

To investigate a possible role for *CYLD* in regulating the IFN- γ signalling pathway, I generated *CYLD*-depleted THP-1 and U937 monocytic cells via lentiviral transduction of a Short Hairpin RNA (ShRNA) targeting *CYLD*. Similarly, a non-targeting ShRNA was transduced in THP-1 and U937 monocytic cells to generate control cell lines. *CYLD* protein levels following ShRNA transduction and puromycin selection were evaluated through WB analysis (Figure 6.1).

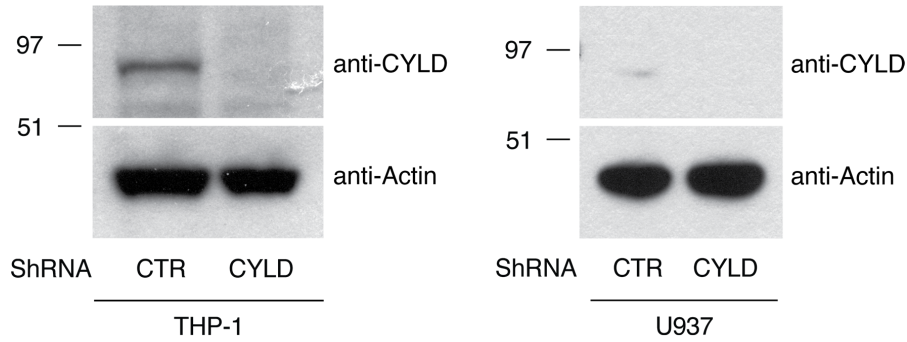


Figure 6.1: CYLD-depleted THP-1 and U937 human monocytic cells. WB analysis of CYLD protein levels in THP-1 and U937 monocytic cells transduced with a CYLD-targeting ShRNA (ShCYLD) or non-targeting ShRNA (ShCTR) and selected with Puromycin (1 μ g/ml, 6 days).

For the purpose of this thesis from now on the following nomenclature will be used:

THP-1 monocytes = THP-1

THP-1 monocytes transduced with control non-targeting ShRNA = THP-1-ShCTR

THP-1 monocytes transduced with CYLD-targeting ShRNA = THP-1-ShCYLD

THP-1-derived macrophages = THP-1^{PMA}

THP-1-derived macrophages with control non-targeting ShRNA = THP-1^{PMA}-ShCTR

THP-1-derived macrophages with CYLD-targeting ShRNA = THP-1^{PMA}-ShCYLD

U937 monocytes = U937

U937 monocytes transduced with control non-targeting ShRNA = U937-ShCTR

U937 monocytes transduced with CYLD-targeting ShRNA = U937-ShCYLD

U937-derived macrophages = U937^{PMA}

U937-derived macrophages with control non-targeting ShRNA = U937^{PMA}-ShCTR

U937-derived macrophages with CYLD-targeting ShRNA = U937^{PMA}-ShCYLD

To explore the role of CYLD in monocyte differentiation and macrophage activation, THP-1-ShCTR and THP-1-ShCYLD cells were differentiated and activated as previously described. Morphological analysis following differentiation showed that THP-1^{PMA}-ShCYLD macrophages exhibited an elongated fibroblastoid morphology

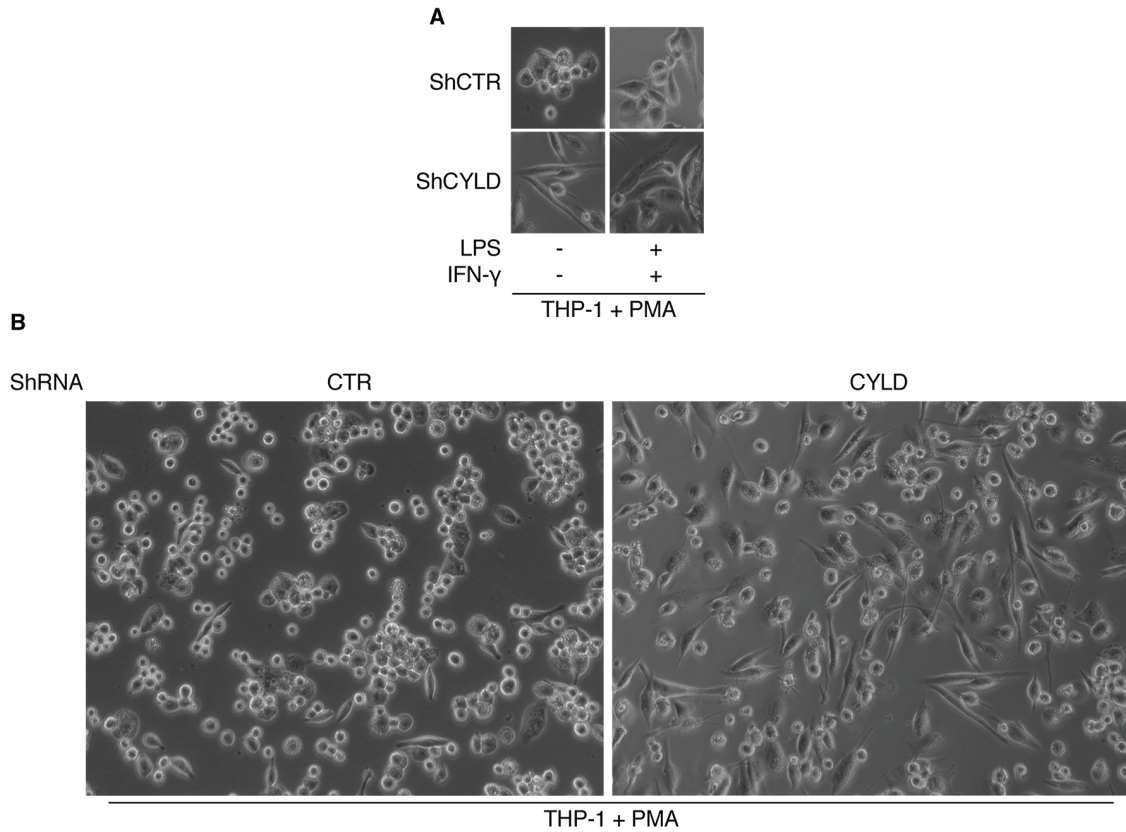


Figure 6.2: Morphological analysis of *CYLD*-depleted macrophages. (A) Bright-field microscopy images for THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages untreated or activated with LPS (50 ng/ml) + IFN-γ (20 ng/ml) for 2 hrs + 22 hrs rest (B) Bright-field microscopy images for THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages.

with pseudopodia, in contrast to the spherical shape of THP-1^{+PMA}-ShCTR cells. The morphological features observed in THP-1^{+PMA}-ShCYLD macrophages can be identified in the reported morphology for LPS + IFN-γ activated THP-1-derived macrophages [141]. Accordingly, THP-1^{+PMA}-ShCTR macrophages stimulated with LPS + IFN-γ displayed a fibroblastoid morphology with pseudopodia, which was enhanced in activated THP-1^{+PMA}-ShCYLD macrophages (Figure 6.2).

6.2.2 Gene expression profiling revealed a pre-activated state in *CYLD*-deficient macrophages

To investigate differences between control and *CYLD*-depleted cells, gene expression analysis was performed through RNA-microarray in monocytic cells, PMA-differentiated cells, and PMA-differentiated cells activated with LPS + IFN-γ.

Global gene expression analysis revealed no major differences comparing control and CYLD-depleted cells at monocytic state, while differences were observed between THP-1^{+PMA}-ShCYLD and THP-1^{+PMA}-ShCTR macrophages following differentiation, and in response to LPS + IFN- γ . In particular, GO analysis of differentially regulated genes comparing THP-1^{+PMA}-ShCYLD and THP-1^{+PMA}-ShCTR macrophages showed an increased expression of genes involved in immune response, defense response and bacterial response. Moreover, GO analysis of differentially regulated genes comparing THP-1^{+PMA}-ShCYLD and THP-1^{+PMA}-ShCTR macrophages activated via LPS + IFN- γ revealed an enhanced expression of genes involved in cell differentiation, cell adhesion, inflammatory response and response to bacterial molecule (Figure 6.3).

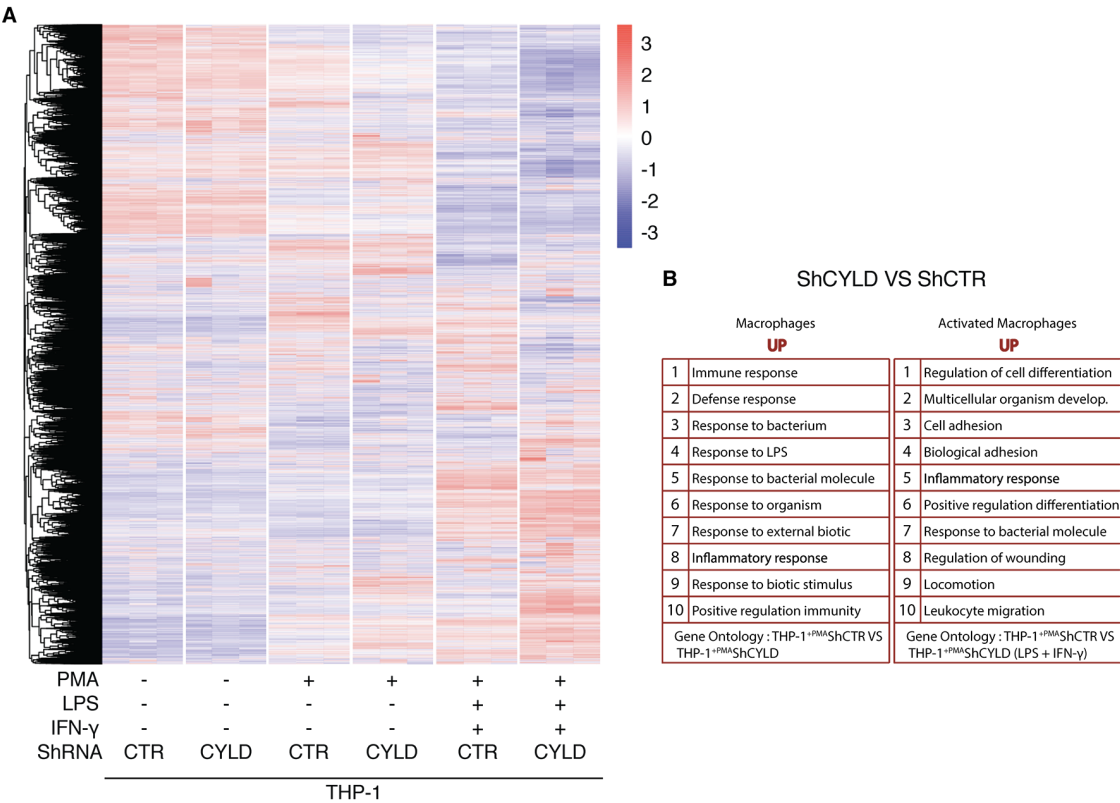


Figure 6.3: Gene expression profiling of control and CYLD-depleted cells. (A) Heat map representation of gene expression profiles for control and CYLD-depleted cells at monocytic state, PMA-differentiated, and following activation with LPS (50 ng/ml) + IFN- γ (20 ng/ml) for 2 hrs + 22 hrs rest. RNA-microarray. Triplicates. (B) GO analysis of differentially expressed genes (upregulated) comparing THP-1^{+PMA}-ShCYLD versus THP-1^{+PMA}-ShCTR macrophages (left) or comparing LPS (50 ng/ml) + IFN- γ (20 ng/ml) treated THP-1^{+PMA}-ShCYLD versus THP-1^{+PMA}-ShCTR macrophages (right).

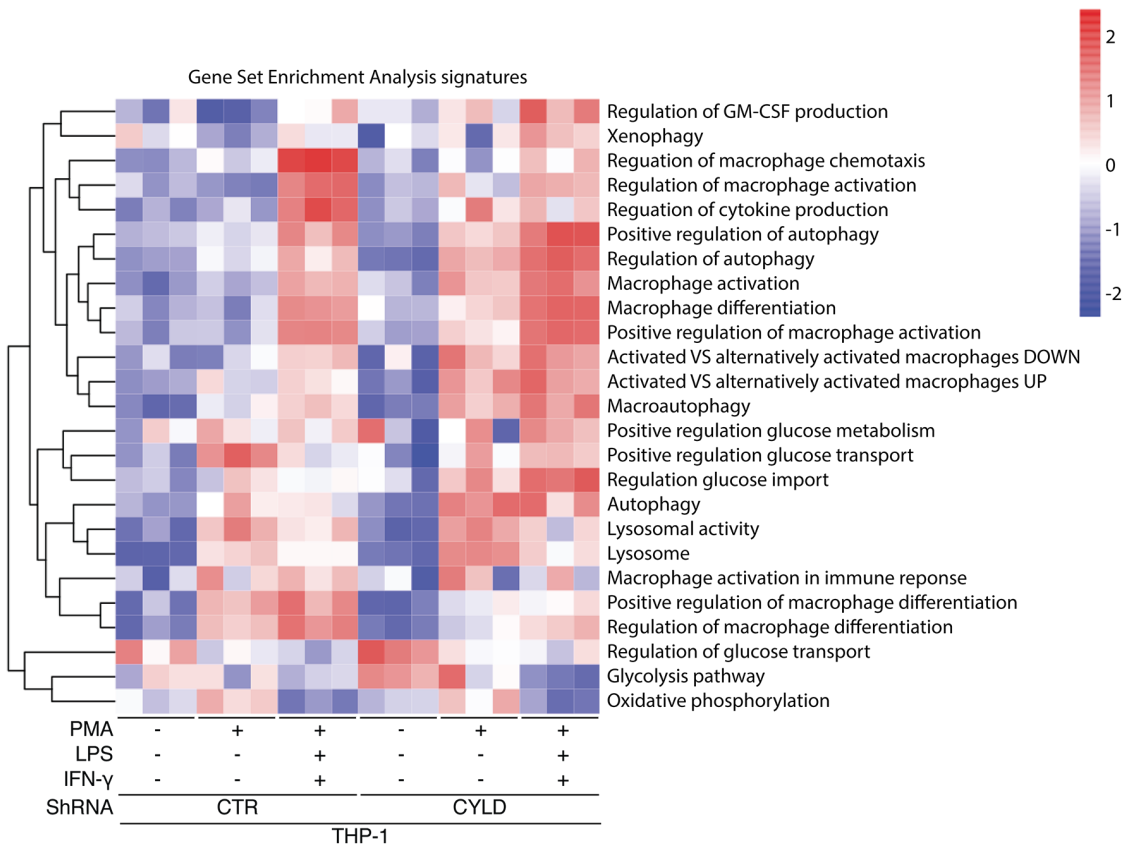


Figure 6.4: Activated macrophage signatures. Heat map of GSEA for macrophage-related gene sets comparing THP-1-ShCYLD and THP-1-ShCTR cells at monocytic state, following PMA-differentiation and subsequent activation with LPS (50 ng/ml) + IFN- γ (20 ng/ml) for 2 hrs + 22 hrs rest, RNA-microarray. Triplicates.

Gene Set Enrichment Analysis (GSEA) for macrophage-related gene sets was performed at monocytic-state, macrophage-state and for activated macrophages in both control and CYLD-depleted cells. GSEA showed an enrichment for gene sets related to macrophage differentiation, glucose metabolism, autophagy and immune response during monocyte-to-macrophage differentiation, while gene sets involved in chemotaxis, macrophage activation, cytokine production and xenophagy were enhanced following LPS + IFN- γ treatment. These expression patterns were similar between control and CYLD-depleted cells across the three cellular states analysed. However, CYLD-depleted cells showed a significantly enhanced expression of these signatures, following PMA-mediated differentiation, and in response to LPS + IFN- γ (Figure 6.4).

I initially focused on characterising the role of CYLD during PMA-mediated

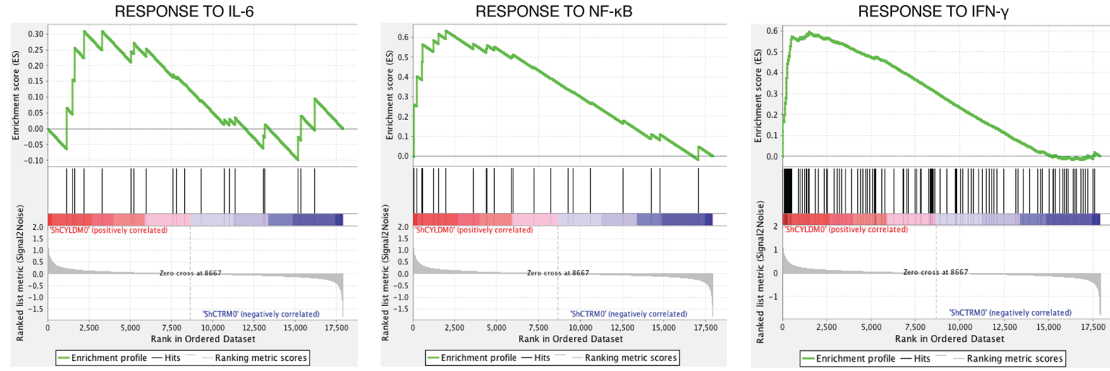


Figure 6.5: GSEA on THP-1+*PMA*-ShCYLD versus THP-1+*PMA*-ShCTR. GSEA for "BIOCARTA IL-6", "BIOCARTA NF- κ B" and "BIOCARTA IFN- γ " pathways on differentially expressed genes comparing THP-1+*PMA*-ShCYLD versus THP-1+*PMA*-ShCTR macrophages, RNA-microarray. Triplicates.

differentiation, and I performed a GSEA for immune signalling pathways on differentially regulated genes in THP-1+*PMA*-ShCYLD compared to THP-1+*PMA*-ShCTR. In agreement with the notion that PMA stimulation activates Protein Kinase C (PKC) and downstream NF- κ B signalling [145], I performed GSEA for the NF- κ B pathway to assess possible differences in its activation. Significant enrichment for NF- κ B GSEA was observed in THP-1+*PMA*-ShCYLD compared to THP-1+*PMA*-ShCTR (Figure 6.5). Moreover, I investigated deregulation of other immune signalling pathways through GSEA, including IL-6 and IFN- γ , comparing THP-1+*PMA*-ShCYLD and THP-1+*PMA*-ShCTR cells. No enrichment for the IL-6 signalling pathway was observed, but the GSEA for the IFN- γ signalling pathway was significantly enhanced in THP-1+*PMA*-ShCYLD compared to THP-1+*PMA*-ShCTR macrophages (Figure 6.5).

6.3 CYLD-depleted macrophages present an enhanced response to IFN- γ

6.3.1 CYLD is a negative regulator of the IFN- γ signalling pathway

The IFN- γ -dependent induction of CYLD, together with an enrichment for the IFN- γ signature identified through GSEA in CYLD-depleted macrophages, prompted

me to investigate further the role of CYLD in this signalling pathway. Similar to the deubiquitinase A20, CYLD upregulation following NF- κ B activation has been linked to its inhibitory function [132, 134]. Therefore, I hypothesised a possible role for CYLD as a negative regulator of the IFN- γ signalling pathway. To investigate this possibility, THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages were stimulated with IFN- γ for various time points. IFN- γ signalling pathway activation was evaluated via STAT1 (Y701) and STAT3 (Y705) phosphorylation. WB analysis displayed an increased induction of phosphorylated STAT1 and STAT3 in CYLD-depleted macrophages compared to control cells, while no differences were observed for total STATs levels. The same experiment was performed in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages, and a similar pattern was observed for STAT1- and STAT3-activating phosphorylation (Figure 6.6 and 9.3).

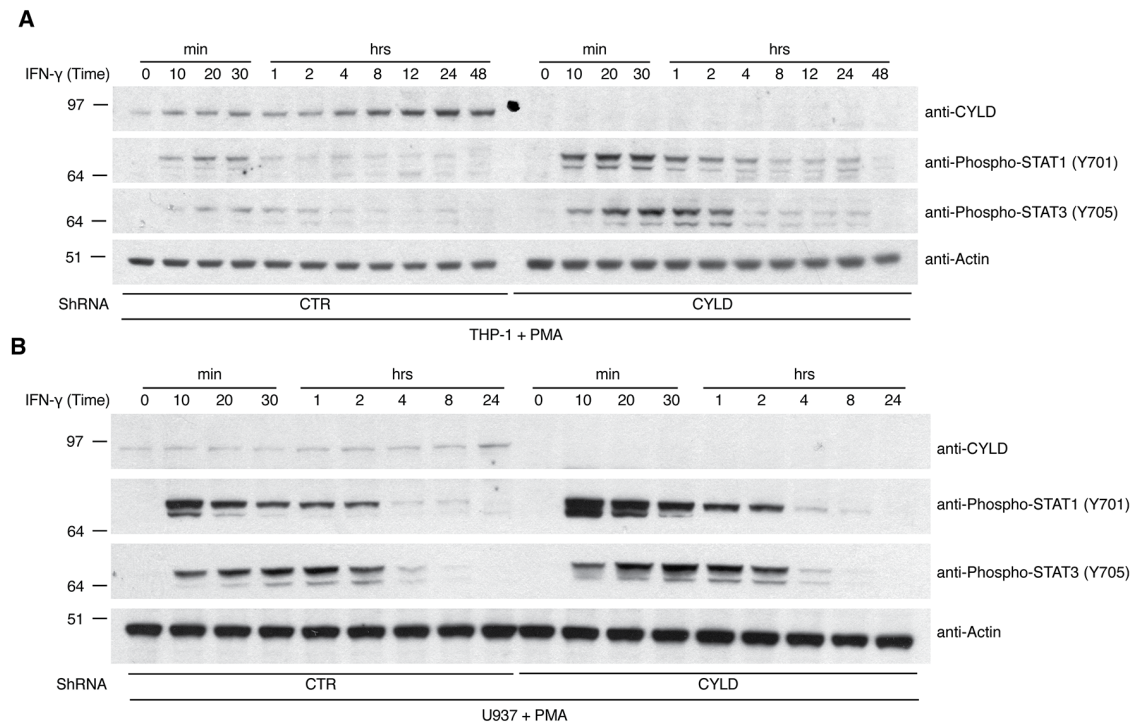


Figure 6.6: CYLD is a negative regulator of the IFN- γ signalling. (A) WB analysis of CYLD, Phospho-STAT1 (Y701) and Phospho-STAT3 (Y705) protein levels in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages following IFN- γ (20 ng/ml) stimulation for indicated times (B) WB analysis of CYLD, Phospho-STAT1 (Y701) and Phospho-STAT3 (Y705) protein levels in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages following IFN- γ (20 ng/ml) stimulation for indicated times.

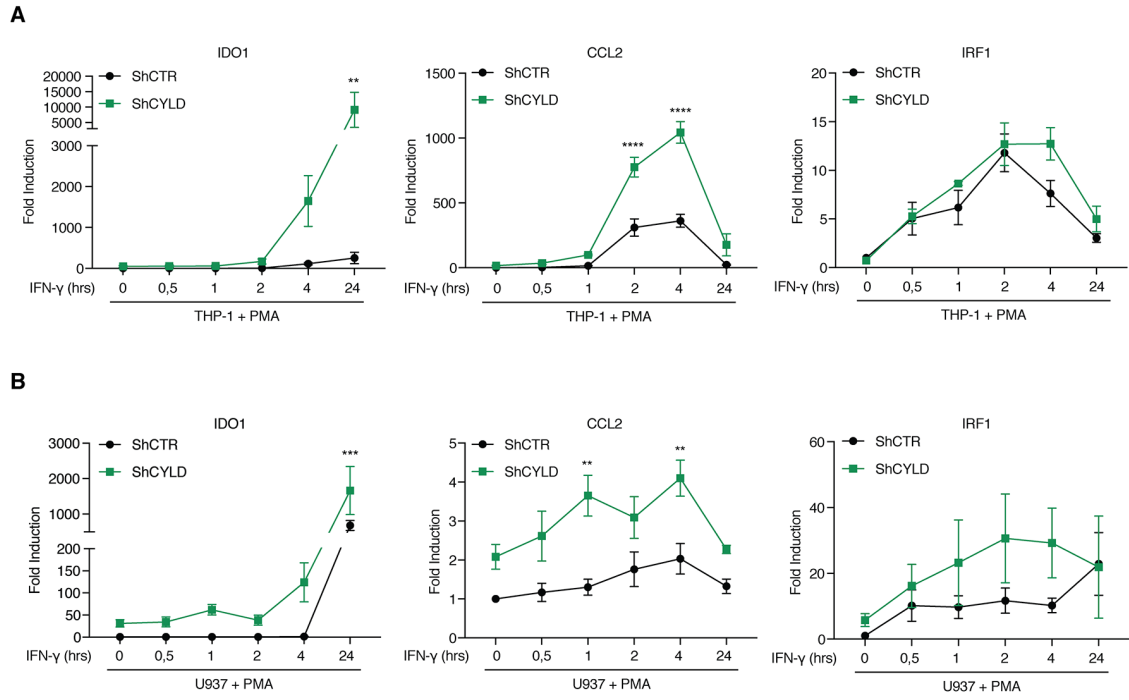


Figure 6.7: CYLD negatively regulates the *IFN- γ* transcriptional response. (A) qRT-PCR for IDO1, CCL2 and IRF1 in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages following *IFN- γ* (20 ng/ml) stimulation for indicated times and normalised to untreated control cells (B) qRT-PCR for IDO1, CCL2 and IRF1 in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages following *IFN- γ* (20 ng/ml) stimulation for indicated times and normalised to untreated control cells. Triplicates. Two Way ANOVA statistics.

Subsequently, I examined whether a stronger activation of STAT1 and STAT3, in *CYLD*-depleted cells, was leading to differences in the downstream transcriptional response. qRT-PCR for IRF1, IDO1 and CCL2 was performed in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages following *IFN- γ* stimulation. qRT-PCR analysis displayed no differences in IRF1 transcriptional dynamic, while significant differences were observed in IDO1 and CCL2 transcription following *IFN- γ* stimulation. A similar pattern was observed in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages treated with *IFN- γ* (Figure 6.7).

To appraise global transcriptional changes in response to *IFN- γ* stimulation, RNA-Sequencing (RNA-Seq) analysis was performed in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages untreated and following *IFN- γ* treatment. Validation of the experimental procedure and RNA-Seq data was initially per-

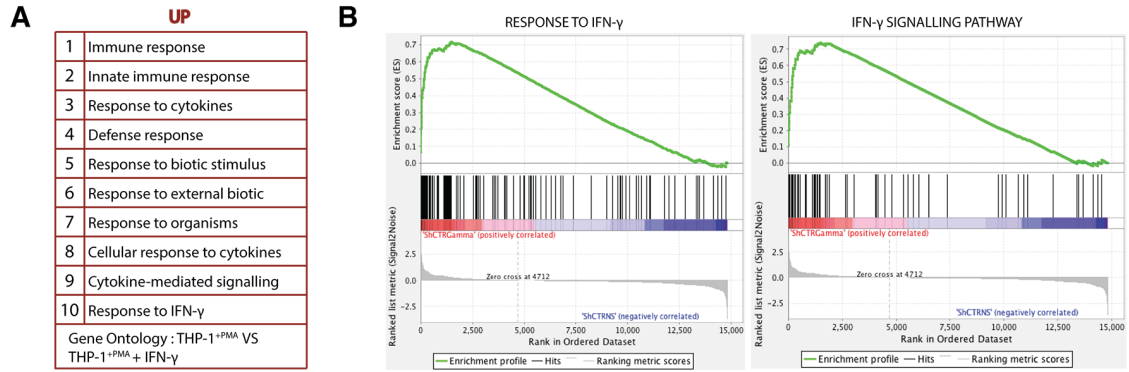


Figure 6.8: Validation of RNA-Seq experimental data. (A) GO on differentially expressed genes comparing THP-1^{PM}A-ShCTR untreated versus IFN-γ treated macrophages, RNA-Seq, triplicates (B) GSEA for IFN-γ-related gene sets on differentially expressed genes comparing THP-1^{PM}A-ShCTR untreated versus IFN-γ treated macrophages. RNA-Seq. Triplicates.

formed analysing THP-1^{PM}A-ShCTR macrophages untreated and following IFN-γ stimulation. GO analysis of differentially expressed genes comparing untreated and IFN-γ treated THP-1^{PM}A-ShCTR macrophages displayed an upregulation of genes involved in immune response, defense response, response to cytokine, and in particular response to IFN-γ. Moreover, GSEA showed an enrichment for IFN-γ-related signatures in treated control cells compared to untreated (Figure 6.8).

To investigate differences in the IFN-γ downstream transcriptional response, GSEA for innate immune and IFN-γ-related gene sets was performed on differentially expressed genes comparing THP-1^{PM}A-ShCYLD versus THP-1^{PM}A-ShCTR macrophages stimulated with IFN-γ. GSEA showed a significant enrichment for innate immune processes and the IFN-γ signatures in CYLD-depleted compared to control macrophages following IFN-γ treatment. These findings suggest CYLD is a negative regulator of the IFN-γ-mediated transcriptional response (Figure 6.9).

RNA-Seq data were further used to validate differences between THP-1^{PM}A-ShCTR and THP-1^{PM}A-ShCYLD macrophages previously identified in RNA-microarray analysis. GO analysis comparing CYLD-depleted and control macrophages showed an enrichment for defense response, cytokine response and immune response genes. In addition, GSEA displayed a significant enrichment in immune response

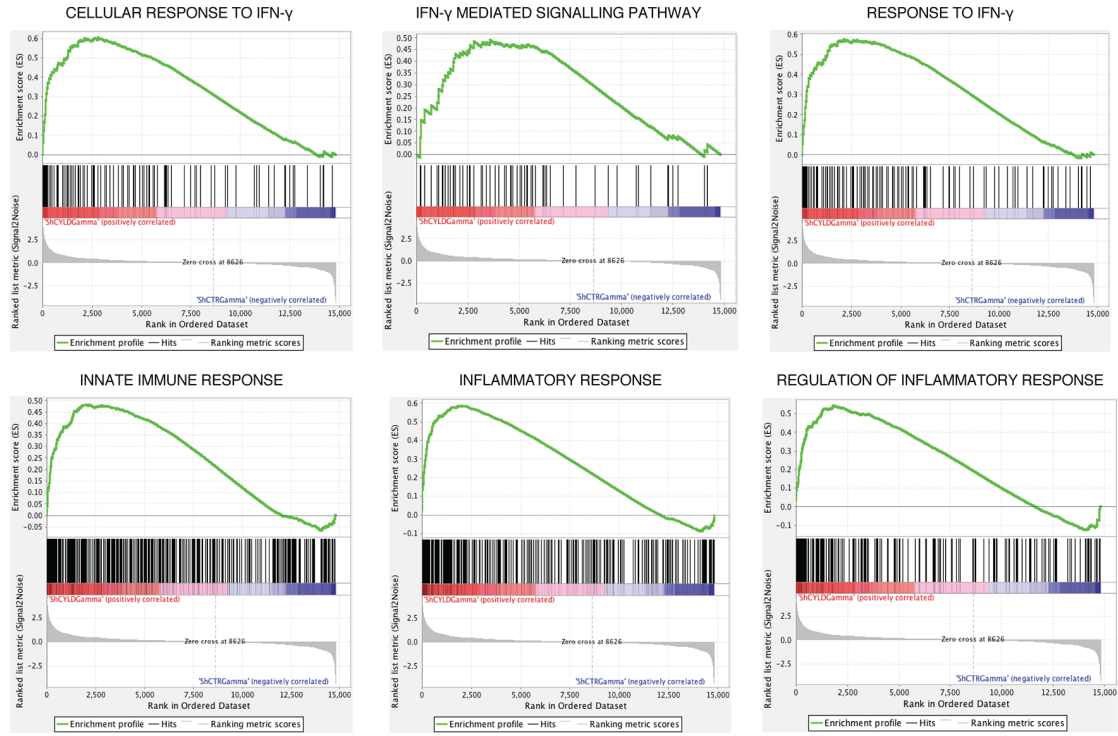


Figure 6.9: Enhanced IFN- γ transcriptional response in *CYLD*-depleted cells. GSEA for innate immune- and IFN- γ -related gene sets on differentially expressed genes comparing THP-1+*PMA*-Sh*CYLD* and THP-1+*PMA*-ShCTR macrophages treated with IFN- γ . RNA-Seq. Triplicates.

and IFN- γ -related signatures in THP-1+*PMA*-Sh*CYLD* compared to THP-1+*PMA*-ShCTR macrophages (Figure 6.10).

6.3.2 *CYLD* negative regulation of the IFN- γ pathway requires its deubiquitinase activity

CYLD's deubiquitinase activity is the mechanism responsible for NF- κ B signalling pathway regulation [8, 33, 35]. As such, I hypothesised that *CYLD* may be regulating IFN- γ signalling pathways via a similar mechanism. To test this, I used *CYLD* KO U2OS cells (U2OS^{*CYLD*-KO}) retrovirally reconstituted with the wild type form of *CYLD* (U2OS^{*CYLD*-WT}) or with the C601A mutant (U2OS^{*CYLD*-C601A}). The C601A is a single amino acid mutation which disrupts the catalytic activity of *CYLD* (Figure 9.5) [23]. In parallel, I used Mouse Embryonic Fibroblasts (MEFs) generated from *CYLD* KO mice (MEF^{*CYLD*-KO}), and transduced to express the human wild

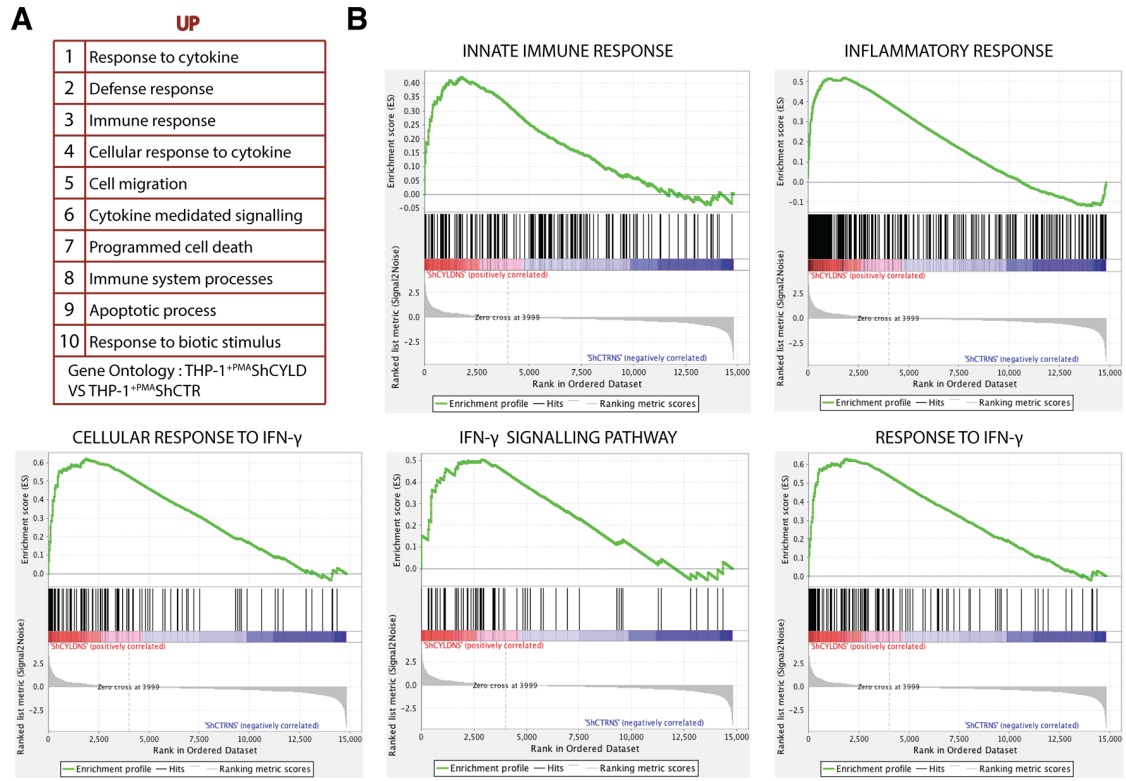


Figure 6.10: *CYLD*-depleted macrophages present increased *IFN-γ* signatures. (A) GO analysis of differentially expressed genes comparing THP-1+*PMA*-ShCYLD and THP-1+*PMA*-ShCTR macrophages. RNA-Seq, triplicates (B) GSEA for immune response, inflammatory response and *IFN-γ*-related signatures on differentially expressed genes comparing THP-1+*PMA*-ShCYLD and THP-1+*PMA*-ShCTR macrophages. RNA-Seq. Triplicates.

type or catalytically inactive form of *CYLD* (MEF^{*CYLD*-WT}, MEF^{*CYLD*-C601A}). All these cells were previously generated in the Gyrd-Hansen lab by Derek Leske.

A qRT-PCR for *IRF1*, *IDO1* and *CCL2* was performed in U2OS^{*CYLD*-KO}, U2OS^{*CYLD*-WT} and U2OS^{*CYLD*-C601A} cells following *IFN-γ* stimulation. Comparable with THP-1- and U937-derived macrophages, an enhanced transcriptional activation of *IDO1* and *CCL2*, but not *IRF1*, was observed in *CYLD* KO cells compared to *CYLD* WT reconstituted cells. Additionally, deregulation of these genes was observed also in cells reconstituted with the C601A catalytically inactive form of *CYLD*, suggesting the requirement of its deubiquitinase activity in regulating the *IFN-γ*-mediated transcriptional response. A similar result was observed in MEF^{*CYLD*-KO}, MEF^{*CYLD*-WT} and MEF^{*CYLD*-C601A}. These cells displayed enhanced transcriptional activation of *IDO1*, *CCL2* and *IRF1* genes in the absence

of CYLD, or in the presence of the catalytically inactive form of the enzyme, following IFN- γ treatment (Figure 6.11).

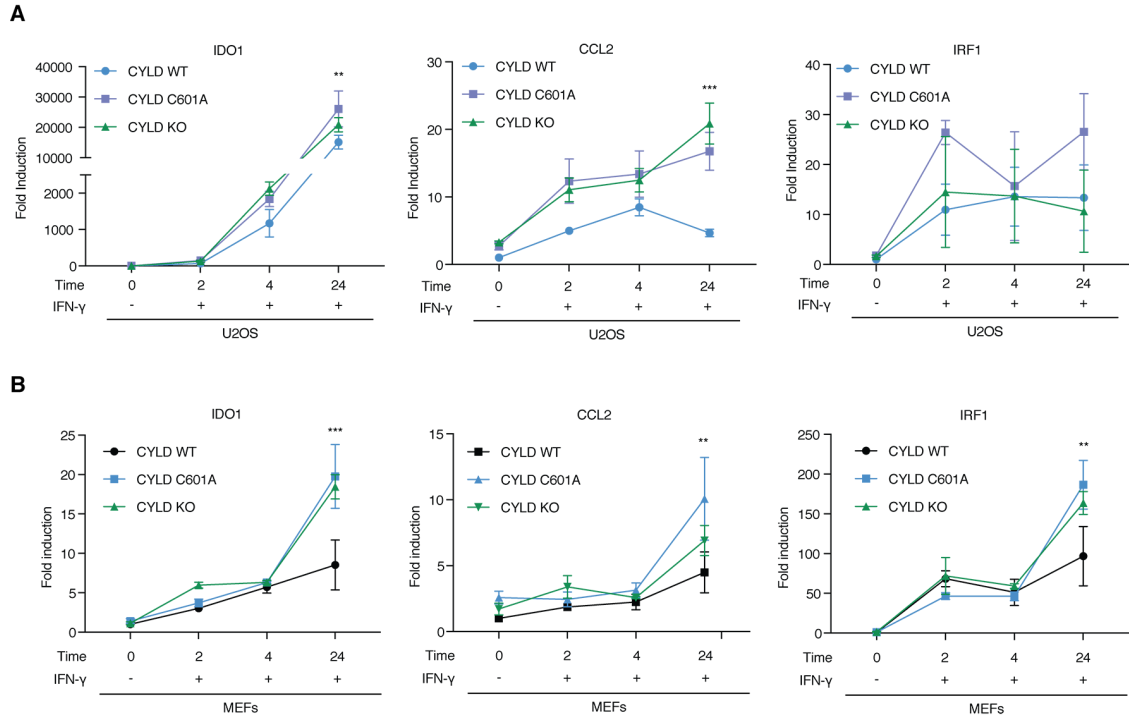


Figure 6.11: CYLD negative regulation of IFN- γ requires its DUB activity. (A) qRT-PCR for IDO1, CCL2 and IRF1 in U2OS^{CYLD-WT}, U2OS^{CYLD-C601A} and U2OS^{CYLD-KO} following IFN- γ (100 ng/ml) stimulation for indicated times and normalised to untreated control cells (B) qRT-PCR for IDO1, CCL2 and IRF1 in MEF^{CYLD-WT}, MEF^{CYLD-C601A} and MEF^{CYLD-KO} following IFN- γ (100 ng/ml) stimulation for indicated times and normalised to untreated control cells. Triplicates. Two Way ANOVA statistics.

6.3.3 Lys63- and Met1-linked ubiquitin chains are involved in IFN- γ signalling

CYLD regulation of the IFN- γ signalling pathway seems to require its enzymatic activity. In line with this, I investigated the direct involvement of Lys63- and Met1-linked Ub chains in the IFN- γ signalling pathway. I generated THP-1 cells with a Doxycycline-inducible expression of GFP-NES (THP-1^{GFP-NES}), GFP-NES-K63-SUB (THP-1^{GFP-NES-K63}), or GFP-M1-SUB (THP-1^{GFP-NES-M1}). qRT-PCR for IDO1, CCL2 and IRF1 was performed in THP-1^{GFP-NES}, THP-1^{GFP-NES-K63} and THP-1^{GFP-NES-M1} PMA-differentiated and challenged with IFN- γ for various

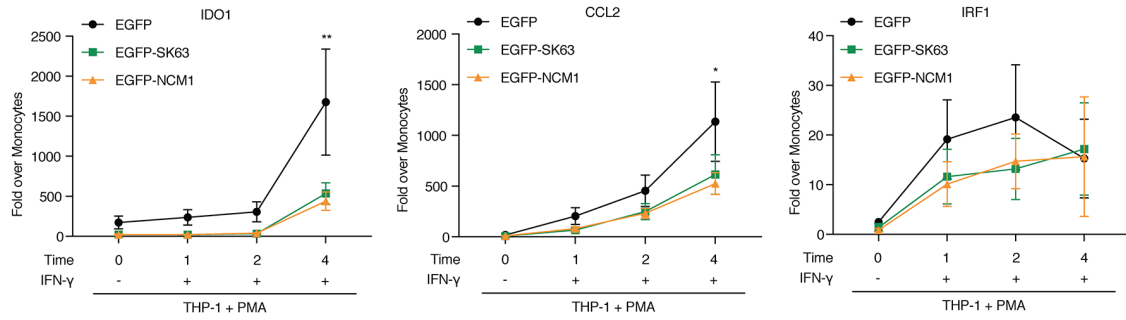


Figure 6.12: Lys63 and Met1 are engaged in $IFN-\gamma$ signalling pathway. qRT-PCR for IDO1, CCL2 and IRF1 in THP-1^{GFP-NES}-, THP-1^{GFP-NES-K63}- and THP-1^{GFP-NES-M1}-derived macrophages incubated with Doxycycline (500 ng/ml, 24 hrs) and treated with $IFN-\gamma$ (20 ng/ml) for indicated times and normalised to untreated control cells. Triplicates. Two Way ANOVA statistics.

time points. Lys63- or Met1-linked Ub chain cytoplasmic interference significantly reduced transcriptional activation of IDO1 and CCL2 following $IFN-\gamma$ treatment, while no differences were observed in IRF1 transcriptional dynamic (Figure 6.12).

6.4 CYLD-depleted macrophages present unwarrented production of $IFN-\gamma$

6.4.1 CYLD-depleted macrophages present enhanced production of pro-inflammatory cytokines

Ahmed *et al.* and Nishanth *et al.* previously showed that CYLD-deficient Bone Marrow-derived Macrophages (BMDMs) display an over-production of pro-inflammatory cytokines in response to $TNF-\alpha$ and *Listeria monocytogenes* infection [45, 51]. Considering the different morphological features, and the diverse gene expression profiles, observed following PMA-mediated differentiation in THP-1-ShCYLD compared to THP-1-ShCTR, I investigated the production of pro-inflammatory mediators in these cells.

Production of CXCL-8, $IL-1\beta$, $IFN-\gamma$ and $TNF-\alpha$ was measured via intracellular cytokine staining and flow cytometry in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages. THP-1^{+PMA}-ShCYLD cells displayed significantly increased production of CXCL-8, $IL-1\beta$ and $IFN-\gamma$ compared to THP-1^{+PMA}-ShCTR cells, while no differences were observed in $TNF-\alpha$ production (Figure 6.13 and 9.4).

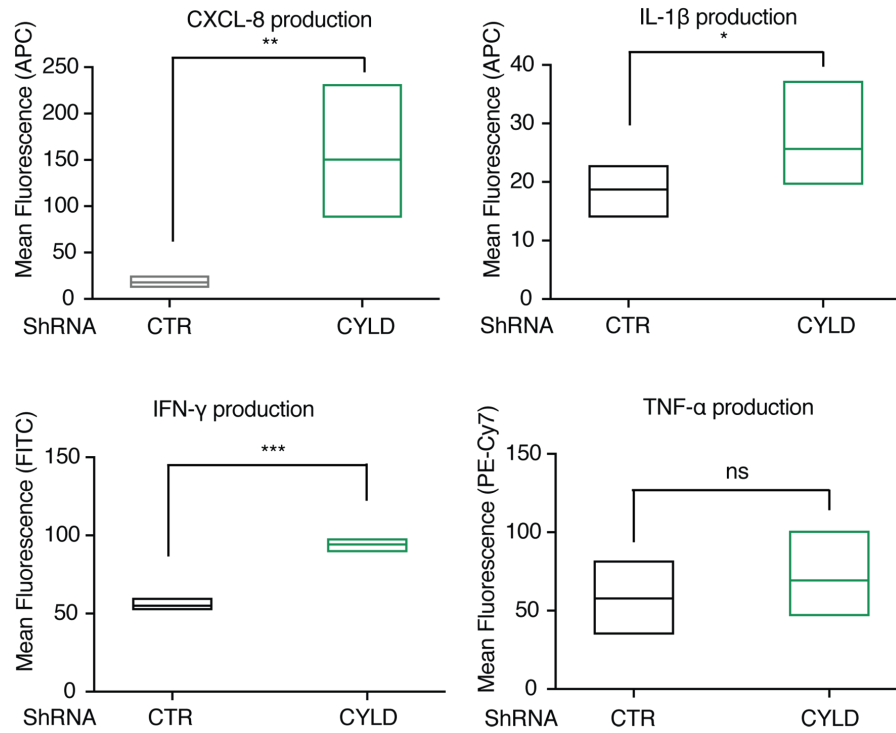


Figure 6.13: Pro-inflammatory cytokine production. Flow cytometry analysis of CXCL-8, IL-1 β , IFN- γ and TNF- α production measured as mean fluorescence in THP-1+PMA-ShCTR and THP-1+PMA-ShCYLD macrophages. Triplicates. Unpaired T test statistics.

In line with this, GSEA of differentially expressed genes comparing THP-1+PMA-ShCYLD and THP-1+PMA-ShCTR macrophages revealed an enrichment in the regulation of IFN- γ production in CYLD-deficient cells (Figure 6.14).

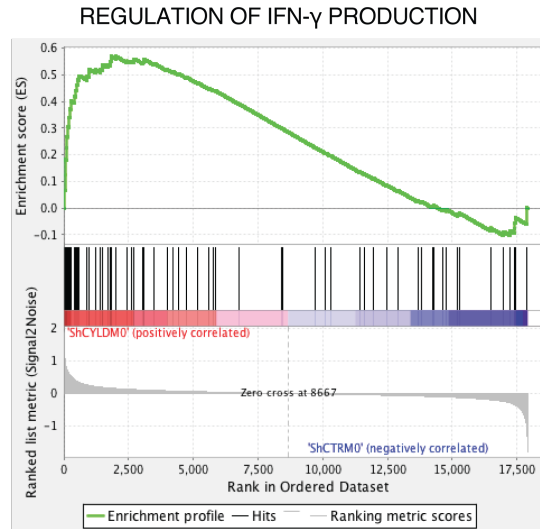


Figure 6.14: Regulation of $IFN-\gamma$ production in CYLD-depleted macrophages. GSEA "Regulation of $IFN-\gamma$ production" of differentially regulated genes comparing THP-1+*PMA*-ShCYLD versus THP-1+*PMA*-ShCTR macrophages from RNA-microarray analysis. Triplicates.

6.5 Discussion

In this chapter I initially investigated the role of the deubiquitinase CYLD during monocyte-to-macrophage differentiation and activation. For this purpose, I generated CYLD-depleted THP-1 and U937 human monocytic cells. My results describe CYLD-deficient macrophages as being in a pre-activated state, partially resembling activated macrophages in terms of morphology and global gene expression profiling. Additionally, RNA-microarray analysis displayed a strong enrichment for innate immune signatures in CYLD-deficient macrophages compared to control cells, including $IFN-\gamma$.

A comparison between $IFN-\gamma$ and $NF-\kappa B$ signalling, together with a strong enrichment for the $IFN-\gamma$ signature in CYLD-depleted macrophages, led me to hypothesise a possible role for CYLD in regulating the $IFN-\gamma$ signalling pathway. Accordingly, I showed that in the absence of CYLD STAT1- and STAT3-activating phosphorylation were increased in response to $IFN-\gamma$ treatment. Additionally, I demonstrated an enhanced transcriptional response for $IFN-\gamma$ late response genes

in CYLD-deficient macrophages and *CYLD* KO epithelial cells. These data suggest that CYLD regulation of the IFN- γ signalling pathway is not a cell type-specific mechanism. Global gene expression analysis, performed through RNA-Seq, also displayed a deregulation of the IFN- γ -dependent transcriptional response in CYLD-deficient macrophages compared to control cells, following IFN- γ treatment.

To understand CYLD negative regulation of this pathway, I tested if its deubiquitinating activity was required through the expression of a catalytically inactive form of CYLD (C601A) in a *CYLD* KO background. Catalytically inactive CYLD mimicked the transcriptional deregulation observed in CYLD-depleted and *CYLD* KO cells in response to IFN- γ , suggesting the need of its deubiquitinase activity to regulate IFN- γ signalling pathway. This aspect was further explored through ectopic expression of Lys63- and Met1-specific Ub-binding domains. I showed that direct cytoplasmic interference of Lys63- or Met1-linked Ub chains impairs the IFN- γ -mediated transcriptional response in macrophages. Interestingly, transcriptional regulation of IFN- γ early responsive genes, such as *IRF1*, was unaffected by the absence of CYLD or interference with Lys63- and Met1-linked Ub chains. These data may suggest that Lys63- and Met1-linked Ub chains are involved in promoting sustained transcription of IFN- γ late response genes.

Lastly, I identified an augmented production of IFN- γ , CXCL-8 and IL-1 β in CYLD-deficient macrophages compared to control cells. In line with this, the GSEA displayed a significant enrichment for the regulation of IFN- γ production in CYLD-depleted compared to control macrophages.

These data highlight the importance of controlling CYLD levels during monocyte-to-macrophage differentiation and in response to IFN- γ or immunoregulatory stimuli activating NF- κ B. Moreover, these data suggest a direct role for CYLD in regulating IFN- γ signalling pathway through its deubiquitinase activity. Unwarrented production of IFN- γ could lead to a potential autocrine priming in CYLD-deficient macrophages. This autocrine priming, in addition to IFN- γ signalling deregulation could be the mechanisms behind the pre-activated state observed in these cells.

7

CYLD suppresses pathogen clearance through regulation of the IFN- γ signalling pathway

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7.1 Introduction

Pathogen sensing and clearance are important functions of innate immune cells. Exposure to immunoregulatory stimuli, such as bacterial products or pro-inflammatory cytokines, induces distinct antimicrobial and inflammatory gene expression programmes in macrophages [65, 95]. IFN- γ , together with PRR signalling or TNF- α , is crucial for complete macrophage activation [77, 112, 114, 115, 146].

I previously identified CYLD as an IFN- γ target gene. Furthermore, I showed its negative regulatory effect on STAT1 and STAT3 phosphorylation and mediated transcriptional response following IFN- γ treatment. Additionally, I characterised CYLD-deficient macrophages as being in a pre-activated state, with increased IFN- γ production compared to control cells. In this chapter, I will investigate the functional consequences of CYLD depletion during bacterial infection. Moreover, I will identify antimicrobial features of CYLD-depleted macrophages and reveal a set of genes involved in the enhanced intracellular bacterial clearance observed in these cells.

7.2 CYLD-deficient macrophages present an improved intracellular bacterial clearance

7.2.1 Depletion of CYLD mimic IFN- γ -dependent pathogen control in macrophages

Nishanth et al. recently demonstrated that *CYLD* KO mice are protected from severe listeriosis and subsequent liver pathology. Moreover, they showed that CYLD-depleted BMDMs present a NF- κ B-dependent enhanced ability to clear intracellular *Listeria monocytogenes*. Furthermore, they observed elevated serum concentrations of IL-6 and IFN- γ in *CYLD* KO mice post *Listeria monocytogenes* infection [51]. In line with this, I investigated if in the *in vitro* system CYLD-deficient macrophages had a higher ability to control intracellular pathogen growth compared to control cells. To test this, THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages were infected with *Salmonella typhimurium* or *Listeria monocytogenes* and clearance of intracellular pathogens was assessed by Colony Forming Units

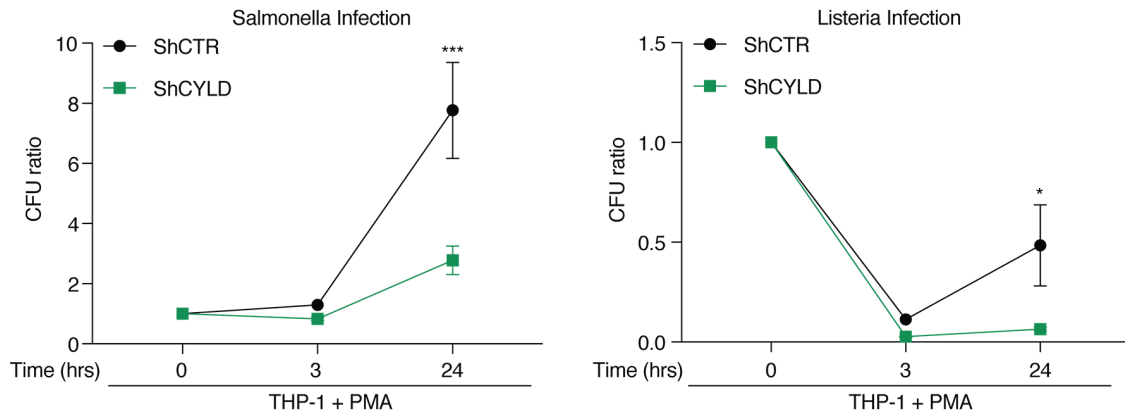


Figure 7.1: Improved pathogen control in CYLD-depleted macrophages. CFUs for THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages infected with *Salmonella typhimurium* or *Listeria monocytogenes* (Multiplicity Of Infection (MOI) = 10) for indicated times and normalised to time zero. Paired T-Test statistics.

(CFUs) assay, 3 and 24 hours post infection. No significant differences were observed at 3 hours post infection with *Salmonella typhimurium* or *Listeria monocytogenes*. However, a significant decrease in CFUs was observed in THP-1^{+PMA}-ShCYLD macrophages 24 hours following *Salmonella typhimurium* or *Listeria monocytogenes* infection in comparison to THP-1^{+PMA}-ShCTR cells (Figure 7.1).

To investigate the reported macrophage-priming effect of IFN- γ in the *in vitro* system, *Salmonella typhimurium* or *Listeria monocytogenes* infections were performed in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages untreated, or primed with IFN- γ for 24 hours ahead of exposure to the pathogens. IFN- γ pre-stimulation conferred a significantly enhanced intracellular pathogen clearance in THP-1-derived macrophages 24 hours post infection with *Salmonella typhimurium* or *Listeria monocytogenes*. This effect was comparable with the improved pathogen control displayed in CYLD-depleted macrophages, while no additional effect was observed in THP-1^{+PMA}-ShCYLD cells pre-treated with IFN- γ (Figure 7.2).

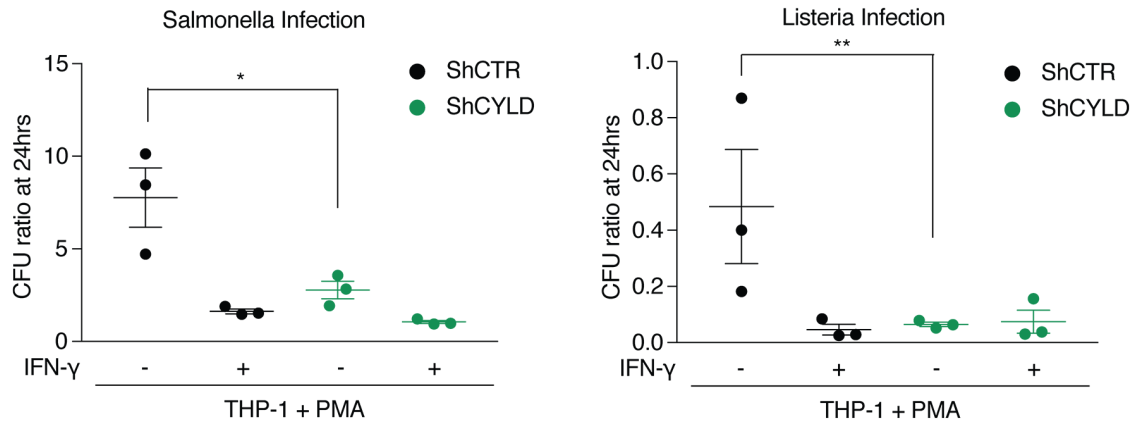


Figure 7.2: CYLD depletion mimic IFN- γ priming in macrophages. CFUs for THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages untreated or IFN- γ (20 ng/ml, 2 hrs + 22 hrs rest) primed and infected with *Salmonella typhimurium* or *Listeria monocytogenes* (Multiplicity Of Infection (MOI) = 10, 24 hrs). Paired T-Test statistics.

7.2.2 Activated macrophage features are enhanced in CYLD-depleted cells

To investigate the deregulated processes responsible for the enhanced bacterial clearance observed in CYLD-deficient macrophages, I performed GSEA for established mechanisms involved in pathogen sensing and clearance, on differentially expressed genes comparing THP-1^{+PMA}-ShCYLD versus THP-1^{+PMA}-ShCTR cells using previous RNA-microarray experiments data. GSEA identified an enriched signature for macroautophagy, xenophagy, lysosomal activity and positive regulation of autophagy in THP-1^{+PMA}-ShCYLD macrophages compared to THP-1^{+PMA}-ShCTR cells (Figure 7.3).

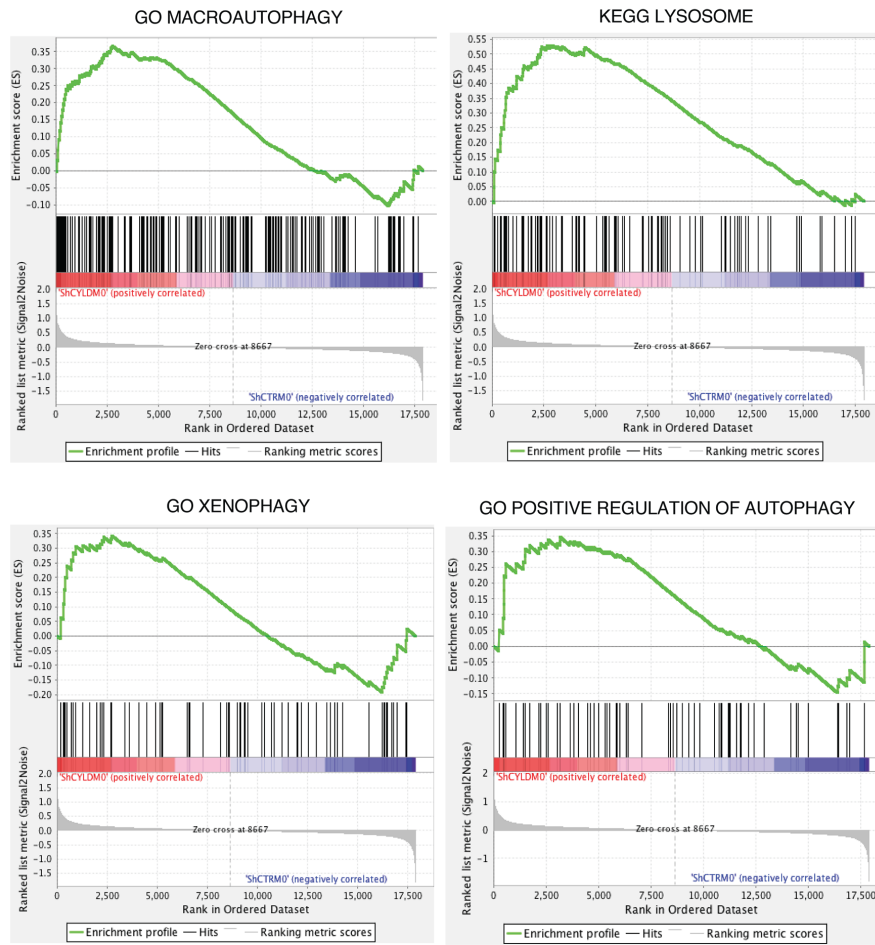


Figure 7.3: GSEA of xenophagy related processes. GSEA for "GO macroautophagy", "KEGG lysosome", "GO xenophagy" and "GO positive regulation of autophagy" on differentially expressed genes comparing THP-1^{+PMA}-ShCYLD versus THP-1^{+PMA}-ShCTR macrophages. RNA-microarray. Triplicates.

7.2.3 CYLD suppresses macroautophagy and xenophagy features in macrophages

I decided to phenotypically validate the differences identified through GSEA comparing THP-1^{+PMA}-ShCYLD versus THP-1^{+PMA}-ShCTR macrophages. Xenophagy can be defined as a specific class of macroautophagy, directed to engulf invading pathogens and eliminate them via the autophagic/lysosomal route [147]. In line with this definition, I initially focused on investigating the bacterial uptake. Analysing the previously described *Salmonella typhimurium* and *Listeria monocytogenes* infection experiments, I observed that CFUs at time point zero were significantly higher in THP-1^{+PMA}-ShCYLD compared to THP-1^{+PMA}-ShCTR cells. Moreover,

in both cell types CFUs increased following IFN- γ pre-treatment. These data suggest that CYLD-depleted macrophages were engulfing *Salmonella typhimurium* or *Listeria monocytogenes* at a higher rate compared to control cells. Additionally, the IFN- γ -dependent increase in the uptake of bacteria for THP-1^{+PMA}-ShCTR macrophages was comparable with the uptake of bacteria for untreated THP-1^{+PMA}-ShCYLD cells.

To further investigate this difference in the uptake capacity, THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages were incubated with FITC-fluorescent latex beads for several time points. Flow cytometry analysis revealed a significant increase in the engulfment of FITC-fluorescent latex beads in CYLD-deficient macrophages compared to control cells (Figure 7.4).

The lysosomal activity and the recruitment to phagosomes are increased in macrophages following activation, and this is a mechanism to improve pathogen clearance [79, 81, 148]. In addition, lysosomal proteases, such as Cathepsin B, H and L or the lysosomal thiol reductase (GILT), are induced by the IFN- γ signalling pathway [149]. To test a possible increase in lysosomal activity within the *in vitro* system, untreated or LPS + IFN- γ activated THP-1-derived macrophages were incubated with DQ-Red-BSA. DQ-Red-BSA is a fluorogenic substrate for proteases, which fluorescence is released as a consequence of lysosomal processing. Flow cytometry analysis revealed an increased lysosomal activity in LPS + IFN- γ treated THP-1-derived macrophages compared to untreated cells. To test a possible difference in the lysosomal activity due to CYLD-depletion in macrophages, THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD cells were incubated with DQ-Red-BSA. Flow cytometry analysis displayed a higher lysosomal activity in THP-1^{+PMA}-ShCYLD macrophages compared to THP-1^{+PMA}-ShCTR cells (Figure 7.5).

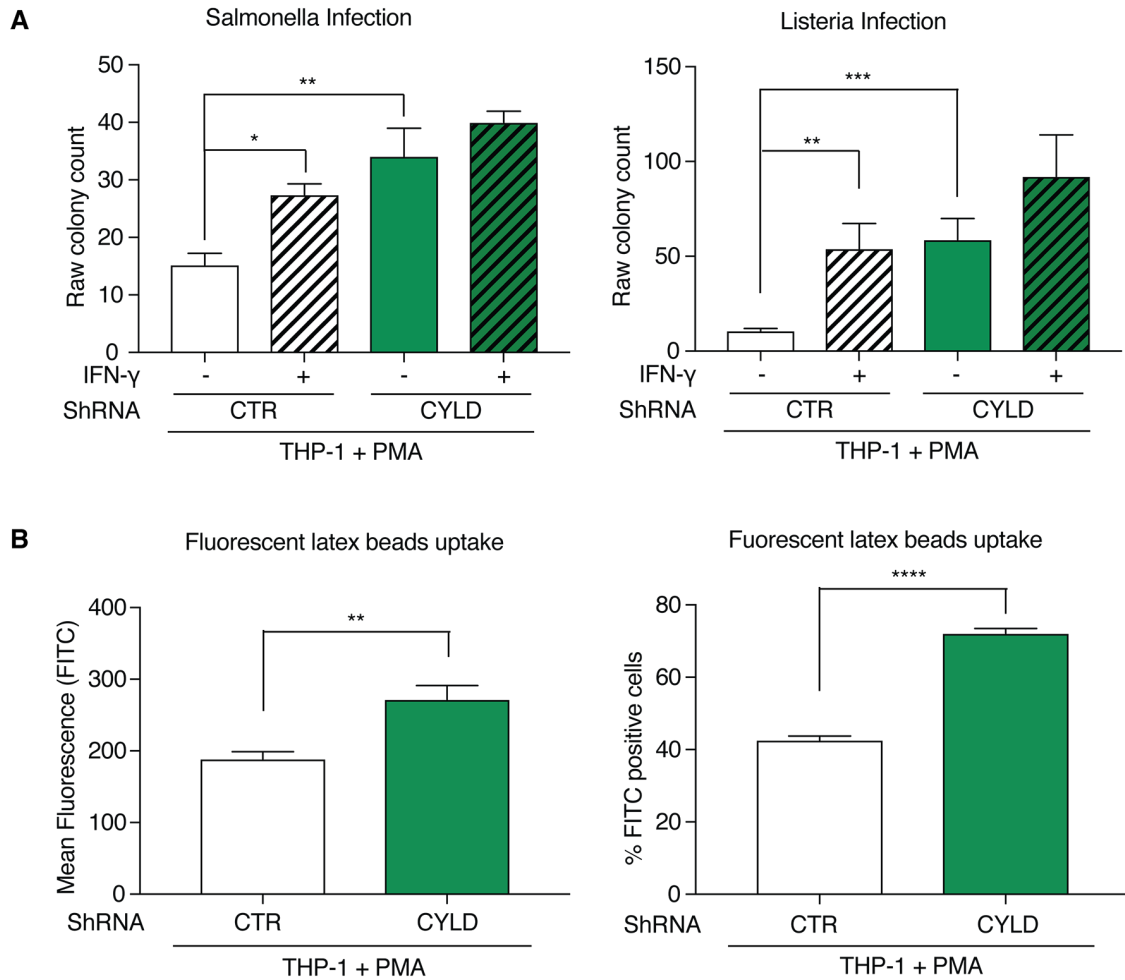


Figure 7.4: CYLD-depleted macrophages have increased phagocytic activity. (A) Raw Colony count of CFUs in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD for *Salmonella typhimurium* or *Listeria monocytogenes* (MOI = 10, 30 mins + Gentamicin treatment (50 μ g/ml, 10 mins)) at time point 0 from figure 7.2 (B) Flow cytometry analysis of THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD incubated with FITC-fluorescent latex beads (10 μ l/ml, 30 mins. Triplicates. Unpaired T-Test statistics.

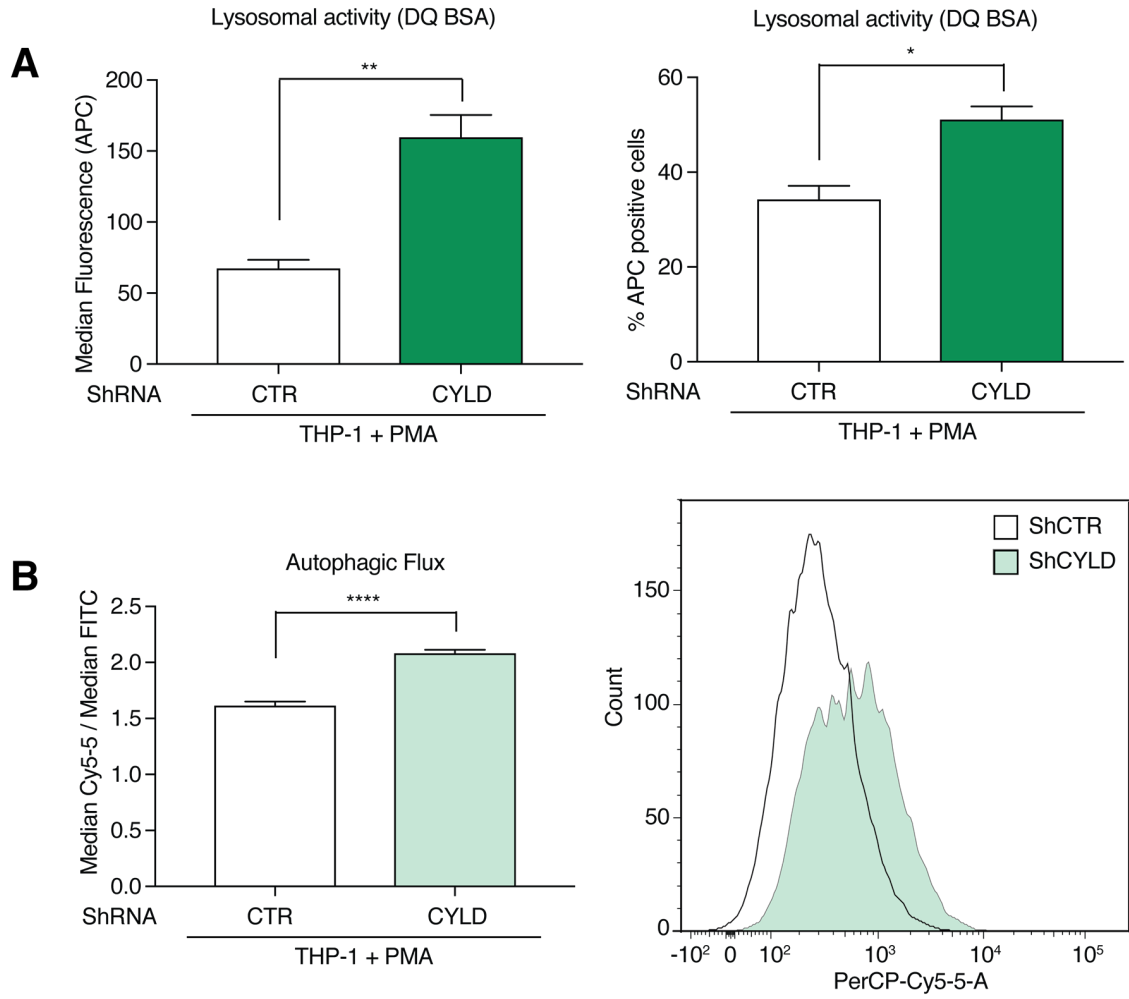


Figure 7.5: CYLD negatively regulates lysosomal activity and autophagy. (A) Flow cytometry analysis of lysosomal activity measured by DQ-Red-BSA treatment (10 μ g/ml, 30 mins) (B) Flow cytometry analysis of mCherry-EGFP-LC3 transduced THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD. Autophagic Flux is defined as median fluorescence mCherry (Cy5-5)/median fluorescence EGFP (FITC) [116]. Representative histograms for mCherry (Cy5-5) channel in mCherry-EGFP-LC3 transduced THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD. Triplicates. Unpaired T-Test statistics.

The autophagic activity was evaluated via transduction of THP-1-ShCTR and THP-1-ShCYLD monocytic cells with an mCherry-EGFP-tagged version of the MAP1LC3B protein (mCherry-EGFP-LC3) [116, 117]. Following stable expression of the mCherry-EGFP-LC3 construct, autophagic flux was assessed via flow cytometry based on mCherry-GFP fluorescence ratio in single cells. Flow cytometry analysis showed an increased autophagic flux in THP-1^{+PMA}-ShCYLD compared to THP-1^{+PMA}-ShCTR macrophages (Figure 7.5).

7.2.4 NOD2, RIPK2 and OPTN are transcriptionally induced by IFN- γ , and upregulated in CYLD-deficient macrophages

Among the differentially regulated genes comparing THP-1^{+PMA}-ShCYLD versus THP-1^{+PMA}-ShCTR macrophages, I identified the NOD2 receptor, the adaptor protein RIPK2, and the xenophagy receptor Optineurin (OPTN). All these candidates were previously described to be important for xenophagy, intracellular bacterial clearance, and have been associated with CYLD function [8, 87, 130, 150]. In addition to the enhanced expression of these genes in THP-1^{+PMA}-ShCYLD compared to control cells, the RNA-microarray analysis showed that NOD2, RIPK2 and OPTN are transcriptionally induced in THP-1^{+PMA}-ShCTR following LPS + IFN- γ treatment. This observation suggests a possible regulation of these genes through IFN- γ signalling. To investigate this possibility, qRT-PCR for NOD2, RIPK2 and OPTN was performed in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages, untreated or following IFN- γ treatment. NOD2, RIPK2 and OPTN mRNA levels were significantly increased in THP-1^{+PMA}-ShCYLD compared to THP-1^{+PMA}-ShCTR macrophages. Furthermore, NOD2, RIPK2 and OPTN were transcriptionally induced following IFN- γ treatment. To validate the induction of NOD2, RIPK2 and OPTN via IFN- γ downstream signalling, I performed qRT-PCR experiments for these genes in THP-1^{+PMA} cells pre-treated with BP1-102 and subsequently stimulated with IFN- γ for various time points. The inhibition of STAT1 and STAT3 activation, by BP1-102, abolished NOD2, RIPK2 and OPTN induction following IFN- γ treatment (Figure 7.6 and 7.7). These experiments were also performed in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages, where an analogous IFN- γ -dependent transcriptional activation of NOD2, RIPK2 and OPTN, together with a significant upregulation of these genes in CYLD-depleted macrophages was observed (Figure 9.6).

To investigate if NOD2, RIPK2 and OPTN transcriptional induction was leading to protein production, WB analysis was performed in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages stimulated with IFN- γ for various time points. WB

analysis displayed increased protein levels for NOD2, RIPK2, and OPTN, between 8 and 24 hours post IFN- γ treatment in THP-1^{+PMA}-ShCTR macrophages. Moreover, NOD2, RIPK2 and OPTN protein levels were higher in THP-1^{+PMA}-ShCYLD compared to THP-1^{+PMA}-ShCTR macrophages, in agreement with previously measured mRNA levels.

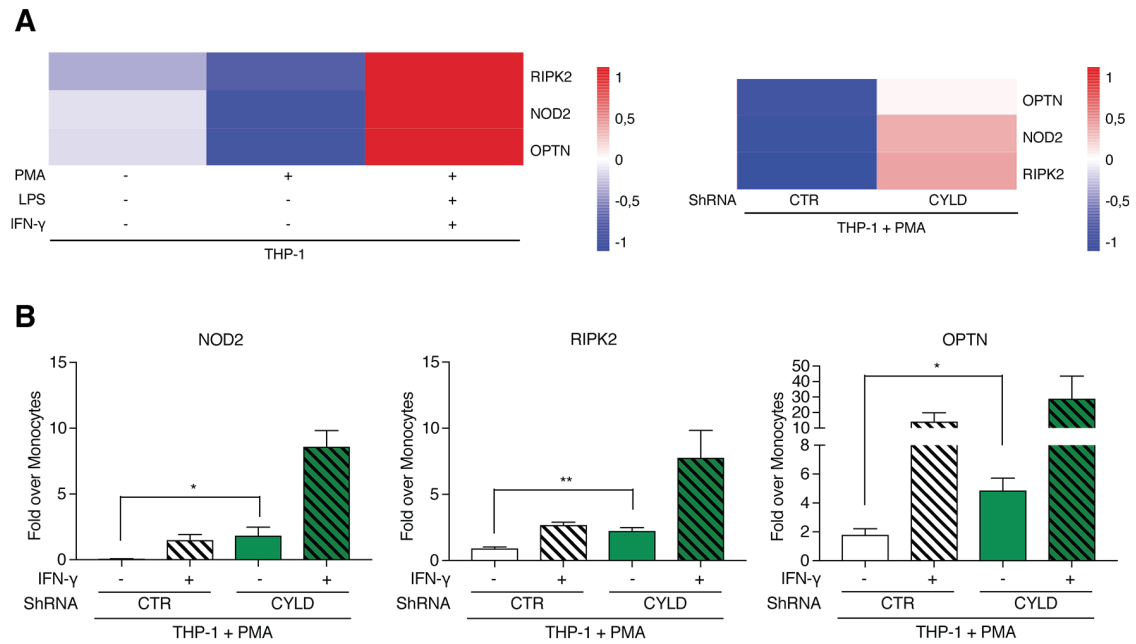


Figure 7.6: NOD2, RIPK2 and OPTN upregulation in CYLD-depleted cells. (A) Heat map for RIPK2, NOD2 and OPTN expression levels in THP-1 cells at monocytic state, PMA-differentiated, or activated with LPS (50 ng/ml) and IFN- γ (20 ng/ml) for 2 hrs + 22 hrs rest from RNA-microarray analysis (left). Heat map for RIPK2, NOD2 and OPTN expression levels in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages from RNA-microarray analysis (right) (B) qRT-PCR for RIPK2, NOD2 and OPTN mRNA levels in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD untreated or stimulated with IFN- γ (20 ng/ml) for 2 hrs + 22 hrs rest and normalised to the control untreated cells. Triplicates. Unpaired T-Test statistics.

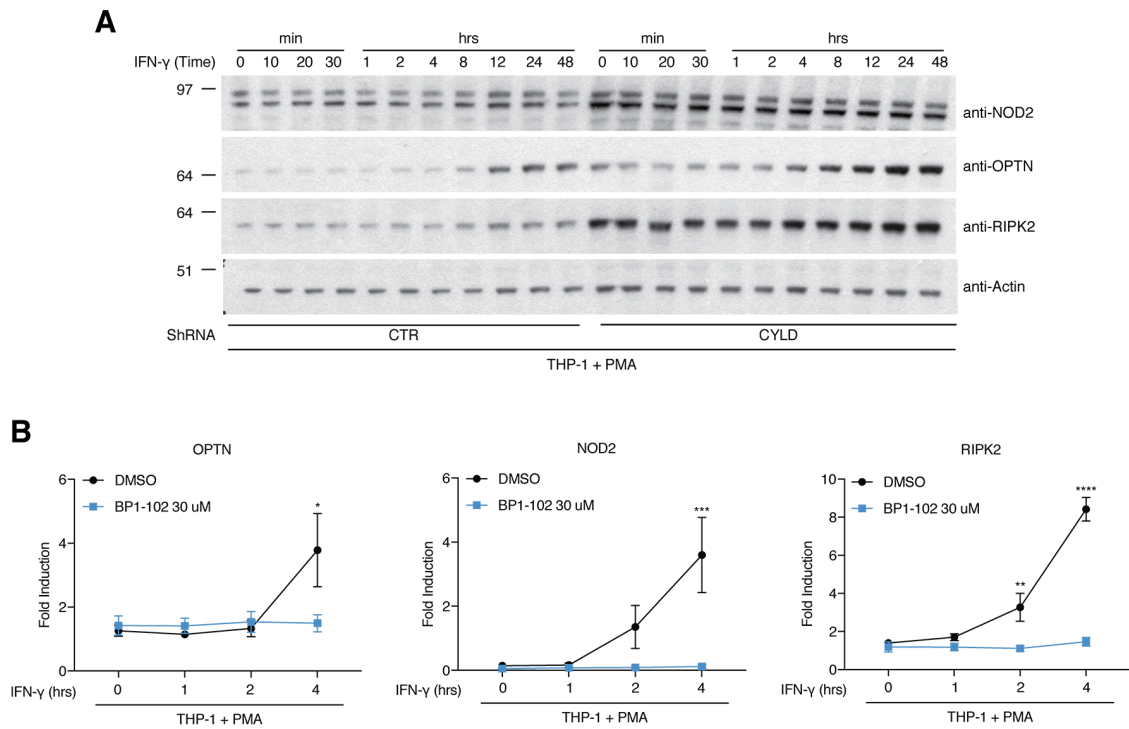


Figure 7.7: NOD2, RIPK2 and OPTN induction following IFN- γ treatment. (A) WB analysis for NOD2, OPTN and RIPK2 levels in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages untreated or stimulated with IFN- γ (20 ng/ml) for indicated times (B) qRT-PCR analysis for OPTN, NOD2 and RIPK2 mRNA levels in THP-1^{+PMA} pre-incubated with BP1-102 (30 μ M) or DMSO and subsequently treated with IFN- γ (20 ng/ml) for indicated times. Triplicates. Two Way ANOVA statistics.

7.3 Improved intracellular bacterial clearance of CYLD-depleted macrophages relies on NOD2 signalling and OPTN

7.3.1 NOD2 signalling pathway contributes to pathogen control in CYLD-deficient macrophages

Schwerd et al. recently demonstrated that NOD2 pre-stimulation by MDP primes monocyte-derived macrophages for an improved clearance of *Salmonella typhimurium*. Moreover, they showed that this effect relies on NOD2-RIPK2 downstream signalling, since XIAP-deficient macrophages do not display an improved pathogen control when pre-treated with MDP [130]. To investigate if NOD2 and RIPK2 upregulation in CYLD-depleted macrophages contributes to their enhanced xenophagy, THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages were treated with GSK-583 and infected with *Salmonella typhimurium*. GSK-583 is an ATP-competitive type I inhibitor, which abrogates XIAP binding to RIPK2 and therefore blocks downstream NOD2 signalling [20]. CFUs analysis showed that the inhibition of the NOD2 signalling pathway do not affect the ability of THP-1^{+PMA}-ShCTR macrophages to restrict intracellular bacterial growth. In contrast, GSK-583 treatment partially reduced the enhanced bacterial clearance of THP-1^{+PMA}-ShCYLD macrophages. These data suggest that the upregulation of NOD2 and RIPK2 in CYLD-deficient cells is increasing xenophagy to some extent. *Salmonella typhimurium* infection was also performed in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages in the presence of the GSK-583 inhibitor, and a similar reduction in the bacterial clearance ability of CYLD-depleted macrophages was observed. In addition, this experiment was also performed in U2OS^{Parental} and U2OS^{CYLD-KO} cells with an analogous result (Figure 7.8).

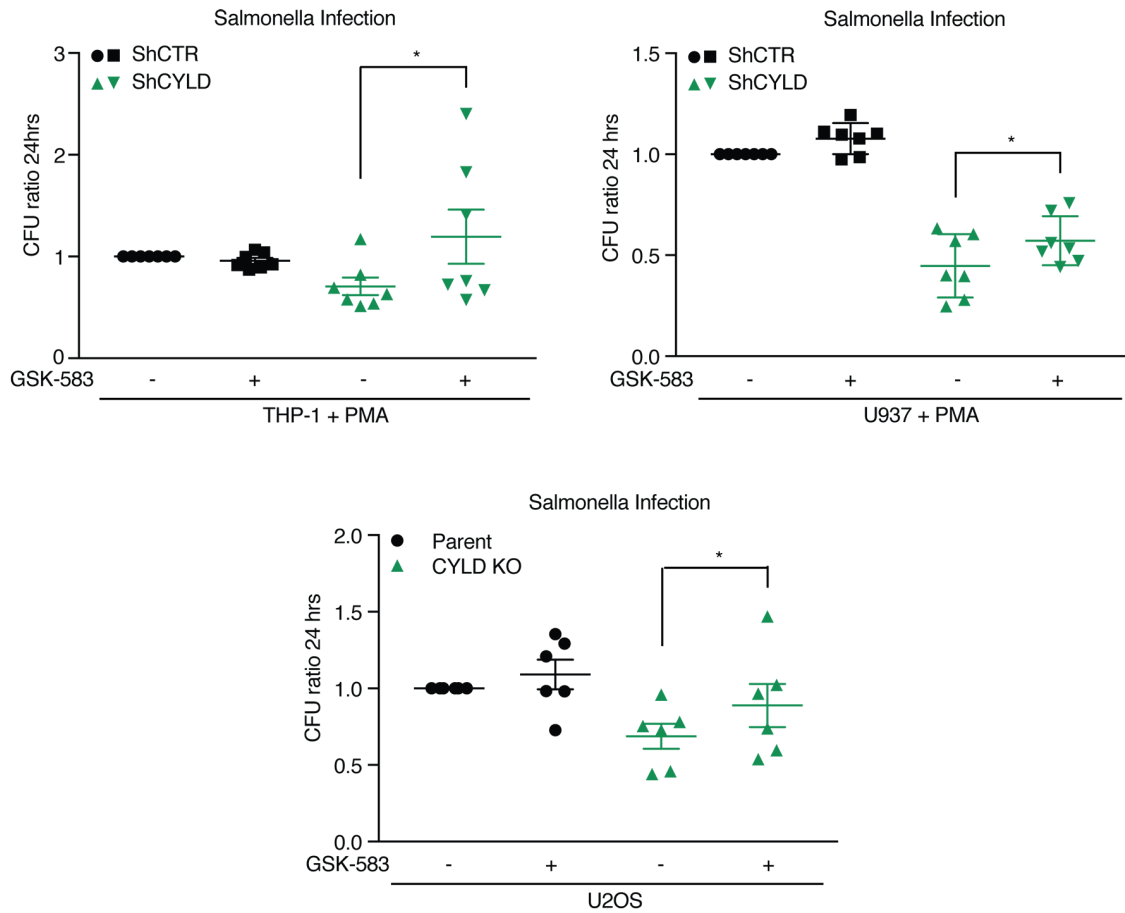


Figure 7.8: NOD2-RIPK2 axis partially contribute to pathogen control. CFUs for THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages, U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages or U2OS^{Parental} and U2OS^{CYLD-KO} cells pre-incubated with GSK-583 (500 nM) and infected with *Salmonella typhimurium* (Multiplicity Of Infection (MOI) = 10), GSK-583 treatment was carried prior infection (1 hr) and for the whole infection period (24 hrs), CFUs were normalised to time point zero and to control untreated cells. Paired T-Test statistics.

7.3.2 OPTN upregulation in CYLD-deficient macrophages is responsible for enhanced bacterial clearance

OPTN was shown to directly interact with CYLD and to negatively regulate the TNF- α and type-I IFN response [150, 151]. Despite this, OPTN-deficient mice do not present impaired NF- κ B signalling following TNF- α or LPS stimulation, but they are more susceptible to *Salmonella typhimurium* infection [87].

In light of increased OPTN levels in CYLD-depleted macrophages, I investigated its possible contribution to the enhanced xenophagic activity observed in these cells. To test this, I generated THP-1-ShCTR-GFP, THP-1-ShCTR-GFP-ShOPTN,

THP-1-ShCYLD-GFP and THP-1-ShCYLD-GFP-ShOPTN cells. In particular, I transduced THP-1 monocytic cells with an EGFP-labelled ShRNA targeting *CYLD* or an EGFP-labelled non-targeting ShRNA, generating THP-1-ShCTR-GFP and THP-1-ShCYLD-GFP respectively. The transduced cells were sorted based on high GFP-expression via flow cytometry and *CYLD* protein levels were evaluated by WB analysis. Subsequently, THP-1-ShCTR-GFP and THP-1-ShCYLD-GFP monocytic cells were transduced with an ShRNA targeting *OPTN* or a non-targeting ShRNA and, following selection, *OPTN* protein levels were evaluated via WB analysis. Afterwards, *Salmonella typhimurium* infection was performed in PMA-differentiated THP-1-ShCTR-GFP, THP-1-ShCTR-GFP-ShOPTN, THP-1-ShCYLD-GFP and THP-1-ShCYLD-GFP-ShOPTN as previously described. CFUs analysis showed no impaired pathogen control in THP-1-ShCTR macrophages *OPTN*-deficient, compared to control cells. Conversely, the enhanced *Salmonella typhimurium* clearance observed in THP-1-ShCYLD macrophages was completely abrogated by the knockdown of *OPTN*. These data suggest that increased *OPTN* levels in *CYLD*-depleted macrophages are critical for their improved intracellular bacterial clearance (Figure 7.9 and 9.11).

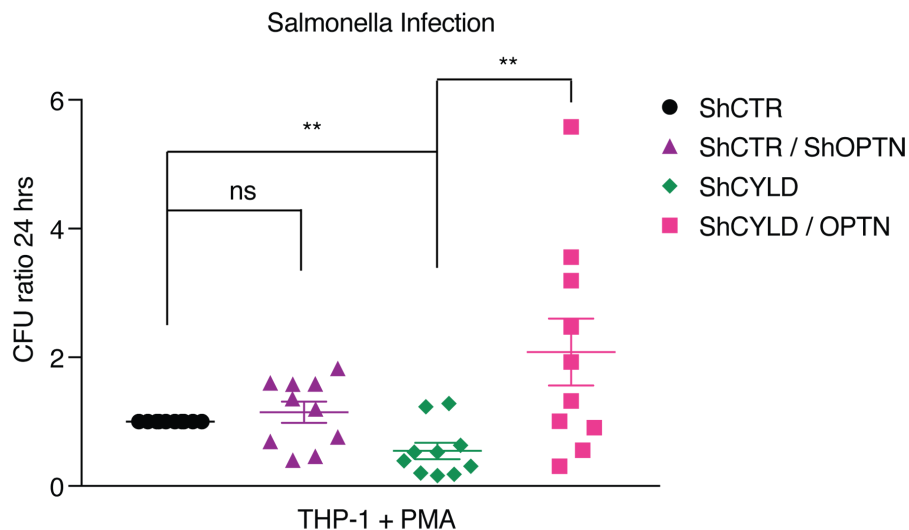


Figure 7.9: *OPTN* is required for pathogen control. CFUs for THP-1-ShCTR-GFP, THP-1-ShCTR-GFP-ShOPTN, THP-1-ShCYLD-GFP and THP-1-ShCYLD-GFP-ShOPTN macrophages infected with *Salmonella typhimurium* (Multiplicity Of Infection (MOI) = 10), CFUs were normalised to time point zero and to control untreated cells. Paired T-Test statistics.

7.4 CYLD suppression of pathogen clearance is mediated by its deubiquitinase activity

7.4.1 CYLD deubiquitinase activity is required to suppress antimicrobial genes expression and bacterial clearance

I previously showed that CYLD deubiquitinase activity is required to regulate IFN- γ downstream transcriptional response. Additionally, I identified different antimicrobial features in CYLD-depleted macrophages, which are regulated by the IFN- γ signalling pathway, including increased levels of the xenophagy-related genes NOD2, RIPK2 and OPTN. Therefore, I investigated if the catalytic activity of CYLD was required to control NOD2, RIPK2 and OPTN upregulation following IFN- γ treatment. To test this, I used U2OS^{CYLD-WT}, U2OS^{CYLD-KO} and U2OS^{CYLD-C601A} cells. qRT-PCR for NOD2, RIPK2 and OPTN was performed on these cells following IFN- γ stimulation. qRT-PCR analysis showed a significant increase in the NOD2, RIPK2 and OPTN mRNA levels following IFN- γ treatment, in U2OS^{CYLD-KO} and U2OS^{CYLD-C601A} compared to U2OS^{CYLD-WT} cells (Figure 7.10).

In addition, to investigate if CYLD catalytic activity was required to suppress bacterial clearance, *Salmonella typhimurium* infection was performed in U2OS^{CYLD-WT}, U2OS^{CYLD-KO} and U2OS^{CYLD-C601A} cells as previously described. CFUs analysis displayed a significant reduction of intracellular *Salmonella typhimurium* in U2OS^{CYLD-KO} cells compared to U2OS^{CYLD-WT}. Furthermore, a significant decrease in bacterial load was observed in U2OS^{CYLD-C601A} cells compared to control, and this reduction was comparable with U2OS^{CYLD-KO} cells (Figure 7.10).

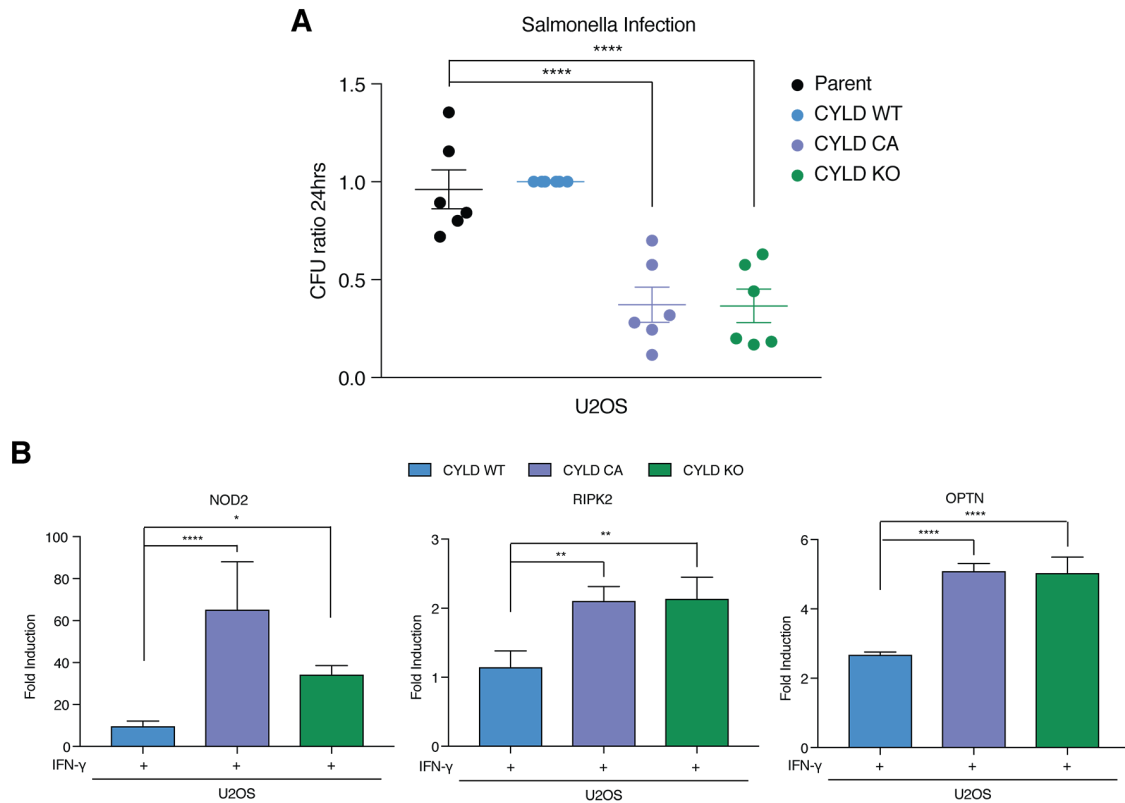


Figure 7.10: CYLD catalytic activity suppresses xenophagy features. (A) CFUs for U2OS^{Parental}, U2OS^{CYLD-WT}, U2OS^{CYLD-KO} and U2OS^{CYLD-C601A} cells infected with *Salmonella typhimurium* (Multiplicity Of Infection (MOI) = 10), CFUs were normalised to time point zero. One Way ANOVA statistics. (B) qRT-PCR for NOD2, RIPK2 and OPTN in U2OS^{CYLD-WT}, U2OS^{CYLD-KO} and U2OS^{CYLD-C601A} cells following IFN- γ (20 ng/ml, 2 hrs + 22 hrs rest) stimulation. Triplicates. Two Way ANOVA statistics.

7.4.2 Lys63- and Met1-linked ubiquitin chains involvement in intracellular bacterial clearance

In addition to their critical role in the regulation of the NF- κ B signalling pathway, Lys63- and Met1-linked Ub chains have been described to be important for recognition and clearance of intracellular bacteria [84–86, 152]. In line with the observation that CYLD catalytic activity is required for the suppression of pathogen clearance, I investigated the involvement of Lys63- or Met1-linked Ub chains in bacterial clearance. To test this, THP-1^{GFP-NES}, THP-1^{GFP-NES-K63} and THP-1^{GFP-NES-M1} were PMA-differentiated and challenged with *Salmonella typhimurium* for 3 and 24 hours. CFUs analysis showed no major differences between THP-1^{GFP-NES}, THP-1^{GFP-NES-K63} and THP-1^{GFP-NES-M1} macrophages at 3 hours post *Salmonella*

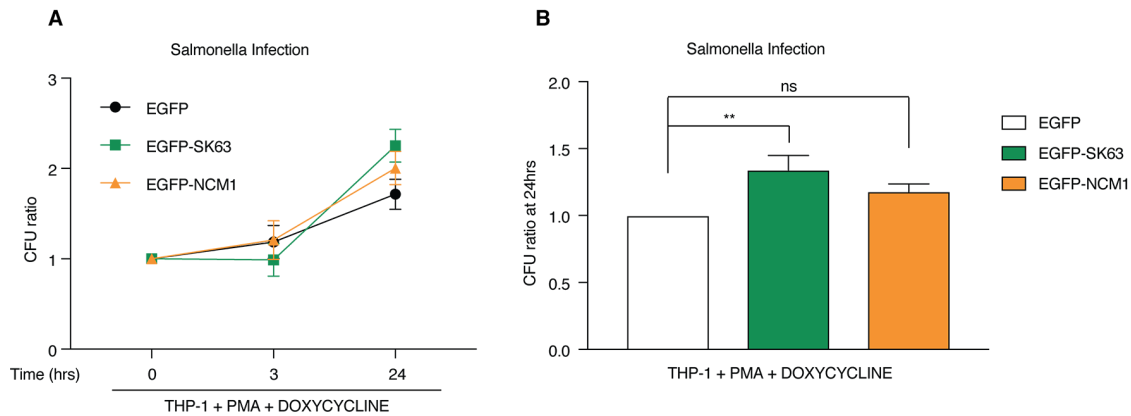


Figure 7.11: Lys63-linked ubiquitin chains role in pathogen control. (A) CFUs for THP-1^{GFP-NES}-, THP-1^{GFP-NES-K63}- and THP-1^{GFP-NES-M1}-derived macrophages incubated with Doxycycline (500 ng/ml, 24 hrs) and infected with *Salmonella typhimurium* (Multiplicity Of Infection (MOI) = 10) for 3 and 24 hrs, CFUs were normalised to time point zero (B) Data from (A) normalised to time point zero and control cells at 24 hrs post infection. One Way ANOVA statistics.

typhimurium infection. However, a significant increase in CFUs was observed in THP-1^{GFP-NES-K63} compared to THP-1^{GFP-NES} control cells 24 hours post infection, while no significant differences were observed in THP-1^{GFP-NES-M1} macrophages compared to control cells. These data suggest that specific Lys63-linked Ub chains interference impairs intracellular bacterial clearance in macrophages, while Met1-linked Ub chains do not appear to be crucial components of this process (Figure 7.11).

7.5 Discussion

In this chapter, I initially identified a deregulation of the xenophagy-related features in *CYLD*-deficient macrophages through global gene expression profiling. Subsequently, I phenotypically validated these characteristics, showing an improved ability in the engulfment of extracellular pathogens, an increased autophagic flux and a higher lysosomal activity for *CYLD*-depleted macrophages compared to control cells. These aspects have been previously reported enhanced in activated macrophages through the IFN- γ signalling pathway, and they are crucial for intracellular pathogen growth restriction [65, 81, 95, 147–149].

In addition, I identified *NOD2*, *RIPK2* and *OPTN* genes to be IFN- γ inducible, and to be significantly upregulated in *CYLD*-depleted macrophages. In line with the

previously described roles for these proteins within innate immunity, I investigated their possible contribution to the enhanced xenophagic activity observed for CYLD-depleted macrophages. Accordingly, I showed a significant requirement for the NOD2 signalling pathway in clearing *Salmonella typhimurium* infection in these cells. However, the inhibition of the NOD2/RIPK2 axis did not completely revert the improved intracellular pathogen control observed in CYLD-depleted cells, suggesting a cumulative effect of several mechanisms leading to this difference. Conversely, generation of CYLD/OPTN double knockdown macrophages completely abrogated the increased *Salmonella typhimurium* clearance of CYLD-deficient macrophages. Nevertheless, the OPTN depletion in control macrophages did not affect pathogen clearance, in contrast with previous reports on *OPTN* KO BMDMs [87, 150, 151].

I demonstrated that the CYLD catalytic activity is important to negatively regulate the IFN- γ signalling pathway, and therefore transcription of xenophagy related genes. Accordingly, I showed that cells expressing a catalytically inactive version of CYLD (C601A) present an improved ability to clear intracellular *Salmonella typhimurium* compared to control, and similarly to *CYLD* KO cells. These data suggest that CYLD controls by hydrolysis Lys63- and Met1-linked Ub chain levels during IFN- γ response and intracellular pathogen clearance. I then investigated the individual role of Lys63- and Met1-linked Ub chains during intracellular pathogen clearance, using linkage-specific Ub-binding domains, and showed that Lys63-linked Ub chains interference impairs *Salmonella typhimurium* clearance in macrophages, while Met1-specific obstruction has no effect.

8

Discussion

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The innate immune system can control and annihilate pathogen infection and colonisation through three main mechanisms: inflammation, programmed cell death and intracellular pathogen clearance [13]. The deubiquitinase CYLD contributes to these three fundamental aspects of immunity. The production of pro-inflammatory cytokines, including TNF- α , IL-1 β , IL-6 and IFN- γ is mainly mediated by the NF- κ B and AP1 TFs. Previous reports have extensively showed how CYLD directs the regulation of this signalling pathway through Lys63- and Met1-linked Ub chain restriction [33, 35–41]. CYLD also promotes programmed cell death (apoptosis and necroptosis) by immune receptors, and has been shown to be cleaved by caspase-8 following LPS in macrophages, possibly as a mechanism to prevent cell death during infection [40, 43, 153]. *CYLD* KO mice are protected from *Streptococcus pneumoniae* and *Listeria monocytogenes* infection, and this enhanced pathogen clearance has been related to increased ROS production and the

deregulation of NF- κ B signalling [49–52]. However, other mechanisms underlying this protective process have not yet been investigated, and the contribution of macrophages to this clearance mechanism is still not clear. IFN- γ promotes macrophage activation and antimicrobial properties, such as macroautophagy and the production of radicals and antimicrobial peptides [77–79, 83]. The activation and regulation of this signalling pathway is therefore crucial for successful pathogen clearance and for the engagement of adaptive immunity [112–115]. In this work, I investigated CYLD regulation during macrophage activation and intracellular pathogen clearance. I identified CYLD to regulate and be regulated by the IFN- γ signalling pathway. In particular, CYLD controls STAT1- and STAT3-activating phosphorylation downstream of IFNGR. Its deubiquitinase activity is required to inhibit the IFN- γ transcriptional response. In addition, I identified CYLD as an IFN- γ target gene that relies on STATs activation. This novel notion of CYLD as a regulator of the IFN- γ signalling pathway provides insight into how this deubiquitinating enzyme can negatively regulate different innate immune pathways. Considering that TNF- α , TLR4 and IFN- γ signalling pathways are involved in macrophage activation, my findings expand the relevance of CYLD in controlling macrophage responses to immunoregulatory stimuli [68–70].

8.1 Transcriptional regulation of CYLD

According to the Human Protein Atlas, CYLD is ubiquitously expressed in human tissues at both the mRNA and protein levels. In line with its role in mediating the inflammatory response, CYLD mRNA levels are higher in primary and secondary lymphoid tissues, such as bone marrow, lymph nodes and the spleen. Moreover, CYLD is highly expressed in testes, where it is reported to regulate germ cell apoptosis and spermatogenesis, although *CYLD* KO mice are fertile and breed normally [44, 45, 48, 153]. CYLD negative regulation of the NF- κ B signalling pathway has been extensively investigated, and increased CYLD mRNA levels in response to NF- κ B-activating stimuli have been described in epithelial cells as a canonical feedback loop that inhibits this signalling pathway [39, 134]. Despite its recognised

role in controlling inflammation and innate immune responses, the regulation of CYLD expression within innate immune cells has not yet been addressed.

I investigated the regulation of CYLD during the differentiation and activation of human macrophages. The *in vitro* model used in this study to address these questions is based on the immortalised human monocytic cell lines THP-1 and U937 differentiated through PMA stimulation, and activated via LPS and IFN- γ treatment. During PMA-mediated monocyte-to-macrophage differentiation, THP-1 cells did not seem to trigger an increase in CYLD mRNA and protein levels. Moreover, TLR4 stimulation through LPS of THP-1-derived macrophages only mildly increased CYLD mRNA and protein levels. This suggests that NF- κ B activation is not sufficient to increase CYLD cellular abundance in these cells. In contrast, I observed enhanced CYLD mRNA and protein levels in monocyte-derived macrophages activated with LPS and IFN- γ , and I identified IFN- γ as the major driver for this. CYLD mRNA and protein levels increased in response to IFN- γ in THP-1- and U937-derived macrophages, as well as PBMC-derived macrophages and epithelial cells. These data suggest that CYLD induction is a general mechanism in response to this cytokine.

Mechanistically, my results suggest that CYLD induction through IFN- γ signalling relies on STAT1 and STAT3 activation. Indeed, the inhibition of these two TFs via fludarabine and BP1-102 abrogates increased CYLD mRNA or protein levels following IFN- γ treatment. However, STAT3-activating stimuli (IL-6 and IL-10) do not induce an increase in CYLD mRNA and protein levels. This indicates a STAT1-dependent regulation of *CYLD*.

LPS + IFN- γ stimulation is widely used as an *in vitro* surrogate to mimic the Th1 response to bacterial infection. This co-stimulation drives specific transcriptional programmes in macrophages, promoting inflammation and bacterial clearance [64, 65]. Therefore, increased levels of CYLD, which is a negative regulator of inflammation, following IFN- γ stimulation may seem counterintuitive to this purpose. However, further characterisation of CYLD induction in response to IFN- γ revealed that CYLD is not part of the primary transcriptional response to this cytokine. This

may indicate the involvement of *de novo*-synthesised TFs allowing its induction. The accumulation of CYLD mRNA begins around 2 hours following stimulation with IFN- γ and persists for 24 hours. Accordingly, CYLD protein accumulation begins around 4 hours post-stimulation and accumulates for up to 48 hours. This dynamic resembles that of other IFN- γ secondary response genes, such as *IDO1*, *CCL2*, *SOCS1*, *IRF2* and *IRF9* (Figure 9.7). IRF1, IRF2 and IRF9 TFs coordinate the secondary transcriptional response, cooperating with STAT1 and STAT3 [79, 83, 95]. Primary IFN- γ response genes resemble the IRF1 transcriptional dynamic, which is a STAT1 direct target. IRF1 is involved in regulating IFN- γ secondary response genes, including *IDO1* [154]. CYLD mRNA levels accumulation mimic *IDO1* transcript accumulation. This may also indicate a possible role for IRF1 in regulating CYLD as well. Additionally, direct comparison of IRF1, IRF2 and IRF9 transcriptional dynamics following IFN- γ treatment revealed that IRF1 induction is compatible with CYLD regulation. In particular, IRF1 transcription was induced around 2 hours prior to CYLD, while IRF2 and IRF9 activation coincided with that of CYLD (Figure 9.7). To test IRF1 engagement in CYLD regulation, I investigated the *CYLD* gene promoter region using the University of California Santa Cruz (UCSC) Genome Browser (genome.ucsc.edu). I analysed publicly available Chromatin Immunoprecipitation and Sequencing (ChIP-Seq) data for STAT1, STAT3 and IRF1 in myeloid cells. ChIP-Seq profiling for IRF1 in K562 human immortalised myelogenous leukaemia cells, following IFN- γ stimulation, showed a strong binding for this TF in the proximity of the CYLD Transcription Start Site (TSS) (Figure 9.8). STAT1 binding to the *CYLD* promoter region also appears to be detectable following IFN- γ stimulation. In addition, STAT1 and STAT3 binding is present in untreated GM-78 human immortalised leukodystrophic cells (Figure 9.8). These observations link CYLD to the IFN- γ 's secondary wave of gene transcription, relying on STAT1 activation and *de novo*-transcribed IRF1. Further experiments, including ChIP-qRT PCR for STAT1 and IRF1, will be important to confirm binding within the *CYLD* promoter region. Furthermore, the generation of IRF1-deficient cells

and subsequent quantification of CYLD mRNA levels following IFN- γ treatment will be important to connect IRF1 with CYLD transcription.

Possible cooperation between STAT1 and IRF1 cannot be excluded. STAT1 can directly remodel the chromatin state via the recruitment of histone acetyltransferases, such as CBP/p300 [95, 155, 156]. This chromatin remodelling step is important to induce the expression of some IFN- γ responsive genes, including the major histocompatibility complex genes [157]. STAT1 binding at the *CYLD* gene promoter region may occur following IFN- γ treatment and promote accessible chromatin via CBP/p300 activity. This could subsequently facilitate IRF1 binding and transcriptional induction of the *CYLD* gene. In addition, the *CYLD* promoter region contains predicted NF- κ B and IRF3 binding sites (data not shown). IFN- γ -dependent chromatin remodelling of the *CYLD* promoter region could potentially lead to stronger and more sustained CYLD induction following NF- κ B- and IRF3-activating stimuli. A similar mechanism has been reported for other NF- κ B target genes, such as *TNF- α* and *IL-6*, where their transcription is increased in IFN- γ -primed macrophages [95]. An inaccessible chromatin state could be the reason behind the mild transcriptional induction that follows NF- κ B-activating stimuli such as LPS.

The intensity of CYLD transcriptional induction could also be dependent on cooperation between different signals and TFs. A classic example of TF cooperation is the *NOS* gene. *NOS* transcription is activated via LPS, and its induction is enhanced by co-stimulation with type I or type II IFNs in a STAT1-dependent manner [105, 107]. An IRF1 binding site has also been identified within the *NOS* promoter region. Indeed, activation of IRF1 together with STAT1 is essential for *NOS* transcriptional induction following IFN- γ stimulation [158]. It will be of interest to investigate if CYLD transcriptional induction is regulated in a similar manner.

8.2 CYLD regulation of the IFN- γ signalling pathway

In addition to IDO1, the accumulation of CYLD mRNA levels mimics the transcriptional dynamic of SOCS1. *SOCS1* is an IFN- γ secondary response gene

and is a negative regulator of the IFN- γ signalling pathway. It is induced in a feedback loop following stimulation and binds the IFNGR1 [137, 159, 160]. This observation, together with the previously reported regulation of NF- κ B signalling through CYLD upregulation, may indicate a possible role for CYLD in inhibiting the IFN- γ signalling pathway [134].

IFN- γ -dependent STAT1- and STAT3-activating phosphorylation, and the downstream transcriptional response, are increased in CYLD-depleted macrophages compared to control cells. Interestingly, deregulation of the IFN- γ transcriptional response seems to be directed to the second wave of target genes. We can infer this from the fact that the IRF1 transcriptional dynamic is unaffected. In contrast, *IDO1*, *CCL2* and macroautophagy-related genes were deregulated by the absence of CYLD. Specifically, transcriptional induction of these second wave genes was stronger and more sustained in macrophages and epithelial cells lacking CYLD.

In contrast, no deregulation of STAT1 and STAT3 phosphorylation was observed in HCT-116 and U2O2 epithelial cells following IFN- γ treatment (data not shown). This could suggest a further step in CYLD control that is specific for immune cells. Accordingly, it can be speculated that there are two possible layers of CYLD regulation in this signalling pathway: an initial direct role on signal transduction and the activation of STATs, followed by a secondary role in controlling the IFN- γ transcriptional response. CYLD-deficient epithelial cells do not have a direct effect on STATs phosphorylation, but they do deregulate the IFN- γ transcriptional response (Figure 6.11). This suggests a specific role for CYLD in controlling STAT1 and STAT3 phosphorylation in immune cells, in addition to a regulation of the IFN- γ transcriptional response.

CYLD catalytic activity is required for the negative regulation of the IFN- γ transcriptional response. In line with this, I showed that Lys63- and Met1-linked Ub chains are important for a complete IFN- γ -mediated transcriptional response, suggesting that the IFN- γ signalling pathway is subject to ubiquitination-dependent regulation. Lys63- and Met1-linked Ub chains are required for PRR complex assembly and downstream signalling [16]. Similarly, these Ub chain types could be

involved in IFNGR complex assembly and activation. In support of this hypothesis, Lys63 chains have recently been shown to be conjugated to JAK2 during GM-CSF-induced activation. This polyubiquitination enhances JAK2 phosphorylation and downstream STAT5-mediated signal transduction [161]. Similarly, JAK2 ubiquitination may occur within the IFNGR complex, where CYLD-dependent fine-tuning of Lys63-linked Ub chains could represent an initial regulatory step for IFN- γ signalling.

Direct ubiquitination of TFs is a mechanism that regulates transcriptional activity [162]. Lys48-linked ubiquitination is a well-known mechanism to target TFs for proteasomal degradation, including NF- κ B and p53 [163, 164]. Furthermore, Lys63-linked Ub chains have been shown to promote the transcriptional activity of the Hypoxia-inducible Factor 1- α (HIF1- α) [165]. *Lawrence et al.* recently reported that the E3 Ub ligase Natural Killer Lytic-associated Molecule (NKLAM) conjugates Lys63-linked Ub chains on STAT1 to positively regulate STAT1-mediated transcriptional activity [166]. Accordingly, CYLD might act as a STAT1 negative regulator, hydrolysing Lys63-linked Ub chains to arrest STAT1 transcriptional activity. However, additional experiments are needed to investigate whether CYLD directly interacts with these substrates (JAK2 and STAT1). In particular, CYLD immunoprecipitation will determine direct CYLD binding to JAK2, STAT1 and other IFNGR components. Furthermore, mass spectrometry analysis of the CYLD interactome performed in U2OS^{Parental} and U2OS^{CYLD-KO} cells by Derek Leske revealed an enrichment for JAK1 peptides, suggesting an interaction between these two proteins. Further experiments are also required to identify ubiquitination targets within the IFN- γ signalling pathway. Tandem Ub-binding Entity (TUBE) pulldown experiments will allow the purification of polyubiquitinated proteins in response to IFN- γ treatment. This experiment will reveal ubiquitinated targets within the IFN- γ signalling pathway. Moreover, a comparison between control and CYLD-depleted macrophages will potentially uncover differences in the ubiquitination state of these targets, linking CYLD deubiquitinase activity to the IFN- γ signalling pathway.

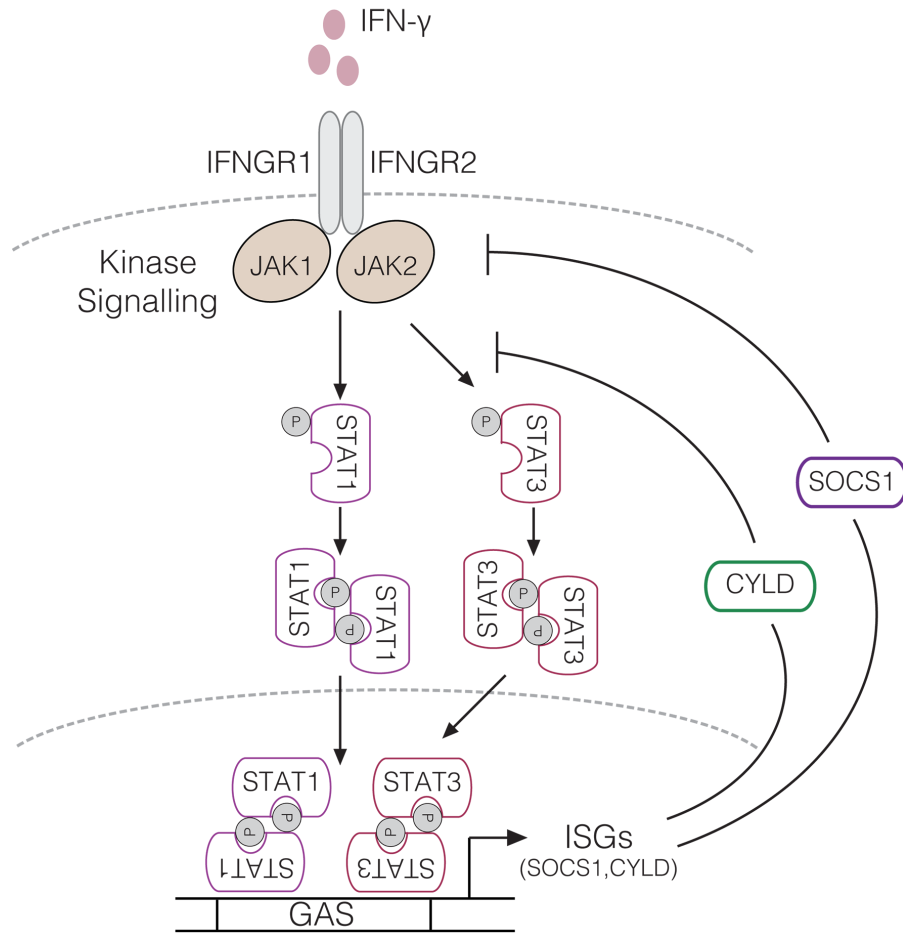


Figure 8.1: IFN- γ signalling pathway. Schematic representation of the IFN- γ signalling pathway including the reported negative regulator SOCS1 and the novel negative regulator CYLD.

8.3 Phenotypic consequences of CYLD depletion

Uncontrolled IFN- γ signalling, together with increased basal production of this cytokine, may contribute to the enhanced antimicrobial transcriptional signatures identified in CYLD-depleted macrophages. Upregulation of macroautophagy, autophagy, lysosomal activity and immunity genes in CYLD-deficient macrophages at steady state resemble the transcriptional signature of control macrophages primed with IFN- γ . Furthermore, transcription of these genes is enhanced in IFN- γ treated CYLD-deficient macrophages.

IL-6, together with IFN- γ , is a crucial pro-inflammatory cytokine that mediates the acute-phase response to pathogens. Conversely, IL-10 is involved in the resolution

of inflammation and is directly induced by IFN- γ [74, 75]. *Nishant et al.* previously observed enhanced intracellular *Listeria monocytogenes* clearance in *CYLD* KO BMDMs, and linked it to impaired NF- κ B signalling, and increased production of ROS, IFN- γ and IL-6. In addition, they described a deregulation of IL-6/STAT3 signalling mediated by *CYLD* in hepatocytes [51]. I did not observe deregulation of the IL-6 signature in *CYLD*-depleted macrophages. Furthermore, production of ROS was not increased in *CYLD*-depleted macrophages compared to control cells (Figure 6.5 and 9.9). I also investigated a possible role for *CYLD* in regulating the IL-6 signalling pathway. Stimulation with IL-6 did not result in enhanced activation of STAT3 in *CYLD*-deficient macrophages compared to control cells, determined by STAT3 phosphorylation and mRNA levels of STAT3 target genes. Similar results were observed following IL-10 stimulation. These data suggest that *CYLD* is not involved in controlling IL-6 and IL-10 signalling pathways in this system (Figure 9.10). The increased serum concentrations of IFN- γ in response to *Listeria monocytogenes* observed by *Nishant et al.* in *CYLD* KO mice have not been further investigated. However, this indicates that *CYLD* KO mice present deregulated IFN- γ production in response to *Listeria monocytogenes*. I showed that *CYLD*-depleted THP-1-derived macrophages present elevated production of IFN- γ , and therefore a similar scenario could occur in *CYLD* KO macrophages in response to *Listeria monocytogenes*. Moreover, the fact that *CYLD*-depleted THP-1-derived macrophages mirror the enhanced intracellular bacterial clearance of *CYLD* KO BMDMs, suggests a similarity between these two systems. Nevertheless, further experiments are needed to explore IFN- γ production and regulation in *CYLD* KO murine macrophages.

IFN- γ is a well-known activating stimulus for macrophages [79, 95]. My results show that deregulation of this signalling pathway through *CYLD* depletion results in increased macroautophagy features, including phagocytosis, autophagic flux and lysosomal activity. These processes have been previously identified to be enhanced in IFN- γ primed macrophages [79, 95]. In line with this, *CYLD*-depleted macrophages display an enhanced ability to restrict intracellular gram-positive

and -negative pathogen growth. Despite the fact that the features reported above appear favourable in the context of bacterial infection, deregulation of the IFN- γ -mediated response has been linked with uncontrolled inflammation [167]. For example, SOCS1 KO in mice causes lymphocyte-dependent perinatal lethality, which is rescued by IFN- γ KO [168]. In a similar manner, the deregulation of NF- κ B activation downstream of the TNFR signalling pathway leads to excessive inflammation and autoimmunity [169]. As previously discussed, some *CYLD* KO mice strains present inflammatory infiltrates in different tissues [44, 45], while other strains do not present an overt phenotype unless challenged with pro-inflammatory stimuli or pathogens [49–52]. These observations have been attributed to NF- κ B signalling pathway deregulation in the absence of *CYLD*. In light of its new role in regulating IFN- γ signalling, further characterisation of the *CYLD* KO mice phenotypes will be important. Indeed, deregulation of the IFN- γ signalling pathway could contribute, together with NF- κ B, to promote inflammation and the reported inflammatory-driven disorders observed in *CYLD* KO mice.

NOD2 and RIPK2 are induced by IFN- γ stimulation, and are upregulated in *CYLD*-deficient macrophages. The NOD2 receptor signalling pathway contributes to intracellular bacterial clearance in two distinct ways. First, it activates the NF- κ B transcriptional response with the production of pro-inflammatory cytokines and antimicrobial peptides [17]. Secondly, it promotes the clearance of cytosolic bacteria via the autophagic-lysosomal machinery [130, 170]. Thus, enhanced levels of NOD2 and RIPK2 could possibly explain the higher xenophagic activity observed in *CYLD*-deficient cells. Published work from the Gyrd-Hansen laboratory has shown a direct role for *CYLD* in controlling the NOD2 signalling pathway via deubiquitination of the adaptor protein RIPK2 [8, 23]. Therefore, in addition to increased cytoplasmic levels of NOD2 and RIPK2, *CYLD*-deficient cells induce stronger downstream activation of the NF- κ B-mediated inflammatory response. However, my results revealed that inhibition of the NOD2/RIPK2 axis in *Salmonella typhimurium* infection has a limited effect on *CYLD*-depleted macrophages clearance. This suggests that

NOD2/RIPK2 upregulation only partially contributes to the enhanced bacterial clearance observed in CYLD-depleted cells.

OPTN mRNA and protein levels are increased in response to IFN- γ treatment and in CYLD-depleted macrophages compared to control cells. OPTN is a NEMO homologous protein, involved in TNF- α -mediated inflammation and xenophagy [87, 171]. In particular, it has been shown to be recruited to the TNF receptor complex, binding CYLD and inhibiting downstream NF- κ B activation [171]. In addition, OPTN is an autophagy receptor that is able to bind Ub-coated bacteria and LC3 family members (MAP1LC3A, MAP1LC3B, GABARAP, GABARAPL1 and GABARAPL2) [172]. OPTN binding to ubiquitinated bacteria is important to promote intracellular clearance via the autophagic route. Accordingly, OPTN KO mice are more susceptible to *Salmonella typhimurium* infection and this effect is not dependent on NF- κ B signalling [87, 173]. In contrast, OPTN depletion in THP-1-derived macrophages does not affect their ability to restrict *Salmonella typhimurium* growth. Conversely, OPTN knockdown completely reverted the enhanced bacterial clearance observed in CYLD-depleted THP-1-derived macrophages. These data suggest that higher OPTN levels together with increased xenophagy in CYLD-depleted macrophages are crucial for intracellular pathogen clearance, while the NOD2 signalling pathway plays a marginal role in this system.

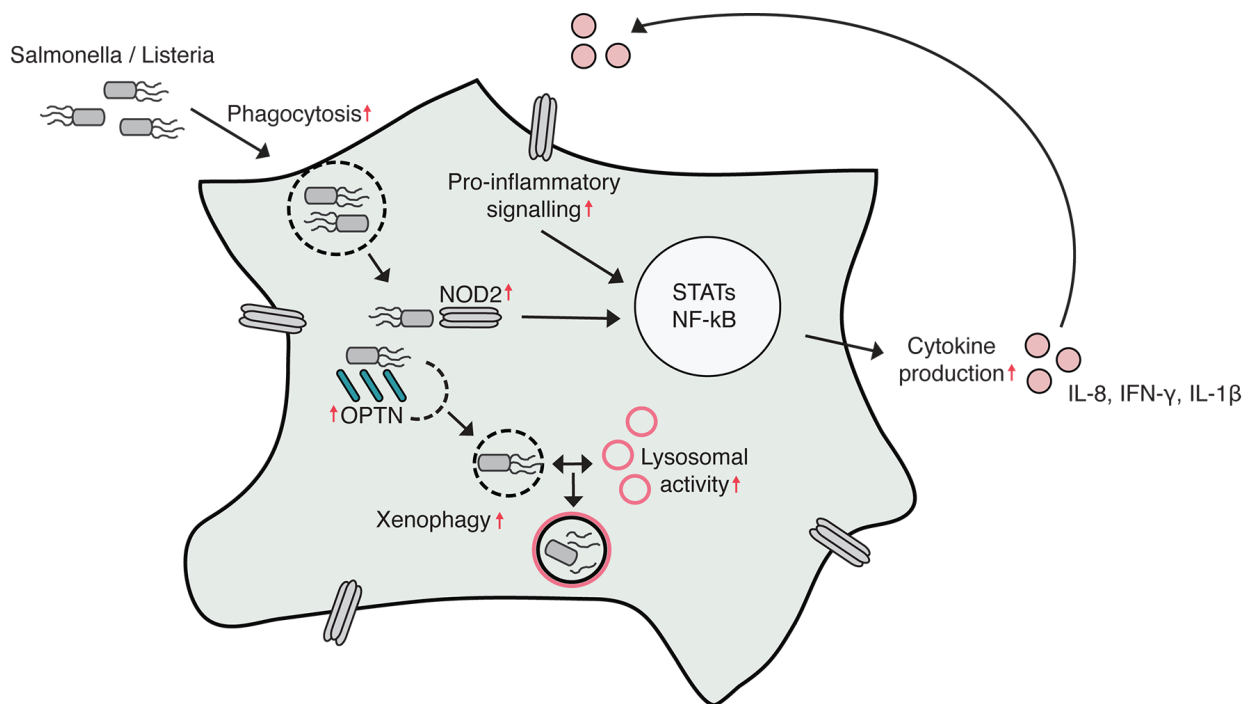


Figure 8.2: CYLD depleted macrophage phenotype. Schematic representation of the processes deregulated by the absence of CYLD in macrophages during bacterial infection. CYLD depletion results in enhanced IFN- γ and NF- κ B signalling, with subsequent upregulation of xenophagic features in macrophages, including phagocytosis, autophagic flux and lysosomal activity. Additionally, enhanced IFN- γ signalling leads to increased NOD2, RIPK2 and OPTN protein levels, which contribute to better sensing and clearance of intracellular pathogens.

8.4 Future perspectives

The increased ability of CYLD-depleted macrophages to clear intracellular pathogen growth seems to be a combination of several antimicrobial and immune mechanisms, which are mainly regulated through the IFN- γ signalling pathway. However, CYLD negative regulation of the NF- κ B signalling pathway could also be involved in *Listeria monocytogenes* and *Salmonella typhimurium* clearance in CYLD-deficient cells. These two signalling pathways are pivotal for macrophage activation, inflammation and bacterial clearance. Therefore, CYLD appears to be a very interesting molecule in the control of these innate immune functions in macrophages. Moreover, CYLD transcriptional regulation has emerged as an important mechanism of inhibition for the IFN- γ -mediated transcriptional response. In an *in vivo* context, this could prevent excessive macrophage-mediated inflammation in response to pathogen sensing or pro-inflammatory stimuli.

Uncontrolled inflammation, as is observed in *CYLD* KO mice, can lead to chronic disorders and eventual malignancies [44, 53]. These observations place CYLD in a fascinating position. On one hand, depletion of this enzyme strongly enhances the ability of the innate immune system to fight infection and colonisation. On the other hand, the absence of CYLD results in chronic inflammation, tissue damage and tumour development. Transient targeting of CYLD activity could represent a possible strategy to boost the immune response in pathogen-related conditions. Moreover, the promotion of macrophage pro-inflammatory features could be important in immunosuppressive contexts, such as the tumour microenvironment, where macrophages tend to acquire the TAMs phenotype. However, in line with the immune-unrelated functions reported for CYLD, the identification of CYLD context-specific interactors and substrates will be essential to avoid the disruption of other cellular activities.

9

Appendices

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9.1 Supplementary figures

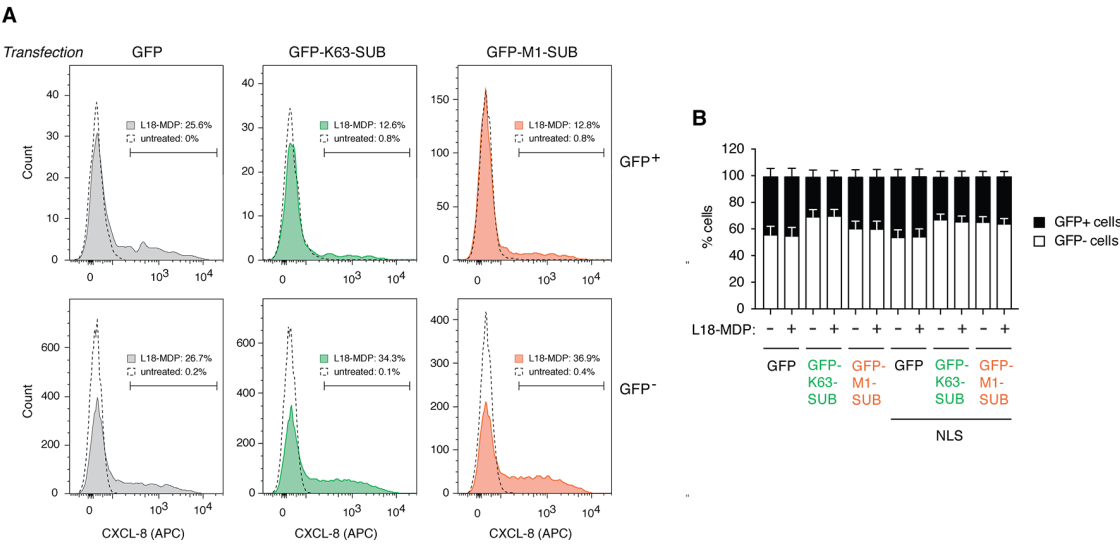


Figure 9.1: CXCL-8 production relies on Lys63- and Met1-linked Ub chains. (A) Representative flow cytometry histograms for CXCL-8 production in GFP+ or GFP- cells following transfection with GFP, GFP-K63-SUB or GFP-m1-SUB and L18-MDP stimulation (200 ng/ml, 6 hrs) (B) Flow cytometry analysis of GFP+ or GFP- cells following transfection with GFP, GFP-K63-SUB or GFP-m1-SUB and L18-MDP stimulation to evaluate the transfection efficiency of the constructs.

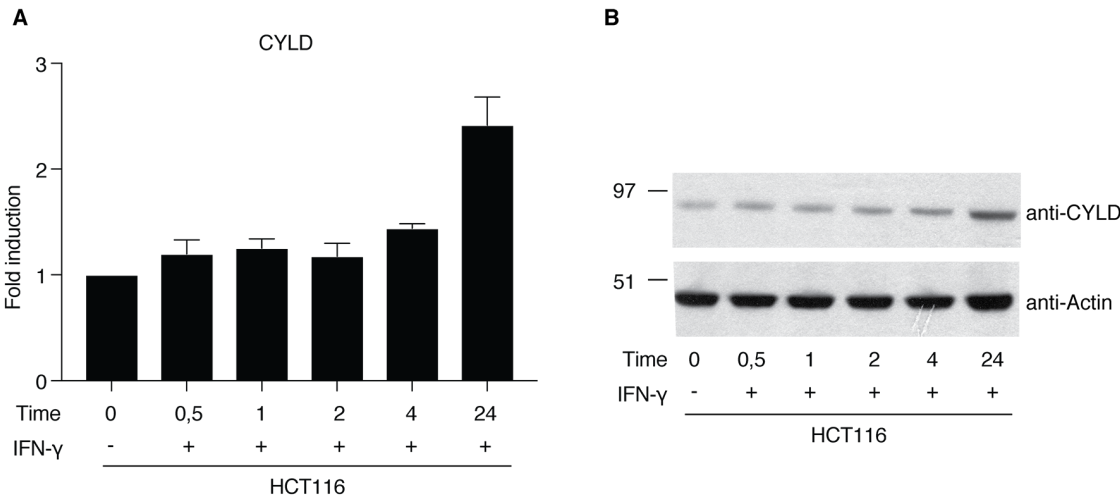


Figure 9.2: CYLD is induced via IFN- γ in epithelial cells. (A) qRT-PCR for CYLD RNA levels on HCT-116 cells treated with IFN- γ (20 ng/ml) for indicated times (B) WB for CYLD protein levels on HCT-116 cells treated with IFN- γ (20 ng/ml) for indicated times.

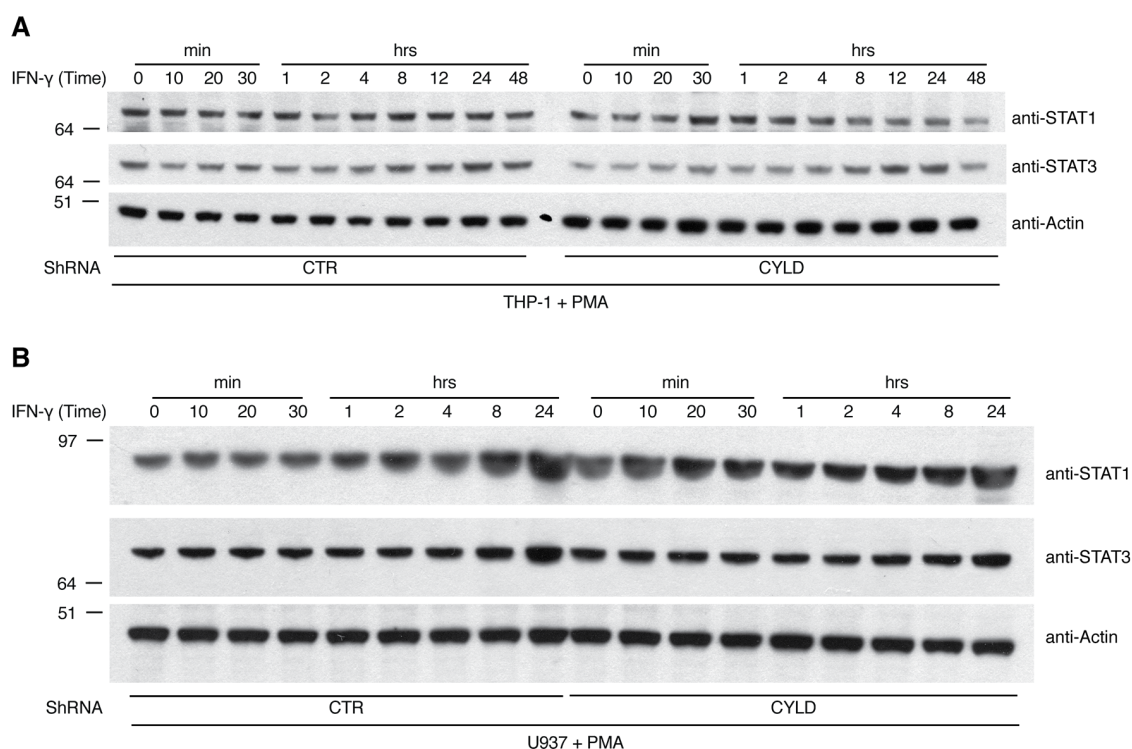


Figure 9.3: CYLD is a negative regulator of IFN- γ signalling. (A) WB analysis of Total-STAT1 and Total-STAT3 protein levels in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages following IFN- γ (20 ng/ml) for indicated times (B) WB analysis of Total-STAT1 (Y701) and Total-STAT3 protein levels in U937^{+PMA}-ShCTR and U937^{+PMA}-ShCYLD macrophages following IFN- γ (20 ng/ml) for indicated times.

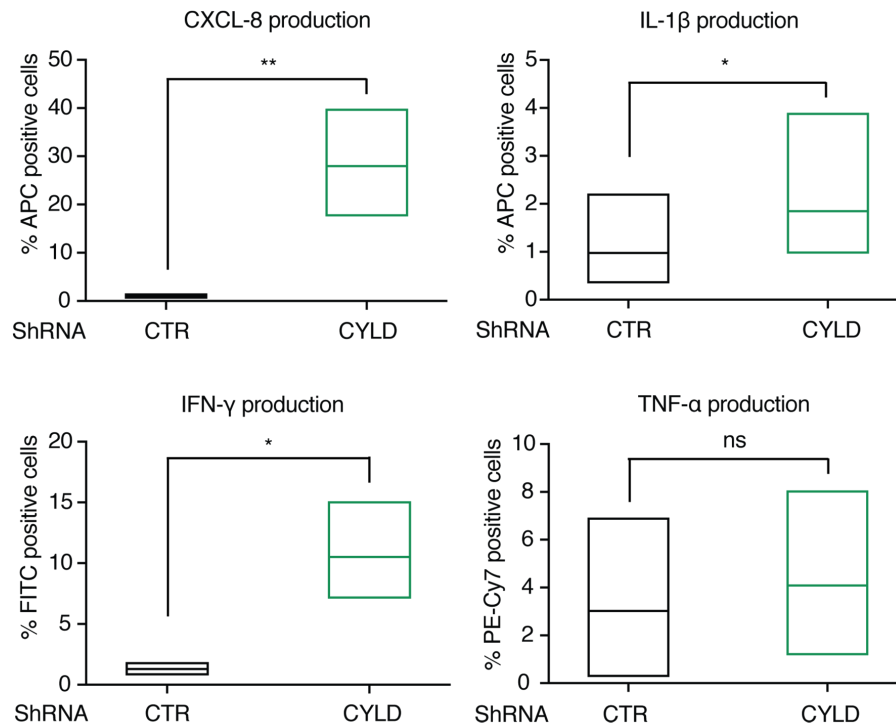


Figure 9.4: Pro-inflammatory cytokine production. Flow cytometry analysis of CXCL-8, IL-1 β , IFN- γ and TNF- α production measured as % of positive cells in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD macrophages. Triplicates. Unpaired T test statistics.

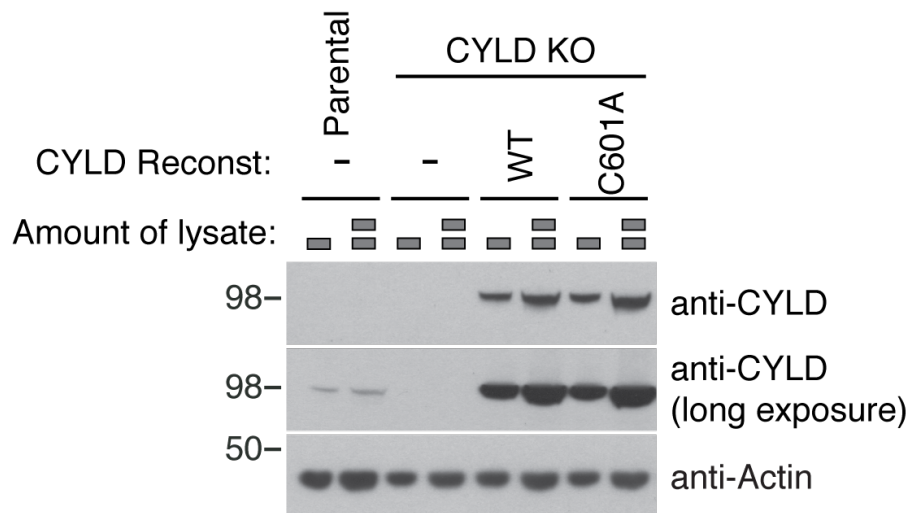


Figure 9.5: CYLD levels in parental and reconstituted U2OS cells. WB analysis of CYLD protein levels in Parental U2OS, U2OS^{CYLD-KO}, U2OS^{CYLD-WT} and U2OS^{CYLD-C601A} reconstituted cells. Data from Derek Leske.

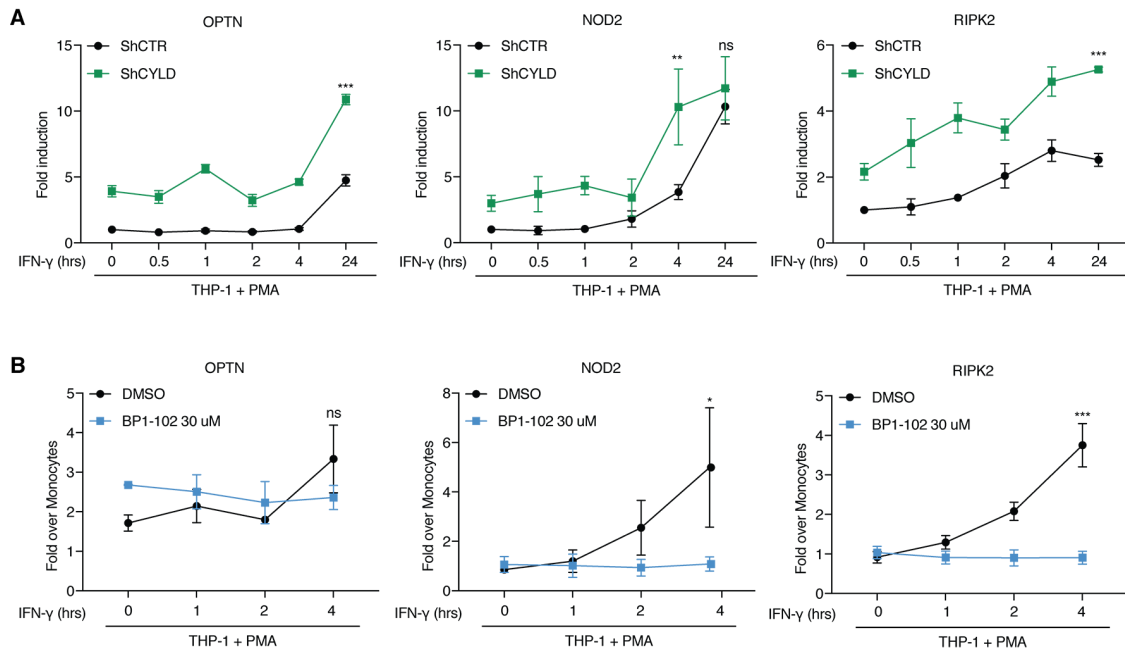


Figure 9.6: NOD2, RIPK2 and OPTN levels in CYLD depleted macrophages. (A) qRT-PCR for RIPK2, NOD2 and OPTN RNA levels in U937+*PMA*-ShCTR and U937+*PMA*-ShCYLD untreated or stimulated with IFN- γ (20 ng/ml) for indicated times and normalised to control untreated cells (B) qRT-PCR for RIPK2, NOD2 and OPTN RNA levels in U937-derived macrophages pre-incubated with BP1-102 (30 μ M) or DMSO, untreated or stimulated with IFN- γ (20 ng/ml) for indicated times and normalised to monocytic untreated cells. Triplicates. Two Way ANOVA statistics.

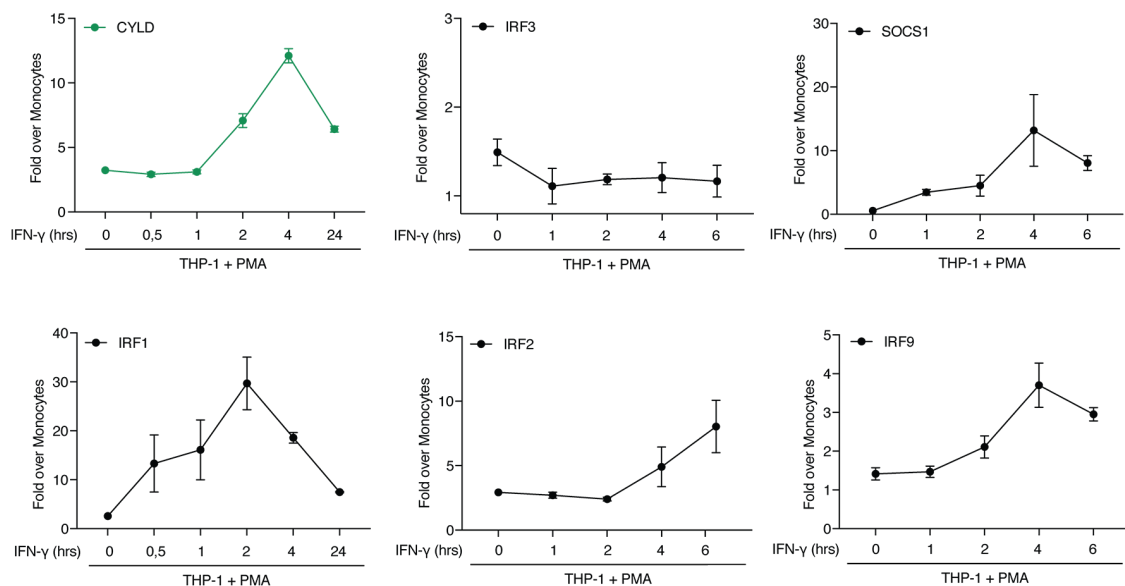


Figure 9.7: IFN- γ responsive genes. qRT-PCR for CYLD, IRF1, IRF2, IRF9, IRF3 and SOCS1 in THP-1-derived macrophages untreated or stimulated with IFN- γ (20 ng/ml) for indicated times and normalised to control untreated cells at monocytic state. Triplicates.

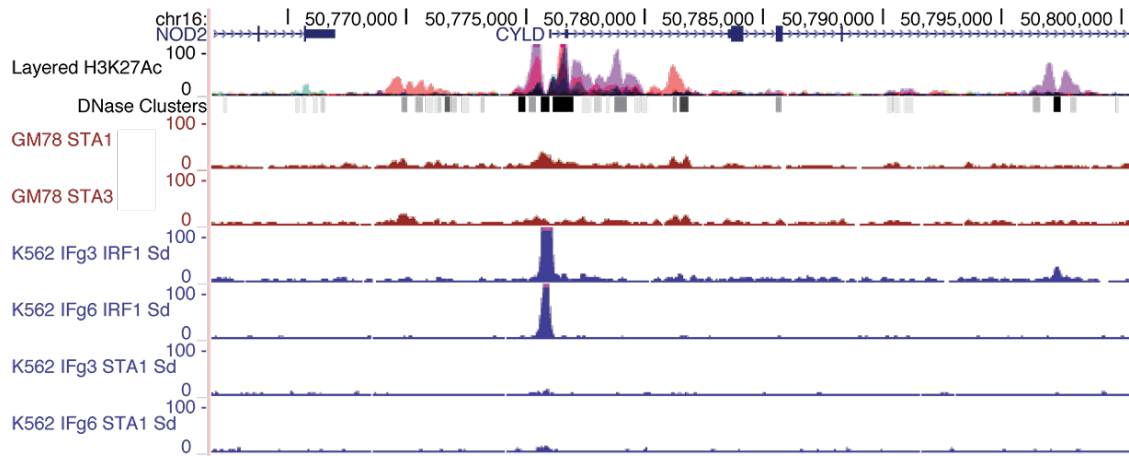


Figure 9.8: STAT1, STAT3 and IRF1 binding at CYLD promoter region. Graphic representation of CYLD gene and promoter region on University of California Santa Cruz (UCSC) Genome Browser (genome.ucsc.edu). Genome position: chr16:50,761,799-50,821,684. Publicly available ChIP-Seq data for STAT1, STAT3 and IRF1 binding are displayed as peaks showing enrichment for these TFs binding (Signal) in the genomic region over untreated sample or over control immunoglobulins. Track number one: GM12878 STAT1 Standard ChIP-seq Signal from ENCODE/SYDH - STAT1 binding in untreated GM-78 cells (GSM935612). Track number two: GM12878 STAT3 IgG-mus ChIP-seq Signal from ENCODE/SYDH - STAT3 binding in untreated GM-78 cells (GSM935557). Track number three: K562 IRF1 Standard IFN γ 30min ChIP-seq Signal from ENCODE/SYDH - IRF1 binding in IFN- γ treated K562 cells (30 mins) (GSM935505). Track number four: K562 IRF1 Standard IFN γ 6hrs ChIP-seq Signal from ENCODE/SYDH - IRF1 binding in IFN- γ treated K562 cells (6 hrs) (GSM935549). Track number five: K562 STAT1 Standard IFN γ 30min ChIP-seq Signal from ENCODE/SYDH - STAT1 binding in IFN- γ treated K562 cells (30 mins) (GSM935487). Track number six: K562 STAT1 Standard IFN γ 6hrs ChIP-seq Signal from ENCODE/SYDH - STAT1 binding in IFN- γ treated K562 cells (6 hrs) (GSM935488).

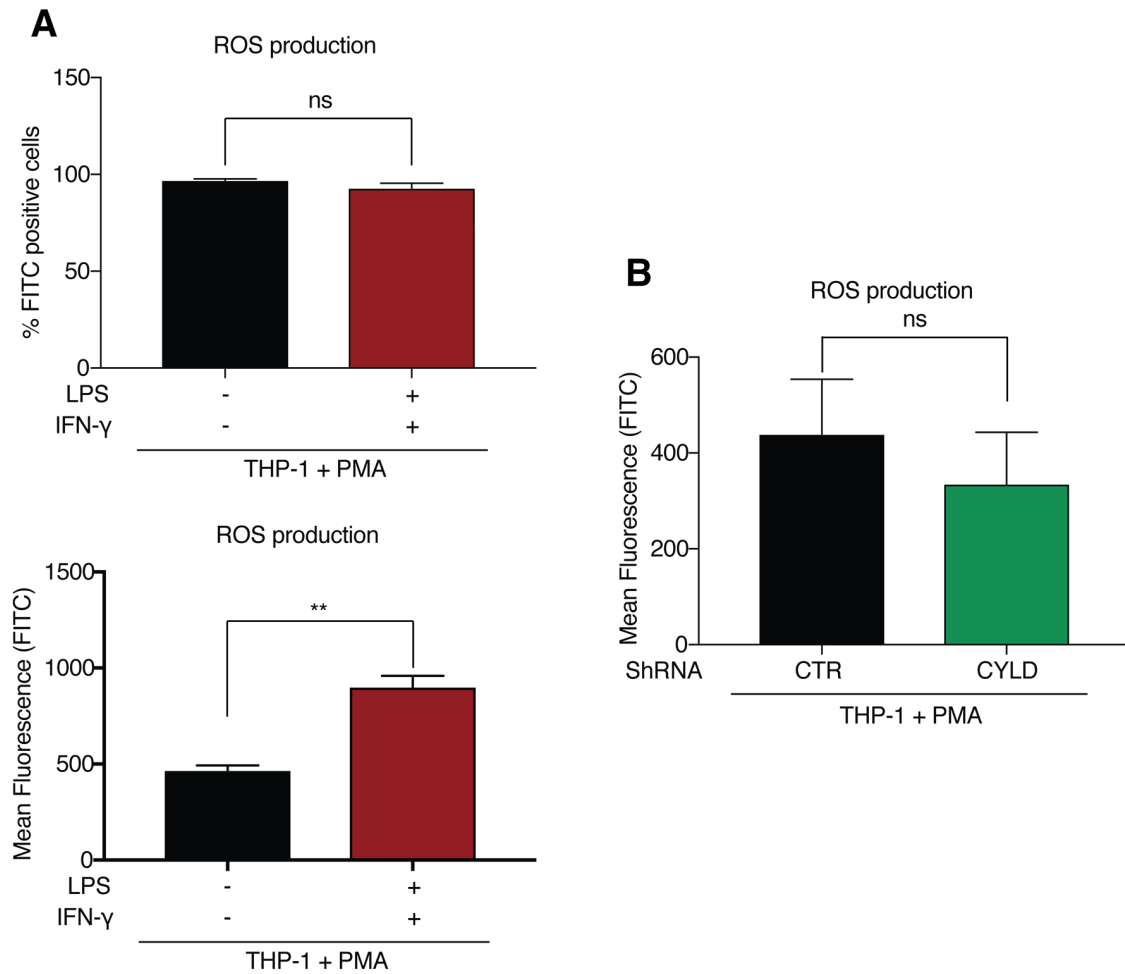


Figure 9.9: ROS production. (A) Flow cytometry analysis of ROS production via ROS Detection Cell-Based Assay Kit (DCFDA) in THP-1 derived macrophages untreated and activated with LPS (50 ng/ml) + IFN- γ (20 ng/ml) treated for 2 hrs + 22 hrs rest. % of FITC fluorescent cells and mean fluorescence of the FITC channel for both conditions. (B) Flow cytometry analysis of ROS production via DCFDA in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD. Mean fluorescence of the FITC channel. Triplicates. One Way ANOVA statistics.

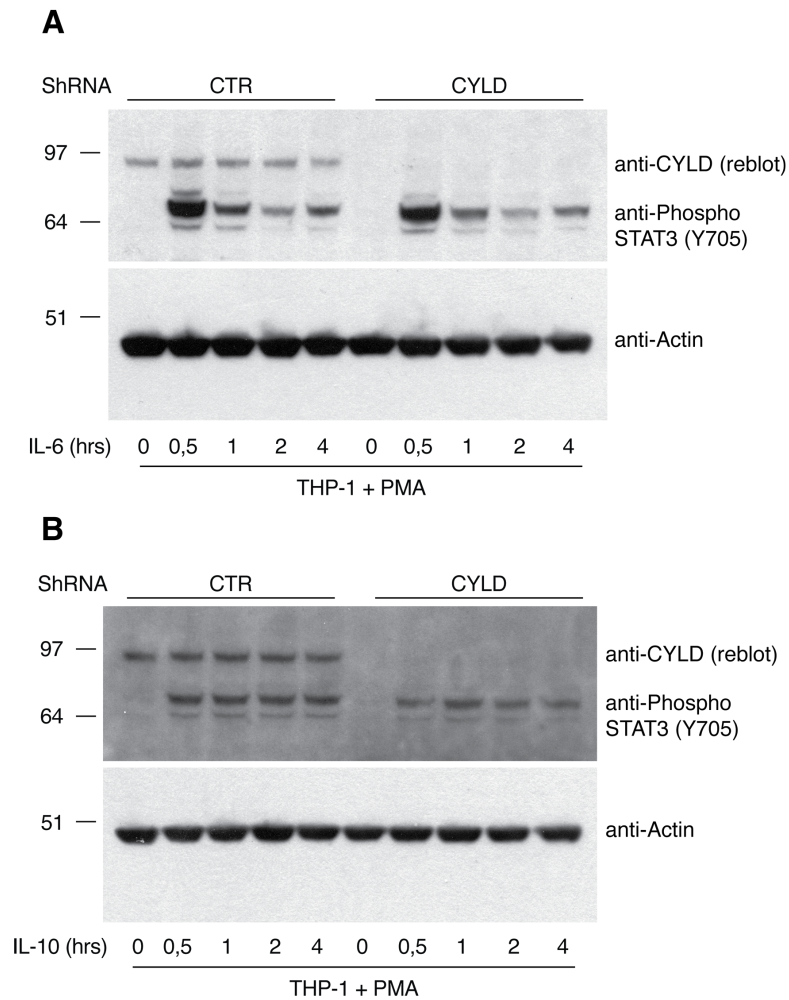


Figure 9.10: IL-6 and IL-10 stimulation. WB for CYLD, Phospho-STAT3 and Actin in (Y705) in THP-1^{+PMA}-ShCTR and THP-1^{+PMA}-ShCYLD stimulated with IL-6 (10 ng/ml) or IL-10 (10 ng/ml) for indicated times.

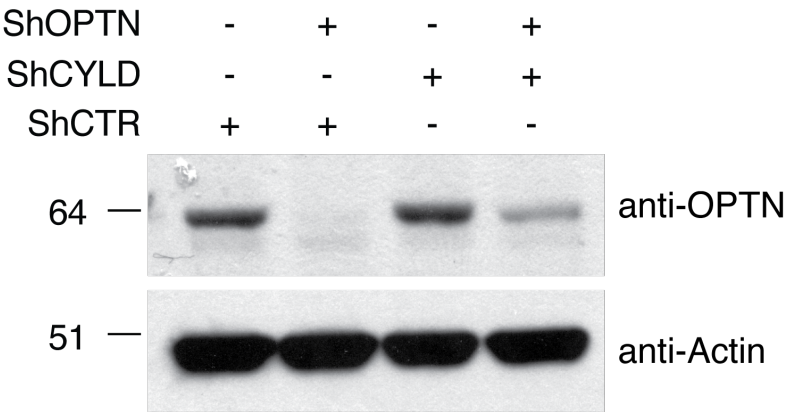


Figure 9.11: OPTN protein levels ShCTR and ShCYLD THP-1. WB analysis of OPTN levels in THP-1-ShCTR-GFP, THP-1-ShCTR-GFP-ShOPTN, THP-1-ShCYLD-GFP and THP-1-ShCYLD-GFP-ShOPTN cells.

9.2 Publications

Selected data from this thesis was published and cited in the following article:

CYLD limits Lys63- and Met1-linked ubiquitin at receptor complexes to regulate innate immune signaling.

*Matous Hrdinka, Berthe Katrine Fiil, **Mattia Zucca**, Derek Leske, Katrin Bagola, Monica Yabal, Paul R. Elliott, Rune Busk Damgaard, David Komander, Philipp J. Jost, Mads Gyrd-Hansen.* Cell reports, 2016

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