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The role of linear ubiquitination in toll-like receptor 3 signalling

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2 LIST OF ABBREVIATIONS

4-OHT	4-hydroxy tamoxifen
AMPK	AMP-activated protein kinase
bp	base pairs
CCL5	chemokine (C-C motif) ligand 5
CD	cluster of differentiation
cFLIP	cellular FLICE-like inhibitory protein
CHX	Cycloheximide
cIAP	cellular inhibitor of apoptosis protein
CLR	C-type lectin receptors
cpdm	chronic proliferative dermatitis mice
CRISPR	clustered regularly interspaced short palindromic repeats
DAMP	damage-associated molecular pattern
DISC	death-inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DUB	deubiquitinating enzyme
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	E3 ubiquitin ligase
ECD	extracellular domain
EGFP	enhanced green fluorescent protein
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FACS	fluorescence activated cell sorting
FADD	Fas-associated protein with death domain
Fc	fragment crystallisable
FCS	fetal calf serum
fl	floxed
G	glycine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase

h	hours
H&E	haematoxylin & eosin
HaCaT	human adult low calcium high temperature
HeLa	Henrietta Lacks
HiCa	high-calcium
HOIL-1	heme-oxidised IRP2 ubiquitin ligase-1
HOIP	HOIL-1-interacting protein
HSV	herpes simplex virus
IAV	influenza A virus
IFN	interferon
IKK	IκB kinase
IL	interleukin
IP	immunoprecipitation
IRES	internal ribosomal entry site
IRF	interferon regulatory factor
IκBα	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha
JNK	c-Jun N-terminal kinases
K	lysine
kDa	kilodalton
L	ligand
LoCa	low-calcium
LPS	lipopolysaccharide
LRR	leucine-rich repeat
LUBAC	linear ubiquitin chain assembly complex
M1	amino terminal methionine; linear linkage
MAPK	mitogen-activated protein kinase
MDA5	melanoma-differentiation associated gene 5
MSCV	murine stem cell virus
Nec-1	Necrostatin-1
NEMO	NF-κB essential modulator
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NLR	NOD-like receptor
OTULIN	OTU deubiquitinase with linear linkage specificity

PAMP	pathogen-associated molecular pattern
PARP	poly-(ADP-ribose) polymerase
PCR	polymerase chain reaction
PI	propidium iodide
PMK	primary murine keratinocytes
Poly(I:C)	polyriboinosinic:polyribocytidylic acid
PRR	pattern-recognition receptor
PTMs	post-translational modifications
RBR	RING – between RING – RING
RHIM	RIP homotypic interaction motif
RIG-I	retinoic-acid inducible gene I
RING	really interesting new gene
RIP	receptor-interacting protein
RLR	RIG-I-like receptor
RNA	ribonucleic acid
RNAi	RNA interference
rpm	rounds per minute
SC	signalling complex
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SHARPIN	SHANK-associated RBCK1 homology-domain-interacting protein
siRNA	small interfering RNA
SM	smac mimetic
ss	single-stranded
TAB	TAK binding protein
TAK	TGF β-activated kinase
TBK	TANK-binding kinase
TCR	T cell receptor
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TLR3-SC	TLR3-signalling complex
TMD	transmembrane domain

TNF	tumour necrosis factor
TNFR	TNF receptor
TNFR1-SC	TNF receptor 1-signalling complex
TRAF	TNF-receptor associated factor
TRIF	TIR domain-containing adapter protein inducing IFN-β
WT	wildtype
zVAD	Z-VAD(OMe)-FMK

3 INTRODUCTION

3.1 PATTERN-RECOGNITION RECEPTORS

It was long thought that recognition of host invasion by the innate immune system occurs in an unspecific manner. This paradigm was challenged in the 1990s by the discovery of specific innate immune recognition of pathogens by Toll-like receptors (TLRs), with TLR4 being the first of all human TLRs discovered [57, 64, 77]. To date, 10 and 12 different TLRs have been identified in human and mouse, respectively [49]. TLRs belong to the superfamily of pattern-recognition receptors (PRRs) comprising different subfamilies of germline-encoded receptors including the TLRs, the NOD-like receptors (NLRs), the C-type lectin receptors (CLRs) and the RIG-I-like receptors (RLRs) [93]. PRRs represent the first line of defence during an immune response and mediate innate immune recognition of diverse pathogens. They recognise so-called pathogen-associated molecular patterns (PAMPs), conserved microbial motifs specific for different groups of pathogens. Whereas NLRs and RLRs reside in the cytosol, CLRs and TLRs are membrane-bound receptors [93].

TLRs are type I transmembrane proteins and comprise an extracellular domain (ECD) with leucine-rich repeats (LRRs), a transmembrane domain (TMD) and an intracellular Toll-interleukin (IL)-1 receptor (TIR) domain [10, 49]. TLRs orchestrate innate immunity to a variety of different exogenous stimuli including infection with bacteria, fungi and viruses [65]. In addition to recognising PAMPs of invading pathogens, TLRs have more recently been implicated to play a role in the recognition of sterile tissue damage by sensing damage-associated molecular patterns (DAMPs), e.g. high-mobility group protein B1, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) [81].

According to their cellular localisation, TLRs can be divided into two groups – whereas TLR1, 2, 4, 5, 6 and 10 are localised on the cellular surface and mainly recognise extracellular lipoproteins, TLR3, 7, 8 and 9 are found in the endosomal compartment of the cell and sense nucleic acids which are delivered to the endosomal compartment by endocytosis [49]. Upon ligand recognition TLRs dimerise [10] resulting in formation of either TLR homo- or heterodimers depending on the respective ligand. TLR2, for example, forms either heterodimers with TLR1

or 6 upon recognition of triacyl lipopetides from gram-negative or diacyl lipopetides from gram-positive bacteria, respectively [49]. Both, TLR4 and 5 form homodimers upon ligand recognition. Whereas lipopolysaccharides (LPS) are sensed by TLR4, TLR5 is activated by flagellin [49]. TLR10 is implicated in mediating innate immunity to influenza infection but its ligand remains to be identified [56].

All endosomal TLRs form homodimers [6]. TLR3 dimerises upon recognition of double-stranded (ds) RNA, whereas single-stranded (ss) RNA is detected by both, TLR7 and 8 [6]. TLR9 recognises DNA [6]. The cellular localisation of the endosomal TLRs is thought to be of importance for discrimination of non-self from self [6, 81].

An overview of the human TLR family is presented in Figure 1.

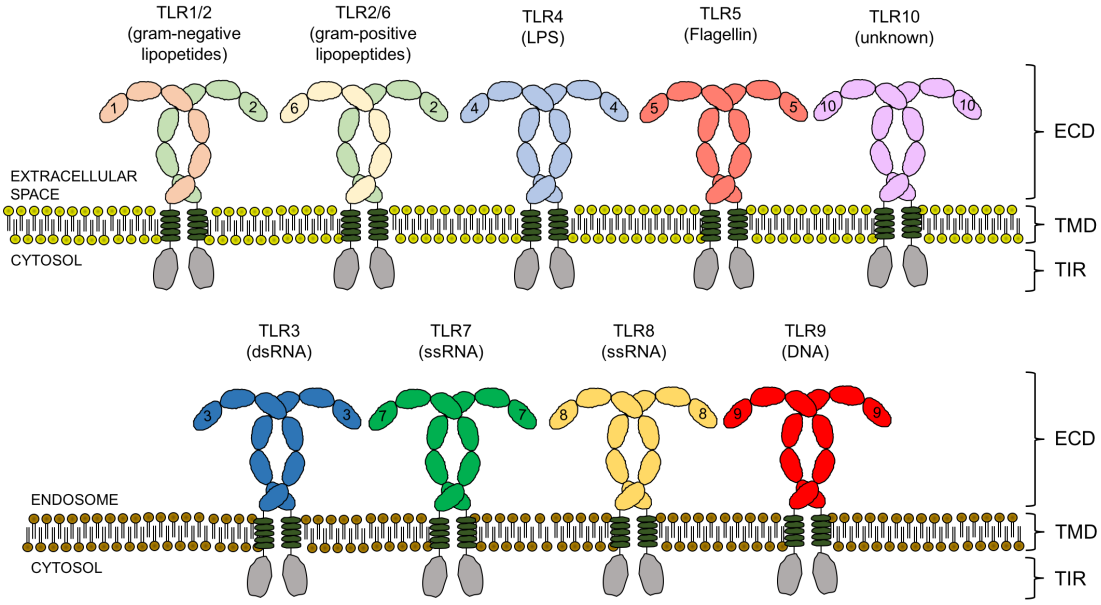

Figure 1. The human TLR family.

The TLR family in humans comprises 10 different TLRs, namely TLR1 - TLR10. TLRs consist of an ECD with LRRs, a TMD and an intracellular TIR domain. All TLRs dimerise upon ligand recognition. TLR1, 2, 4, 5, 6 and 10 belong to the group of cell surface TLRs. TLR2 is known to form heterodimers with either TLR1 or TLR6 resulting in recognition of lipopetides from gram-negative or gram-positive bacteria, respectively. LPS is recognised by TLR4 homodimers whereas flagellin is sensed by TLR5. The ligand of TLR10 remains to be identified.

TLR3, 7, 8 and 9 are localised in the endosomal compartment of the cell and, thus, form the group of endosomal TLRs. Their respective ligands are delivered to the endosomes by endocytosis. All endosomal TLRs form homodimers upon ligand recognition. TLR3 is known to sense dsRNA, whereas ssRNA is detected by TLR7 and 8. DNA is recognised by TLR9.

TLR, toll-like receptor; ECD, extracellular domain; LRRs, leucine-rich repeats; TMD, transmembrane domain; TIR, Toll-IL-1 receptor; LPS, lipopolysaccharides; ds, double-stranded; ss, single-stranded; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

3.1.1 TLR3 SIGNALLING

TLR3 is, like all other TLRs, a type I transmembrane protein. Its ECD is localised in the endosomal compartment and mediates recognition of dsRNA which is either generated by viruses during their replication cycle as PAMP [2] or released from damaged cells as DAMP [5, 12, 16, 48]. Whereas it is clear that TLR3 is involved in the host response to viral infection, its precise role is controversial and remains rather poorly defined [75]. TLR3-deficient patients have been identified to be highly susceptible to herpes simplex virus (HSV) 1 encephalitis [112]. In addition, presence of TLR3 seems to be crucial for fighting infections with influenza A virus (IAV) as a patient suffering from IAV-associated encephalopathy was identified to harbour a mutation in the *Tlr3* gene [40] and children with TLR3 polymorphisms developed IAV-induced pneumonia [26]. Contrary to that, TLR3 deficiency was proposed to protect mice from IAV-induced lethal hyperinflammation [55]. In addition to TLR3's complex function in sensing viral infections, its role in tissue damage is also not fully understood. Whereas wound healing and skin regeneration were shown to critically depend on TLR3-mediated inflammation [54, 70], TLR3 has also been shown to mediate deleterious effects of tissue damage [5, 16].

Recognition of dsRNA or its synthetic analogue polyriboinosinic:polyribocytidylic acid (poly(I:C)) by TLR3 results in receptor dimerization leading to a horseshoe-shaped formation of two TLR3 receptors complexed with dsRNA thereby allowing subsequent recruitment of the adaptor protein TIR-domain-containing adapter-inducing interferon (IFN)-β (TRIF) via its TIR domain to the TIR domain of TLR3 [58, 62]. This triggers activation of the following downstream signalling branches:

Firstly, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) signalling via recruitment of receptor-interacting protein (RIP) 1 (or RIP3) which binds to TRIF via its RIP homotypic interaction motif (RHIM) domain. Subsequently, tumour necrosis factor (TNF) receptor-associated factor (TRAF) 6 is recruited which in turn enables recruitment of the TGF β-activated kinase (TAK)/TAK binding protein (TAB) and IκB-kinase (IKK) complexes [47, 66].

Secondly, induction of type I IFNs via activation of IFN regulatory factor (IRF) 3 by TRAF3, TANK-binding kinase (TBK) 1 and IKKε [30, 36].

Thirdly, cell death induction via RIP1, Fas-associated protein with death domain (FADD) and Caspase-8/-10, negatively regulated by cellular inhibitor of apoptosis proteins (cIAPs) 1 and 2, and differentially regulated by cellular FLICE-like inhibitory protein (cFLIP) isoforms [27, 28, 107].

TLR3 has been identified to induce different types of cell death, namely apoptosis or necroptosis, depending on the cellular context [27, 28]. Both, apoptosis and necroptosis are considered programmed forms of cell death, i.e. they are induced in a regulated manner by ligation of receptors known to be capable of inducing cell death [98]. Caspase activity is required for apoptosis induction. On the contrary, necroptosis induction depends on the kinase activity of RIP1 and RIP3, and mainly, if not exclusively, occurs when caspases are absent or their activity is impaired [91, 98].

The TLR3 signalling pathway and its components are illustrated in Figure 2.

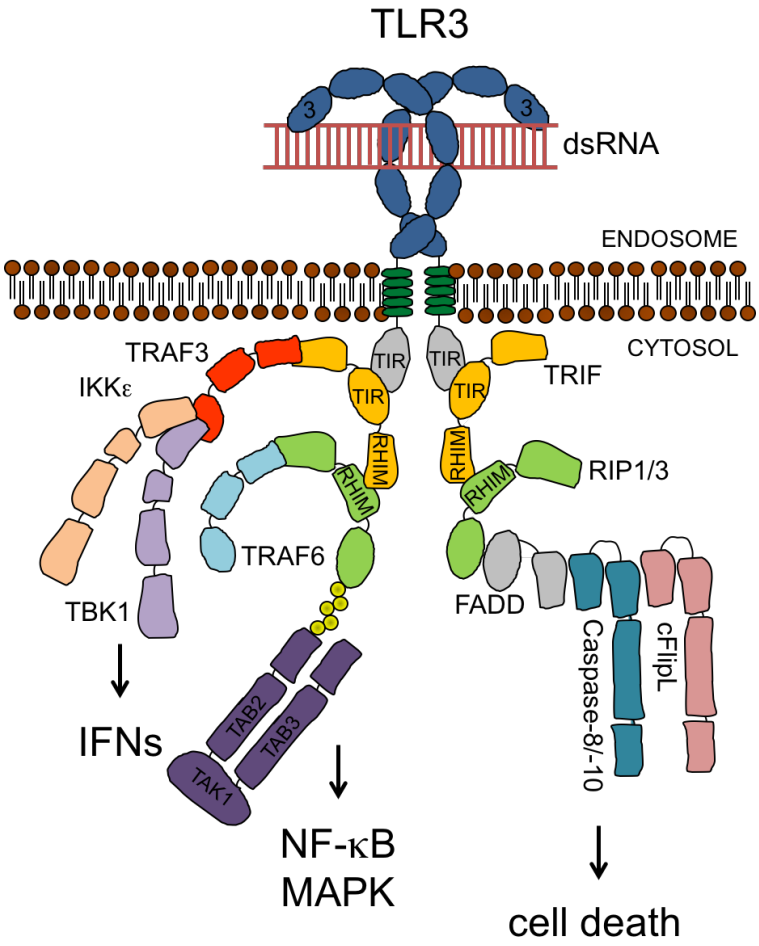

Figure 2. The TLR3 signalling pathway.

TLR3 is localised in the cell's endosomal compartment where it senses extracellular dsRNA that is delivered to the endosome by endocytosis. Upon ligand recognition, TLR3 dimerises resulting in recruitment of the adaptor protein TRIF via its TIR domain to the TIR domain of TLR3. Upon TRIF binding, further downstream signalling events are initiated: (i) RIP1 (or RIP3) is recruited via its RHIM domain to the RHIM domain of TRIF. Recruitment of TRAF6 results in ubiquitination of RIP1 thereby allowing recruitment of the TAK/TAB complex leading to induction of NF-κB and MAPK target genes. (ii) TRAF3 is recruited via TRIF resulting in induction of IFNs via recruitment of IKKε and TBK1. (iii) Recruitment of FADD to RIP1 leads to assembly of Caspase-8/-10 and cFLIP isoforms culminating in cell death induction.

TLR, toll-like receptor; ds, double-stranded; RNA, ribonucleic acid; TRIF, TIR-domain-containing adapter-inducing IFN-β; TIR, Toll-IL-1 receptor; RIP, receptor interacting protein; RHIM, RIP homotypic interaction motif; TRAF, TNF-receptor associated factor; TAK, TGF β-activated kinase; TAB, TAK binding protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK, mitogen-activated protein kinase; IFNs, interferons; IKK, IκB kinase; TBK1, TANK-binding kinase 1; FADD, Fas-associated protein with death domain; cFLIP, cellular FLICE-like inhibitory protein.

3.2 UBIQUITINATION

Ubiquitination (also known as ubiquitylation or ubiquitinylation) is an energy-dependent, post-translational modification process in which the ~ 8 kilodalton (kDa) protein ubiquitin is covalently attached to one or more amino acid residues of a substrate protein [114]. Ubiquitin molecules are preferentially attached to lysine (K) residues but also other amino acid residues can serve as adapters for ubiquitin molecules [13, 105, 109]. The process of ubiquitination is a sequence of three events known as activation, conjugation and ligation, involving three different types of enzymes: the first step is adenosine triphosphate-dependent and carried out by a ubiquitin-activating enzyme (E1) resulting in formation of a thioester linkage between ubiquitin and the E1 [114]. The next step catalyses the transfer of ubiquitin from the E1 to the active-site cysteine of a ubiquitin-conjugating enzyme (E2) [114]. The third and final step of ubiquitination is executed by an E3 ubiquitin ligase (E3) resulting in the formation of a(n) (iso)peptide bond between the carboxy terminal glycine of ubiquitin and an amino acid residue of a target protein [20, 39]. The substrate protein can also be ubiquitin itself, which will result in formation of a ubiquitin-linkage or di-ubiquitin [114]. Ubiquitin has seven K residues, opening the possibility of forming seven different linkage types. Depending on the localisation of the K residue they are termed K6, K11, K27, K29, K33, K48 and K63 [114]. In addition, a donor ubiquitin can also be attached to an acceptor ubiquitin via the amino terminal methionine (M1) resulting in formation of M1- or linear linkages [51], reviewed in [114].

Different functions can be attributed to the different linkage types [52]. The well-studied K48- and K63-linkages target proteins for proteasomal degradation and are required for cell signalling events, respectively [18, 19]. Functions attributed to the other, so-called ‘atypical’ ubiquitin linkages are very diverse. K6-linkages have been implicated in DNA damage responses [68, 72, 110] and K11-linkages were shown to mediate proteasomal degradation during mitosis, but also to play a role in membrane trafficking and, most recently, in TNF signalling [9, 24, 32, 34, 108]. K27-linkages were identified to be involved in mitochondrial damage responses [31, 33]. Both, K29- and K33-linkages were implicated in the regulation of AMP-activated protein kinase (AMPK)-related protein kinases [1]. K33-linkages were further shown to be important for the regulation of T cell receptor (TCR) responses [44]. M1- or linear ubiquitin linkages are exclusively mediated by an E3 called linear ubiquitin

chain assembly complex (LUBAC). This linkage type has been implicated in the regulation of signalling pathways activated by various ligands, amongst them TNF, IL-1β, cluster of differentiation (CD) 40 ligand, muramyl dipeptide and LPS [21, 32, 35, 45, 96, 97], reviewed in [114]. The different linkage types and their yet identified biological functions are summarised in Figure 3, adapted from [114].

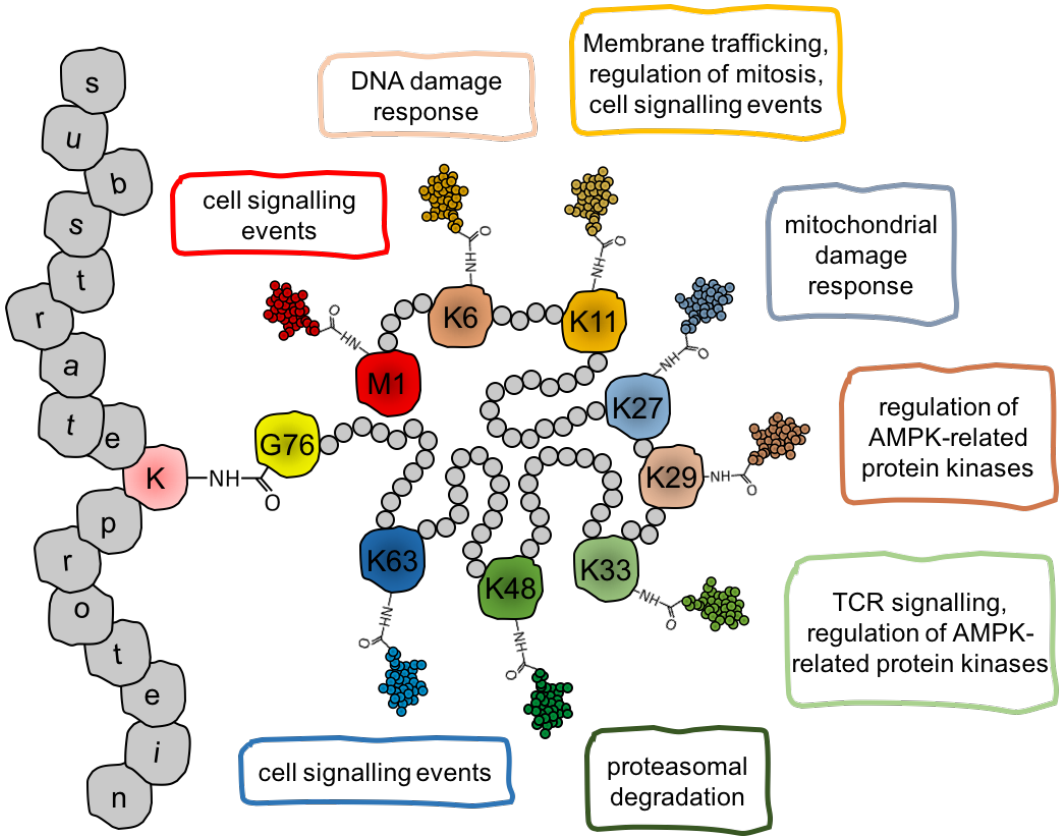

Figure 3. The different inter-ubiquitin linkage types and their biological functions.

The ~ 8 kDa protein ubiquitin is attached to a substrate protein during an energy-dependent process involving three different types of enzymes called E1, E2 and E3 resulting in formation of a(n) (iso)peptide bond between the carboxy-terminal G of ubiquitin and an amino acid residue of a target protein, in most cases a K residue. Ubiquitin itself comprises seven internal K residues via which inter-ubiquitin linkages can be formed, namely K6-, K11-, K27-, K29-, K33-, K48- and K63-linkages. In addition, a di-ubiquitin can also be formed via the amino terminal M resulting in M1- or linear linkages. The well-studied K48- and K63-linkages have been implicated in proteasomal degradation and cell signalling events, respectively. All other linkage types have not been studied in much detail, yet certain biological functions have been attributed to these linkage types as summarised above. This figure was adopted from [114].

kDa, kilodalton; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, E3 ubiquitin ligase; G, glycine; K, lysine; M, methionine; AMPK, AMP-activated protein kinase; TCR, T cell receptor; DNA, deoxyribonucleic acid.

3.2.1 LINEAR UBIQUITINATION

In 2006, M1- or linear linkages were identified to be catalysed by a complex called LUBAC, consisting of the two E3 ubiquitin ligases heme-oxidised IRP2 ubiquitin ligase-1 (HOIL-1) and HOIL-1-interacting protein (HOIP) [51]. Both, HOIL-1 and HOIP belong to the family of really interesting new gene (RING) – between RING – RING (RBR) E3 ubiquitin ligases which is defined by the catalytically active RBR domain mediating linkage formation [89]. Although both, HOIL-1 and HOIP possess an RBR domain, recent studies identified HOIP, and not HOIL-1, being the catalytically active LUBAC component mediating linear linkage formation [32, 51]. In 2011, three independent groups identified SHANK-associated RBCK1 homology-domain-interacting protein (SHARPIN) as third component of LUBAC making it a tripartite complex [32, 45, 97]. To date, LUBAC remains the sole complex known to assemble linear ubiquitin linkages under native conditions.

A physiological function of this complex was not discovered until 2009, when Tokunaga *et al.* identified LUBAC to be required for IL-1β-induced NF-κB activation [96]. In the very same year, Haas *et al.* discovered that LUBAC forms part of the native TNF-receptor (TNFR) 1-signalling complex (SC) thereby regulating TNF-mediated gene activation whilst preventing TNF-induced cell death [35].

Since then, LUBAC has been shown to be essential for the regulation of signalling processes upon stimulation with different stimuli [21, 32, 35, 45, 79, 96, 97].

3.2.2 LUBAC DEFICIENCY *IN VIVO*

3.2.2.1 SHARPIN DEFICIENCY

Mice lacking SHARPIN protein expression due to a spontaneous mutation in the *Sharpin* gene develop severe chronic skin inflammation at 5 to 6 weeks of age, and have therefore been termed chronic proliferative dermatitis mice (*cpdm*) [42]. The inheritance of the mutation occurs autosomal recessive. Lesions are mainly located on the animal's trunk and are defined by epidermal hyperplasia, hyper- and parakeratosis and hyperproliferation [42]. Furthermore, single cell necrosis and formation of microabscesses can be found in the epidermis of *cpdm* mice [42]. Granulocyte, eosinophil and macrophage infiltration is an additional characteristic of *cpdm* epidermis [42]. Of note, Gerlach *et al.* discovered that TNF deficiency rescued *cpdm* dermatitis, and suggested that skin inflammation is a consequence of aberrant TNF-induced cell death in the absence of SHARPIN [32]. Recently, two

independent groups provided the genetic proof for this hypothesis as genetic ablation of cell death pathway components was sufficient to prevent dermatitis development in *cpdm* mice [53, 80].

Apart from their overt skin inflammation, *cpdm* mice suffer from similar lesions in the mouth, esophagus and fore stomach. In addition, immune cell infiltration of liver, lung and joints has been described [42] and perturbed architecture of lymphoid tissues, absence of Peyer's patches and splenomegaly are additional characteristics of the *cpdm* phenotype [43, 88].

3.2.2.2 HOIL-1 DEFICIENCY

Mice lacking HOIL-1 have been described to not present with an overt phenotype [96]. Interestingly, however, it has previously been described that loss-of-expression and loss-of-function mutations in the *HOIL1* gene in human patients resulted in a controversial phenotype defined by chronic autoinflammation, immunodeficiency with recurrent invasive bacterial infections and muscular amylopectinosis [7]. More recently, two independent studies identified truncating mutations in *HOIL1* to be causative for a phenotype encompassing signs of amylopectinosis, muscular weakness and progressive cardiomyopathy [71, 103].

3.2.2.3 HOIP DEFICIENCY

In 2013, two independent groups suggested that mice expressing a mutant form of HOIP lacking its catalytic activity, and thus the capability of forming linear linkages, die around embryonic day 11 [25, 86].

More recently, Peltzer *et al.* showed that HOIP-deficient mice die at E 10.5 due to excessive endothelial cell death induced by TNFR1 during embryogenesis [74] establishing the importance of linear ubiquitination for the prevention of cell death during embryonic development.

In addition, an analysis of mice lacking HOIP's catalytic activity specifically in B cells revealed that linear ubiquitin linkages are required for B cell development and activation [86].

In humans, HOIP deficiency has previously been shown to result in a phenotype consisting of immunodeficiency and autoinflammation similar to that of HOIL-1-deficient patients [8].

3.3 CENTRAL QUESTION

Deregulation of TLR signalling pathways has been implicated in the pathogenesis of a number of different disorders [49, 81]. DAMPs released from damaged or dying cells are thought to contribute to disease development [81]. The fact that deregulated TLR signalling can result in a host-harming outcome drew attention to identification of crucial regulators of TLR signalling pathways. Different molecular mechanisms have been identified which ensure tight control of TLR signalling, one of them being the attachment of post-translational modifications (PTMs) to proteins [14, 69].

Whilst TLR3 is a crucial mediator of various physiological processes ensuring host-protective immunity and proper wound healing [54, 112], the outcome of TLR3 signalling can also be deleterious resulting in inflammation, increased susceptibility to certain viral infections and, consequently, increased host lethality [12, 16, 55]. Thus, tight regulation of TLR3 signalling is required to mediate host-protective immunity whilst preventing damaging hyperinflammation. It is therefore important to fully understand how the TLR3 signalling output is differentially regulated, i.e. which factors are responsible for achieving a balanced and adequate response to TLR3 stimulation. One such factor is represented by PTMs such as phosphorylation and ubiquitination which have both been implicated to play a role in the regulation of TLR3 signalling [100]. As it is unclear why TLR3 signalling can be both, host-protective or host-harming, it is likely that there are additional, yet unidentified, factors that control the different TLR3 signalling outputs and may be altered under pathophysiological conditions.

Recently, one such factor has been identified for the TNF signalling pathway. Linear ubiquitination mediated by LUBAC crucially regulates innate immune signalling by TNF. Absence of LUBAC results in markedly reduced TNF-induced cytokine signalling but enhanced TNF-induced cell death [32, 35]. Extrapolating this information to the TLR3 signalling pathway and its controversial role in viral infection and tissue damage, the question arose whether linear ubiquitination may be a yet unidentified, but key player in the regulation of TLR3 signalling.

Thus, the central question of this thesis is whether linear ubiquitination plays a role in TLR3 signalling and, if yes, if TLR3 signalling requires presence of linear ubiquitination to maintain a balanced TLR3 signalling output thereby providing a possible explanation why TLR3 signalling can be host-protective in some scenarios whilst being host-harming in others.

4 MATERIAL & METHODS

4.1 ANTIBODIES

Table 1. Antibodies used in experimental set-ups within this MD thesis project.

α-Actin	mouse IgG1	Sigma-Aldrich, Munich, Germany
α-Caspase-3	goat	R&D Systems, Minneapolis, US
α-Caspase-8	goat	Santa Cruz Biotech., Dallas, US
α-Caspase-8 (C15)	mouse IgG2b	Enzo Life Sciences, Farmingdale, US
α-cFLIP (NF6)	mouse IgG1	Enzo Life Sciences, Farmingdale, US
α-cIAP1/2	mouse IgG2a	R&D Systems, Minneapolis, US
α-ERK	rabbit	Cell Signaling, Danvers, US
α-FADD	mouse IgG1	Enzo Life Sciences, Farmingdale, US
α-HOIL-1 (2D2)	mouse IgG2a	selfmade (generated from 8-week-old BALBc mice immunised with recombinant full-length GST-tagged HOIL-1 protein) [35]
α-human-HOIP	rabbit	Sigma-Aldrich, Munich, Germany
α-IκBα	rabbit	Cell Signaling, Danvers, US
α-JNK	rabbit	Cell Signaling, Danvers, US
α-mouse-HOIP	rabbit	custom-made by Thermo Fisher Scientific, Waltham, US
α-PARP	mouse IgG1	BD Bioscience, Franklin Lakes, US
α-pERK	rabbit	Cell Signaling, Danvers, US
α-pIRF3 (Ser396)	rabbit	Cell Signaling, Danvers, US
α-pIκBα	mouse IgG1	Cell Signaling, Danvers, US
α-pJNK	rabbit	Cell Signaling, Danvers, US
α-RIP1	mouse IgG2a	BD Bioscience, Franklin Lakes, US
α-SHARPIN	mouse IgG1	selfmade (generated from 8-week-old BALBc x C57BL6 mice immunised with a KHL-coupled peptide (GPDAEAQLRRLQLSADC)) [32]
α-SHARPIN	rabbit	Proteintech, Chicago, US

α-TLR3	mouse IgG1	kindly provided by S. Lebecque, Lyon [27]
α-TRAF6 (C-term)	rabbit	Epitomics, Burlingame, US
pTBK1	rabbit	Cell Signaling, Danvers, US
GAPDH	mouse IgG1	Abcam, Cambridge, UK
Flag	mouse IgG1	Sigma-Aldrich, Munich, Germany
M1 (1E3)	rabbit	Merck-Millipore, Darmstadt, Germany
TRIF	rabbit	Cell Signaling, Danvers, US
NEMO (FL419)	rabbit	Santa Cruz Biotech., Dallas, US
goat α-mouse IgG1	HRP	Southern Biotech, Birmingham, US
goat α-rabbit IgG (H+L)	HRP	Southern Biotech, Birmingham, US
goat α-mouse IgG2a	HRP	Southern Biotech, Birmingham, US
goat α-mouse IgG2b	HRP	Southern Biotech, Birmingham, US

4.2 CONSUMABLES

Table 2. Consumables used in experimental set-ups within this MD thesis project.

6 well plate	TPP, Trasadingen, Switzerland
12 well plate	TPP, Trasadingen, Switzerland
96 well plate	TPP, Trasadingen, Switzerland
1-200 µl pipette tips for gel loading	VWR, Radnor, US
1-10µl tips	STARLAB (UK) Ltd, Milton Keynes, UK
1-200µl tips	STARLAB (UK) Ltd, Milton Keynes, UK
1250µl tips	STARLAB (UK) Ltd, Milton Keynes, UK
75 cm² flasks with filter cap	TPP, Trasadingen, Switzerland
150 cm² flasks with filter cap	TPP, Trasadingen, Switzerland
5ml serological pipettes	STARLAB (UK) Ltd, Milton Keynes, UK
10ml serological pipettes	STARLAB (UK) Ltd, Milton Keynes, UK
25ml serological pipettes	STARLAB (UK) Ltd, Milton Keynes, UK
Cell strainer, 100 µm	BD Bioscience, Franklin Lakes, US
DMEM	Invitrogen, Karlsruhe, Germany

Dulbecco's PBS (DPBS)	Invitrogen, Karlsruhe, Germany
Falcons 15ml	TPP, Trasadingen, Switzerland
Falcons 50ml	TPP, Trasadingen, Switzerland
MEM Eagle-EBSS	Lonza, Basel, Switzerland
MOPS buffer	Invitrogen, Karlsruhe, Germany
Penicillin-Streptomycin	Invitrogen, Karlsruhe, Germany
Reagent reservoir	VWR, Radnor, US
SeeBlue® Plus2 Pre-Stained marker	Invitrogen, Karlsruhe, Germany
SYBR® Safe DNA Gel Stain	Life technologies, Carlsbad, US
Tissue culture dish Ø 60 mm	TPP, Trasadingen, Switzerland
Tissue culture dish Ø 100 mm	TPP, Trasadingen, Switzerland
Tissue culture dish Ø 150 mm	TPP, Trasadingen, Switzerland
Trypsin-EDTA (10x)	Life technologies, Carlsbad, US
Borax Anhydrous	Sigma-Aldrich, Munich, Germany
8-Strip PCR caps	STARLAB (UK) Ltd, Milton Keynes, UK
0.2ml 8-Strip PCR tubes	STARLAB (UK) Ltd, Milton Keynes, UK
FACS tubes	BD Bioscience, Oxford, UK
Cell scraper	TPP, Trasadingen, Switzerland
OptiMEM I (reduced-serum medium)	Invitrogen, Karlsruhe, Germany
1.5 ml Eppendorf savelock tubes	Eppendorf, Hamburg, Germany
2.0 ml Eppendorf savelock tubes	Eppendorf, Hamburg, Germany
Amersham Hyperfilm ECL	GE Healthcare, Munich, Germany
Whatman paper	Whatman, London, UK
ECL membrane hybond (0.45µm)	GE Healthcare, Munich, Germany
2-log DNA Ladder (0.1–10.0kb)	New England Biolabs, Ipswich, US
PBS, pH 7.4	Invitrogen, Karlsruhe, Germany
NuPAGE® Novex 4-12% Bis-Tris gel	Invitrogen, Karlsruhe, Germany
cryogenic vials (1.5ml)	Nalgene, Rochester, US
Trypsin	Mediatech Inc., Manassas, US
Fetal calf serum	Sigma-Aldrich, Munich, Germany

100x Antibiotic-Antimycotic	Invitrogen, Karlsruhe, Germany
Corning® 96 well plates, opaque bottom	Corning, Corning, US
keratinocyte serum free media	Invitrogen, Karlsruhe, Germany
TrypLE express trypsin	Invitrogen, Karlsruhe, Germany

4.3 CHEMICALS

Table 3. Chemicals used in experimental set-ups within this MD thesis project.

Sodium azide	Sigma-Aldrich, Munich, Germany
Trizma® base	Sigma-Aldrich, Munich, Germany
2-Propanol	Merck, Darmstadt, Germany
Ethanol	VWR, Radnor, US
Propidium iodide	Sigma-Aldrich, Munich, Germany
(Z)-4-Hydroxytamoxifen (4-OHT)	Sigma-Aldrich, Munich, Germany
TWEEN® 20	Sigma-Aldrich, Munich, Germany
Dimethylsulfoxide (DMSO)	Sigma-Aldrich, Munich, Germany
Powdered milk	Roth, Karlsruhe, Germany
Glycine	Sigma-Aldrich, Munich, Germany
EDTA	Sigma-Aldrich, Munich, Germany
Methanol	VWR, Radnor, US
NaCl	Roth, Karlsruhe, Germany
Na_2HPO_4	Sigma Aldrich, Munich, Germany
KCl	Sigma-Aldrich, Munich, Germany
KH_2PO_4	Sigma-Aldrich, Munich, Germany
Glycerol	VWR, Radnor, US
Triton-X 100	Sigma-Aldrich, Munich, Germany
$CaCl_2$	Sigma-Aldrich, Munich, Germany
$MgCl_2$	Sigma-Aldrich, Munich, Germany
Tris-HCl	Sigma-Aldrich, Munich, Germany
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Munich, Germany

Formalin	Sigma-Aldrich, Munich, Germany

4.4 REAGENTS

Table 4. Reagents used in experimental set-ups within this MD thesis project.

MYCOALERT assay control set	Lonza, Basel, Switzerland
MYCOALERT detection kit	Lonza, Basel, Switzerland
Western Lightning® ECL	Perkin Elmer, Wellesley, US
Western Lightning® Plus-ECL	Perkin Elmer, Wellesley, US
Super Signal West Femto	Life technologies, Carlsbad, US
Phosphatase Inhibitor Cocktail 2	Sigma-Aldrich, Munich, Germany
5 PRIME MasterMix	5 Prime, Wilford, UK
7-Cl-O-Nec-1	Merck Millipore, Darmstadt, Germany
BCA Protein Assay Kit	Thermo Fisher Scientific, Waltham, US
Poly(I:C) HMW	InvivoGen, San Diego, US
Reagent Diluent Concentrate 2	R&D Systems, Minneapolis, US
Smac Mimetic 083 (SM083)	kindly provided by M. Bolognesi
Substrate Reagent Pack	R&D Systems, Minneapolis, US
z-VAD(OMe)-FMK	Abcam, Cambridge, UK
Lipofectamine 2000 transfection reagent	Invitrogen, Karlsruhe, Germany
human αCCL5/RANTES DuoSet ELISA	R&D Systems, Minneapolis, US
human α-IL-6 DuoSet ELISA	R&D Systems, Minneapolis, US
human α-IL-8 DuoSet ELISA	R&D Systems, Minneapolis, US
human α-TNF DuoSet ELISA	R&D Systems, Minneapolis, US
murine αCCL5/RANTES DuoSet ELISA	R&D Systems, Minneapolis, US
murine αCCL20 DuoSet ELISA	R&D Systems, Minneapolis, US
human IFN-β VeriKine ELISA kit	PBL Assay Science, Piscataway Township, US
siGENOME Non-Targeting siRNA #3	Thermo Fisher Scientific, Waltham, US
ProteinG Sepharose	GE Healthcare, Munich, Germany
human HOIP siRNA (sequence #4)	Thermo Fisher Scientific, Waltham, US

human HOIL-1 siRNA (ON-TARGETplus SMARTpool)	Thermo Fisher Scientific, Waltham, US
human TLR3 siRNA (ON-TARGETplus sequences #5-8)	Thermo Fisher Scientific, Waltham, US
human SHARPIN siRNA (ON-TARGETplus SMARTpool)	Thermo Fisher Scientific, Waltham, US
CellTiter-Glo® assay	Promega, Madison, US
BglI	New England Biolabs, Ipswich, US
NEBuffer 3.1	New England Biolabs, Ipswich, US
M2 beads	Sigma-Aldrich, Munich, Germany
Protein G Sepharose 4 Fast Flow	GE Healthcare, Munich, Germany
Dispase II	Sigma-Aldrich, Munich, Germany
Agarose	Sigma-Aldrich, Munich, Germany
Chelex-100 beads	Sigma-Aldrich, Munich, Germany
Bovine serum albumine	Sigma-Aldrich, Munich, Germany
Puromycin	Sigma-Aldrich, Munich, Germany

4.5 EQUIPMENT & DEVICES

Table 5. Equipment & devices used in experimental set-ups within this MD thesis project.

Multiskan Ascent Platereader	Thermo Fisher Scientific, Waltham, US
BD Accuri C6	BD Bioscience, Oxford, UK
Mastercyler	Eppendorf, Hamburg, Germany
Powerpac 3000	Bio-Rad, Hercules, US
Sonicator Q125	Qsonica, Newtown, US
Thermomixer comfort	Eppendorf, Hamburg, Germany
Mithras LB 940	Berthold Tech., Bad Wildbad, Germany
Axioskop 40	Zeiss, Oberkochen, Germany
MoFlo FACS	Beckman Coulter, Munich, Germany

4.6 BUFFERS

Table 6. Buffers used in experimental set-ups within this MD thesis project.

Blocking buffer	5% milk powder 0.05% TWEEN 20 in PBS
Freezing medium	90% FCS 10% DMSO
Propidium iodide (PI) buffer	1µg/ml PI in PBS
Stripping buffer	50mM Glycine in H_2O (pH 2.2)
Transfer buffer	192mM Glycine 25mM Tris-Base 0.01% EDTA 20% Methanol
Wash buffer	0.05% TWEEN 20 in PBS
PBS (pH 7.4)	137mM NaCl 8.1mM Na_2HPO_4 2.7mM KCl 1.5mM KH_2PO_4
Lysis buffer (western blot)	30mM Tris-Base (pH 7.4) 120mM NaCl 2mM EDTA 2mM KCl 10% Glycerol 1% Triton X-100 protease inhibitor phosphatase inhibitor

Lysis buffer (IP) – buffer 1	100mM NaCl 40mM Tris-HCl (pH 7.5) 1mM $CaCl_2$ 1mM $MgCl_2$ protease-inhibitor 0.5% Triton X-100
Lysis buffer (IP) – buffer 2	100mM NaCl 40mM Tris-HCl (pH 7.5) 1mM $CaCl_2$ 1mM $MgCl_2$ protease-inhibitor 1% Triton X-100 0.1% SDS
HiCa-Medium (1.3 mM Ca^{2+})	S-MEM (Lonza) 8% chelexed FCS 1% penicillin-streptomycin 1.3mM Calcium
LoCa-Medium (0.05 mM Ca^{2+})	S-MEM (Lonza) 8% chelexed FCS 1% penicillin-streptomycin
Alkaline lysis reagent (25mM NaOH/0.2mM EDTA), pH 12	1g NaOH 0.074g Na_2EDTA 1l H_2O
Tris HCl buffer (40mM Tris HCl), pH 5	6,3g Tris HCl 1l H_2O

4.7 MICE

Tlr3$^{-/-}$ (9675, B6N.129S1-Tlr3^{tm1Flv}/J), *cpdm* (7599, C57BL/KaLawRij-*Sharpin*^{cpdm}/RijSunJ) and Tamoxifen-inducible Cre (4682, B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J) mice were obtained from The Jackson Laboratory, Bar Harbor, US. *Hoip*-floxed mice were generated as previously described [74]. *Hoip*-floxed mice were crossed with transgenic mice expressing the Tamoxifen-inducible loxP-deleter Cre recombinase (4682) to generate Tamoxifen-inducible HOIP-deficient mice. TLR3-deficient mice (9675) were interbred with *cpdm*$^{+/-}$ mice (7599) to generate *Tlr3*$^{+/-}$.*cpdm*$^{+/-}$ mice. These were further interbred to generate wildtype (WT), *Tlr3*$^{-/-}$, *cpdm*$^{-/-}$ and *Tlr3*$^{-/-}$.*cpdm*$^{-/-}$ littermates. All mice were crossed for at least five generations before the experimental studies. All mice were typed by polymerase chain reaction (PCR) analysis. Colonies were fed ad libitum. All animal experiments were conducted under an appropriate UK project license in accordance with the regulations of UK home office for animal welfare according to the ASPA (Animals (Scientific Procedures) Act 1986).

4.8 CELL LINES

4.8.1 HACAT CELLS

HaCaT (human adult low calcium high temperature) cells are a spontaneously transformed aneuploid immortal keratinocyte line from adult human skin with full epidermal differentiation capacity that had first been described in 1988 [11].

4.8.2 HT29 CELLS

The human colorectal adenocarcinoma cell line HT29 was established in 1964 from the primary tumour of a 44-year-old Caucasian female with colorectal adenocarcinoma [102].

4.8.3 PHOENIX-AMPHO CELLS

Phoenix is a retrovirus producer cell line. The line is based on the HEK293T cell line, a human embryonic kidney cell line [73].

4.8.4 HELA CELLS

Henrietta Lacks (HeLa) cells are derived from the cervical cancer of Henrietta Lacks who died of her cancer in 1951 [87].

4.8.5 HEK293FT CELLS

The human HEK293FT cell line is derived from human embryonic kidney cells transformed with simian virus 40 large T antigen.

4.9 CULTURE OF CELL LINES

HaCaT, HeLa, Phoenix-AMPHO, HEK293FT and HT29 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Cells were kept in 5% CO_2-humidified atmosphere at 37°C and were split twice weekly. In order to split cells medium was taken off the cells, cells were rinsed once with 1x PBS, and 1x trypsin (2x trypsin for HaCaT cells) was added until cells started to detach from the surface. Trypsin was then inactivated by adding equal amounts of medium supplemented with 10% FCS, cells were then transferred to a 50ml tube and subsequently centrifuged at 1200 rounds per minute (rpm) for 5 minutes. Supernatant was discarded and cells were re-suspended in fresh medium supplemented with FCS. Cells were then split 1 in 10 by transferring the respective amount of cells to a new flask and fresh medium was added. All cell lines were tested on a regular basis and determined to be mycoplasma free using MycoAlert Mycoplasma Detection Kit from Lonza.

4.9.1 FREEZING OF CELLS

1.5 x 10^6 cells were re-suspended in freezing medium (FCS containing 10% dimethyl sulfoxide (DMSO)), transferred to a cryogenic vial and stored at -80°C in an isopropanol box for at least 24 hours (h) before being further transferred into a liquid nitrogen tank for long-term storage.

4.9.2 THAWING OF CELLS

Frozen cells were thawed by re-suspending them in warm medium and transferring them to a 15 ml tube. Cells were then centrifuged for 5 minutes at 1200 rpm. Supernatant including DMSO was discarded and cells were re-suspended in fresh medium and transferred to a tissue culture flask. Medium was changed the following day to remove dead cells.

4.10 PRIMARY MURINE KERATINOCYTES (PMKS)

4.10.1 ISOLATION AND CULTURE OF PMKS FROM NEWBORN MICE

Primary murine keratinocytes (PMKs) were isolated according to the protocol established and described in detail by Lichti *et al.* from newborn mice at the age of 0 – 2 days [61]. In summary, newborn mice were humanely killed. Mice were then rinsed with ethanol twice for 2 minutes before the skin was carefully separated from the rest of the body in one piece. The skin was floated on trypsin at 4°C overnight. The following day, epidermis was separated from dermis, and keratinocytes were

isolated using high-calcium medium (HiCa medium = 1.3mM Ca^{2+}). After isolation, keratinocytes were counted and seeded at equal numbers in low-calcium medium (LoCa medium = 0.05mM Ca^{2+}). 24h after plating, dead cells were removed by washing once with DPBS and fresh LoCa medium was added. Medium was changed every other day until cells reached 70 – 90% confluency, usually between day 3 – 5 after plating. For PMKs from Tamoxifen-inducible Cre *Hoip*$^{fl/fl}$ newborn mice, 4-hydroxy tamoxifen (4-OHT) was added to the medium at a concentration of 1µM directly after isolation for at least 72h. 24h prior to stimulation with poly(I:C), cells were washed with DPBS to remove residual 4-OHT and fresh medium without 4-OHT was added.

4.10.2 Isolation and culture of PMKs from adult mice

Tails of mice were wiped with 70% Ethanol, then removed at base and carefully incised using a scalpel making a longitudinal incision down the tail, skin was subsequently removed from the tails using tweezers. Skin was then incubated overnight at 4°C in keratinocyte serum free medium with dispase II at a final concentration of 2.1U/ml to cleave epidermal-dermal junctions. The following day, epidermis was separated from dermis. Epidermis was then incubated for 15 minutes in 2ml TrypLE express trypsin to isolate keratinocytes. After addition of medium, cells were centrifuged at 600rpm for 10 minutes. Supernatant was removed and the pellet was re-suspended in medium. Floating dead cells were removed the following day. Keratinocytes were then grown to 70-90% confluency until further experiments were performed, usually after 3-5 additional days in culture.

4.10.3 FCS preparation for isolation and culture of PMKs

For preparation of chelexed FCS 3.3g of Chelex 100 beads/100ml FCS were used. Chelex 100 beads were re-suspended in 0.1M NaH_2PO_4 (pH 7) and mixed by shaking for 30 minutes at room temperature. Subsequently, beads were separated from the supernatant by centrifugation for 1 minute at 1000rpm, supernatant was discarded. The remaining beads were re-suspended in 25ml 70% Ethanol and mixed by shaking for 30 minutes at room temperature. Next, beads were centrifuged for 1 minute at 1000rpm, afterwards supernatant was discarded. Beads were then re-suspended in DPBS. 4ml beads in DPBS were added to 40ml FCS. Beads and FCS were gently mixed on wheel for 8h at 4°C. FCS was separated from beads by

centrifugation at 1000rpm for 1 minute. Chelexed FCS was collected, sterile-filtered and stored at -20°C until further use.

4.11 HaCaT cells stably expressing HOIP or HOIPmut

Sequences coding for HOIP were cloned into the transfer vector murine stem cell virus (MSCV)-internal ribosomal entry site (IRES)_enhanced green fluorescent protein (EGFP). Vectors containing either HOIP variants or non-coding sequence (empty vector) were transfected into Phoenix-AMPHO cells using Lipofectamine 2000 according to the manufacturer's instructions. The following day, medium was replaced and retrovirus-containing supernatant was collected after an additional 24h. The supernatant was supplemented with Polybrene (6µg/ml) and added to HaCaT cells seeded one day before in 6 wells at a seeding density of 2 x 10^5 cells/well. Subsequently, cells were centrifuged at 1250 x g for 45 minutes. Finally, successfully reconstituted GFP-positive cells were isolated by cell sorting.

4.12 Generation of HOIP-deficient cells

For production of HOIP-deficient HeLa and HaCaT cells, Lenti clustered regularly interspaced short palindromic repeats (CRISPR) version 2 vector, from Feng Zhang (Addgene plasmid # 52961) [85], targeting the following sequence was used: 5'- CGAGATGTGCTGCGATTATA.

Lentiviral particles were produced in HEK293FT cells upon co-transfection of LentiCRISPR vector together with packaging vectors psPAX2 and pMD2G. Cells were infected as in the case of retroviral transduction and subjected to puromycin (2µg/ml) selection for one week. Single cell cloning was achieved by limiting dilution and HOIP-deficient cells were validated by western blotting.

4.13 Cell lines stably expressing Flag-tagged TLR3

For tagging of exogenously expressed proteins a C-terminal tandem affinity purification-tag consisting of 2x Strep-tag II sequence followed by a PreScission cleavage site and 1x Flag-tag was used. Coding sequences for TLR3 fused at the C-terminus to a tandem affinity purification-tag were inserted into the retroviral MSCV-IRES_EGFP vector. These vectors were transfected using Lipofectamine 2000 in Phoenix-AMPHO cells. One day after transfection, the medium was replaced and subsequently collected at day three. Viral supernatants were passed through 0.45µm filter, added to HaCaT or HeLa cells in the presence of Polybrene

at 6µg/ml before cells were subjected to spin-infection (2500rpm, 45 minutes, 30°C). EGFP-positive cells were sorted to more than 95% purity.

4.14 CELL VIABILITY ASSAY

Cell viability was determined using CellTiter-Glo® assay according to the manufacturer's instructions.

4.15 CELL DEATH ASSAY

Cell death was determined by fluorescence activated cell sorting (FACS) analysis. Briefly, cells were seeded in 12 wells at a seeding density of 0.5×10^4 cells/well. The following day cells were stimulated. 24h later supernatants were transferred to FACS tubes. Cells were washed once with 1x PBS and trypsin was added to the cells. Upon detachment, cells were re-suspended in medium and collected in the respective FACS tube. FACS tubes were centrifuged for 5 minutes at 1800rpm. Supernatants were carefully discarded and pellets were re-suspended in PI buffer. After an incubation of at least 15 minutes at 4°C, samples were measured by FACS for PI positivity. Percentage of PI-positive cells equates to the number of dead cells.

4.16 WESTERN BLOT ANALYSIS

Cells were seeded in 6 wells at a seeding density of 3×10^5 cells/well. The next day, cells were stimulated or left untreated. Cells were washed twice with ice-cold PBS and then lysed in lysis buffer (western blot). Protein concentration was determined and lysates were subsequently denatured in reducing sample buffer at 95°C for 10 minutes before separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent blotting on membranes. After the transfer, membranes were blocked in blocking buffer for at least 1h at room temperature. Incubation with the primary antibodies was either performed at 4°C overnight or for 1h at room temperature. Membranes were washed for 3x10 minutes using wash buffer before incubation with the secondary antibody for 1h at room temperature. Prior to re-probing with the next primary antibody, membranes were stripped with stripping buffer and blocked again in blocking buffer.

4.17 IMMUNOPRECIPITATION (IP)

0.5–1x10^7 cells were seeded per condition. The following day, cells were stimulated as indicated in 8ml DMEM supplemented with 5% FCS. Cells were washed twice with ice-cold PBS. Next, cells were harvested in 0.5ml lysis buffer (IP) – buffer 1. Cells were lysed for 20 minutes on wheel at 4°C. Lysates were subsequently cleared by centrifugation at 13,000rpm for 10 minutes and transferred to a new eppendorf tube. The remaining pellets were re-suspended in 0.5ml of lysis buffer (IP) – buffer 2 and subjected to sonification prior to centrifugation at 13,000rpm for 20 minutes. The cleared lysate was added to the lysate of centrifugation step 1. 30µl of lysates were subsequently stored at -20°C for analysis of whole cell lysates. 10µl M2-beads for Flag-IP or 10µl Protein-G beads that have been pre-coupled to either Caspase-8 antibody or to SHARPIN antibody were added to each sample. Samples and beads were incubated at 4°C for at least 24h. Samples were washed at least 5 times with lysis buffer (IP) containing 1% Triton X-100. After the last centrifugation step, beads were sucked dry and re-suspended in 2x reducing sample buffer, boiled for 10 minutes at 95°C and separated by SDS-PAGE prior to subsequent analysis by western blot.

4.18 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Cells were stimulated as indicated for 4h (IFN-β) or for 24h (IL-6, IL-8, TNF, chemokine (C-C motif) ligand (CCL) 5 and CCL20). Cell supernatants were subsequently collected and stored at -20°C until further analysis. Cell viability was determined in parallel using CellTiter-Glo® assay. The respective ELISA was performed according to manufacturer's instructions.

4.19 RNA INTERFERENCE

Cells were plated in 6-well plates at a seeding density of 1.2 x 10^5 cells/well 24h prior to transfection with the respective small interfering RNAs (siRNAs) using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. Cells were reseeded 48h after transfection and subjected to further experimental analysis 24h later.

4.20 GENOTYPING

Ear samples were lysed using 75µl Alkaline lysis reagent per sample. Samples were heated to 95°C for 5 – 30 minutes. 75µl Tris HCl buffer was added to neutralise pH.

4.20.1 *CPDM* PCR

Primers (sense primer AGACCCTGCCTCACCTGTGGTGCTC and antisense primer GAAAGTTGATTCCAGAATCGGGCCCACACTCGCCGCCC) were added together with 5PRIME Mastermix to each sample and the polymerase chain reaction (PCR) was run in the PCR machine for 40 cycles (15 minutes at 95°C, 30 seconds at 94°C, 30 seconds at 61.3°C and 30 seconds at 72°C). A restriction enzyme specifically cleaving the mutated allel was added (0.5µl of Bgl I and 1.5µl of NEBuffer 3.1 per sample) and samples were incubated for at least 3h at 37°C. Afterwards samples were run in a 3-4% agarose gel with Borax buffer.

Expected bands: WT = 196 base pairs (bp), *cpdm* = 161 bp.

4.20.2 *TLR3* PCR

Primers (mutant primer GCC AGA GGC CAC TTG TGT AG, WT primer GCA ACC CTT TCA AAA ACC AG and common primer AAT TCA TCA GTG CCA TGA GTT T) were added together with 5PRIME Mastermix to each sample and PCR was run (1 minute at 94°C, 35 x (30 seconds at 94°C, 1 minute at 60°C, 1 minute at 72°C), 2 minutes at 72°C). Afterwards samples were run in a 1-2% agarose gel with Borax buffer.

Expected bands: WT = 341 bp, mutant = 208 bp.

4.21 SEVERITY SCORING OF DERMATITIS

Severity of dermatitis was assessed at the age of 8 weeks on 6 different body parts (head, neck, anterior trunk, posterior trunk, forelegs and hind legs) in a blinded manner. The score was obtained by grading each of the six above-named body parts. Lesion absent (0 points) or lesion detectable (1 point), accounting to a maximum of 6 points/mouse. This score was adopted from the Basic Clinical Scoring System score [90].

4.22 HISTOLOGY

For preparation of skin, liver and spleen tissue sections, mice were sacrificed according to Guidance on Operation of Animals [Scientific Procedures] Act 1986. Skin, liver and spleen were removed, fixed in 10% formalin for one week and then transferred to 70% ethanol. Paraffin embedding, preparation of sections and haematoxylin & eosin (H&E) staining of tissues were performed by histological services at the National Heart & Lung Institute, Imperial College London, South Kensington Campus, London SW7 2AZ.

4.23 STATISTICAL ANALYSIS

Data were analysed using GraphPad Prism 6 software (GraphPad Software). Statistical significance between groups was determined using unpaired Student's t-test. A p-value of < 0.05 was considered significant and indicated with *, $p < 0.01$ = ** and $p < 0.001$ = ***.

5 RESULTS

5.1 TLR3 SIGNALLING IN HACAT CELLS

To address the question whether linear ubiquitination plays a role in the regulation of TLR3 signalling, a cellular system was required in which poly(I:C) induces all known TLR3 signalling outputs. The immortalised human keratinocyte line, HaCaT, was chosen for further analysis. To control whether the effect exerted by poly(I:C) on HaCaT cells was due to stimulation of TLR3, RNA interference (RNAi)-mediated suppression of TLR3 was performed.

5.1.1 POLY(I:C) INDUCES GENE ACTIVATION IN A TLR3-DEPENDENT MANNER

HaCaT cells, either control-transfected or transfected with siRNA targeting TLR3, were stimulated and gene activatory signalling was assessed by western blot (Figure 4). This analysis revealed that poly(I:C) stimulation induced phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor alpha (IκBα) and its subsequent degradation over time in control-transfected cells (Figure 4). In addition, poly(I:C) induced phosphorylation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) (Figure 4). IRF3, involved in the transcription of IFNs, was also phosphorylated following stimulation with poly(I:C). Of note, RNAi-mediated suppression of TLR3 abolished poly(I:C)-induced activation of the above-named signalling pathways indicating that presence of TLR3 was required to mediate these effects (Figure 4). Analysis of TLR3 protein expression confirmed that it was largely diminished in HaCaT cells transfected with siRNA targeting TLR3 as compared to control-transfected cells.

Thus, presence of TLR3 is essential for poly(I:C)-induced activation of NF-κB, MAPK and IRF3 signalling in HaCaT cells.

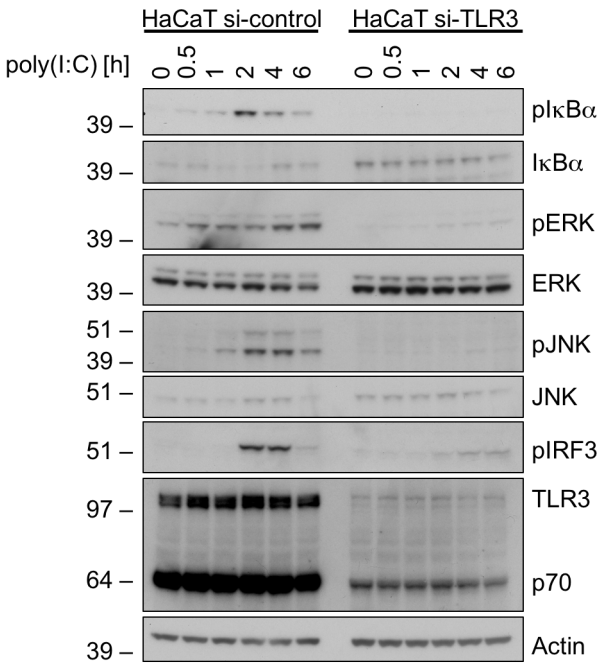

Figure 4. Poly(I:C) induces gene activation in HaCaT cells in a TLR3-dependent manner.

HaCaT cells were transfected with control or TLR3 siRNA. 72h after transfection cells were stimulated with 5µg/ml poly(I:C) for the indicated times. Cells were subsequently lysed and subjected to analysis by western blot. Induction of pIκBα, pERK, pJNK and pIRF3 and total amounts of IκBα, ERK and JNK were determined. Protein amount of TLR3 full length and its 70 kDa cleavage fragment was assessed for knockdown efficiency. Actin serves as loading control. Molecular weight markers are indicated on the left. Western blots are representative of two independent experiments.

Poly(I:C), polyriboinosinic:polyribocytidylic acid; HaCaT, human adult low calcium high temperature; TLR, toll-like receptor; siRNA, small interfering RNA; h, hours; p, phospho; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; IRF, interferon regulatory factor; kDa, kilodalton.

Gene activation ultimately culminates in the secretion of cytokines and chemokines. Thus, levels of the cytokines IL-6 and TNF (Figure 5A) and of the chemokines IL-8 and chemokine (C-C motif) ligand 5 (CCL5) (Figure 5B) were determined in the supernatants of HaCaT cells following poly(I:C) stimulation. Poly(I:C) induced substantial secretion of the above-named cytokines and chemokines in a concentration-dependent manner (Figure 5). Importantly, this poly(I:C)-induced response was completely blunted in HaCaT cells in which TLR3 protein expression was inhibited by RNAi (Figure 5).

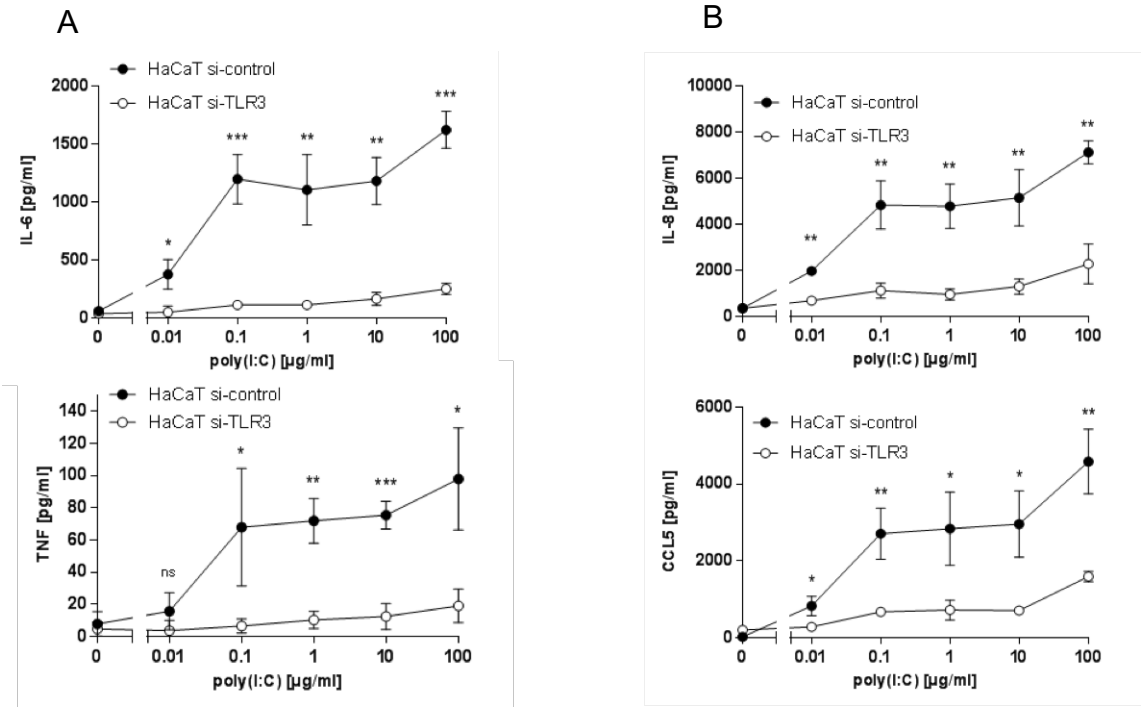

Figure 5. Poly(I:C) induces secretion of cytokines and chemokines via activation of TLR3 in HaCaT cells.

HaCaT cells were transfected with control or TLR3 siRNA. After 72h cells were stimulated with the indicated concentrations of poly(I:C). Cell supernatants were collected after 24h. (A) Concentrations of the cytokines IL-6 and TNF were determined by ELISA. (B) Concentrations of the chemokines IL-8 and CCL5 were determined by ELISA. Values are presented as means ± SD of at least three independent experiments performed in triplicates. *p < 0.05; **p < 0.01, ***p < 0.001, unpaired Student's t-test.

Poly(I:C), polyriboinosinic:polyribocytidylic acid; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; h, hours; IL, interleukin; TNF, tumour necrosis factor; ELISA, enzyme-linked immune sorbent assay; CCL, chemokine (C-C motif) ligand; SD, standard deviation; ns, not significant.

5.1.2 POLY(I:C) INDUCES CELL DEATH VIA TLR3

In addition to gene activation, poly(I:C)-induced cell death was assessed in HaCaT cells as additional known TLR3-mediated signalling output. HaCaT cells were transfected with control or TLR3 siRNA. Subsequently, cells were stimulated with poly(I:C) and cell death induction was assessed. Poly(I:C) induced cell death in control-transfected HaCaT cells in a concentration-dependent manner. In cells, in which expression of TLR3 was inhibited by RNAi, poly(I:C)-induced cell death was markedly decreased (Figure 6). Thus, also cell death induction by poly(I:C) critically depends on TLR3 presence in HaCaT cells.

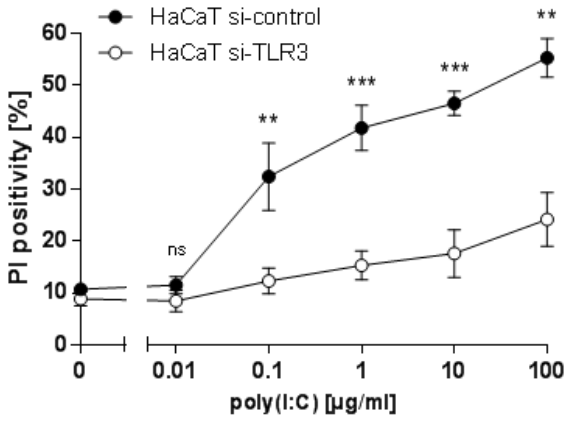

Figure 6. Poly(I:C) induces cell death via TLR3 in HaCaT cells.

HaCaT cells were transfected with control or TLR3 siRNA. After 72h cells were stimulated with the indicated concentrations of poly(I:C) and analysed for PI positivity by FACS 24h later. All values are presented as means ± SD of at least three independent experiments performed in triplicates. ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test.

Poly(I:C), polyriboinosinic:polyribocytidylic acid; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; h, hours; PI, propidium iodide; FACS, fluorescence-activated cell sorting; SD, standard deviation; ns, not significant.

Next, the question was addressed which kind of cell death is induced by poly(I:C) stimulation in HaCaT cells. Therefore, the RIP1 kinase inhibitor 7-Cl-O-Nec-1 (Nec-1) or/and the pan-caspase inhibitor Z-VAD(OMe)-FMK (zVAD) were applied in order to block necroptotic or/and apoptotic cell death. Prior to stimulation with poly(I:C), HaCaT cells were incubated with either Nec-1, zVAD or a combination thereof. Subsequently, cell death was assessed by analysing cells for PI positivity by FACS. Whereas blockage of RIP1 kinase activity by Nec-1 did not significantly block poly(I:C)-induced cell death, zVAD was very efficient at doing so. The combination of Nec-1 and zVAD, however, was even more effective in preventing cell death induction following stimulation with poly(I:C) than zVAD alone suggesting that poly(I:C)-induced cell death is apoptotic but can, via inhibition of caspase activity, partially be converted to necroptosis (Figure 7).

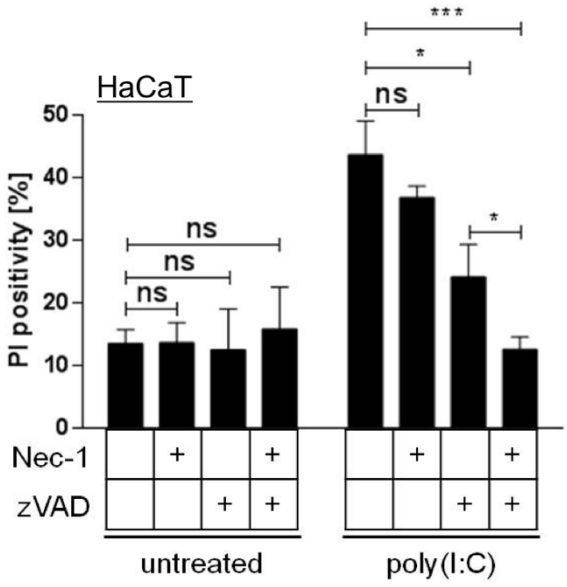

Figure 7. TLR3-induced cell death in HaCaT cells is mainly apoptotic.

Prior to stimulation with 50µg/ml poly(I:C), HaCaT cells were incubated for 1h with 10µM Nec-1, 20µM zVAD or a combination thereof. After 24h cells were analysed for PI positivity by FACS. All values are presented as means ± SD of at least three independent experiments performed in triplicates. * $p < 0.05$; *** $p < 0.001$, unpaired Student's t-test.

TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; poly(I:C), polyriboinosinic:polyribocytidylic acid; h, hour; Nec-1, 7-Cl-O-Nec-1; zVAD, Z-VAD(OMe)-FMK; PI, propidium iodide; FACS, fluorescence-activated cell sorting; SD, standard deviation; ns, not significant.

5.1.3 POLY(I:C) INDUCES FORMATION OF A SIGNALLING COMPLEX VIA TLR3

To test whether presence of TLR3 is required for poly(I:C)-induced formation of a signalling complex, HaCaT cells were transfected with control or TLR3 siRNA. Immunoprecipitation (IP) of Caspase-8 was performed in presence versus absence of TLR3 in HaCaT cells stimulated or not with poly(I:C) (Figure 8). In order to stabilise the signalling complex, this IP was performed in presence of the pan-caspase inhibitor zVAD. Poly(I:C) stimulation induced association of the E3 ubiquitin ligases TRAF6 and cIAP1/2 with Caspase-8. Along with these proteins, RIP1 was recruited to Caspase-8 in a poly(I:C) stimulation-dependent manner.

Importantly, RNAi-mediated suppression of TLR3 abolished association of these proteins with Caspase-8 (Figure 8) demonstrating that poly(I:C)-induced formation of this complex critically depends on presence of TLR3.

Taken together, these experiments clearly show that HaCaT cells serve as a suitable cellular system to study the TLR3 signalling pathway in detail as presence of TLR3 is essential for poly(I:C)-mediated gene activation and the subsequent secretion of cytokines and chemokines, for poly(I:C)-induced cell death and for formation of a poly(I:C)-induced signalling complex.

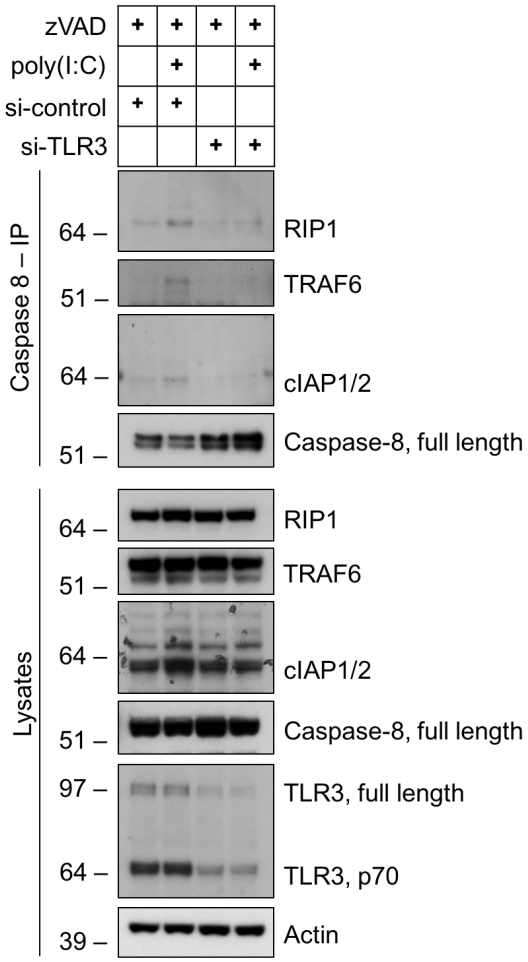

Figure 8. Poly(I:C) induces formation of a signalling complex via TLR3.

HaCaT cells were transfected with control or TLR3 siRNA. After 72h HaCaT cells were incubated with 20µM zVAD prior to stimulation with 25µg/ml poly(I:C). After 2h of stimulation Caspase-8 was immunoprecipitated and analysis of co-immunoprecipitated proteins by immunoblotting was performed as indicated. Actin serves as loading control. TLR3 immunoblotting of lysates was performed for demonstration of knockdown efficiency.

Poly(I:C), polyriboinosinic:polyribocytidylic acid; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; h, hours; zVAD, Z-VAD(OMe)-FMK; RIP, receptor interacting protein; TRAF, TNF receptor associated factor; cIAP, cellular inhibitor of apoptosis protein; IP, immunoprecipitation.

5.2 LUBAC CRUCIALLY DETERMINES TLR3 SIGNALLING OUTPUTS

Having established a cellular system in which all known TLR3 signalling outputs were induced by poly(I:C) stimulation in a TLR3-dependent manner, next, TLR3 signalling outputs were assessed in presence versus absence of the three different LUBAC components SHARPIN, HOIL-1 and HOIP.

To study the impact of linear ubiquitination on TLR3 signalling, RNAi-mediated suppression of each LUBAC component was performed in HaCaT cells (Figure 9). Analysis of protein expression of all three LUBAC components by western blotting upon transfection with control siRNA or siRNA targeting SHARPIN, HOIL-1 or HOIP revealed that silencing of any of the three LUBAC components affected protein stability of the other two (Figure 9).

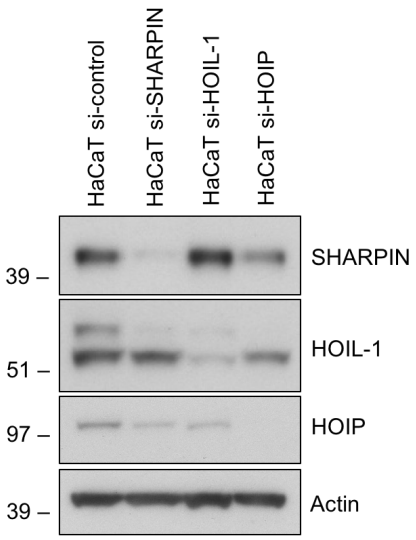

Figure 9. LUBAC components maintain each other's protein stability.

HaCaT cells were transfected with control siRNA or siRNA targeting SHARPIN, HOIL-1 or HOIP. After 72h, cells were lysed and subjected to further analysis by western blot. Actin serves as loading control. Molecular weight markers are indicated on the left.

LUBAC, linear ubiquitin chain assembly complex; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; h, hours.

5.2.1 LUBAC MAINTAINS TLR3-MEDIATED GENE ACTIVATION

Next, TLR3-mediated gene activation in presence versus absence of LUBAC components was investigated. HaCaT cells were transfected with control siRNA or siRNA targeting SHARPIN (Figure 10A) or HOIP (Figure 10B). Subsequently, known TLR3-triggered gene-activatory signalling pathways were evaluated upon stimulation with poly(I:C). Of note, silencing of either SHARPIN or HOIP prevented phosphorylation of IκBα. Concomitantly, absence of SHARPIN or HOIP resulted in diminished and delayed degradation of IκBα. In addition to NF-κB, also MAPK signalling pathways were assessed. Interestingly, absence of either SHARPIN or HOIP resulted in diminished phosphorylation of ERK following poly(I:C) stimulation as compared to control RNAi-transfected cells. In addition, poly(I:C)-induced JNK phosphorylation required presence of HOIP (Figure 10B). Total amounts of ERK and JNK were not affected following stimulation, neither in control-transfected cells nor in cells transfected with RNAi targeting SHARPIN or HOIP. In addition, phosphorylation of IRF3 was analysed in order to assess the impact of LUBAC components on IFN induction. Phosphorylation of IRF3 was, if at all, marginally reduced in the absence of SHARPIN (Figure 10A), but no differences were observed in the absence of HOIP (Figure 10B) as compared to control-transfected cells.

Thus, LUBAC components are required to maintain activation of NF-κB and MAPK signalling pathways following stimulation with poly(I:C) whereas activation of IRF3 seems to occur independently of LUBAC presence in HaCaT cells.

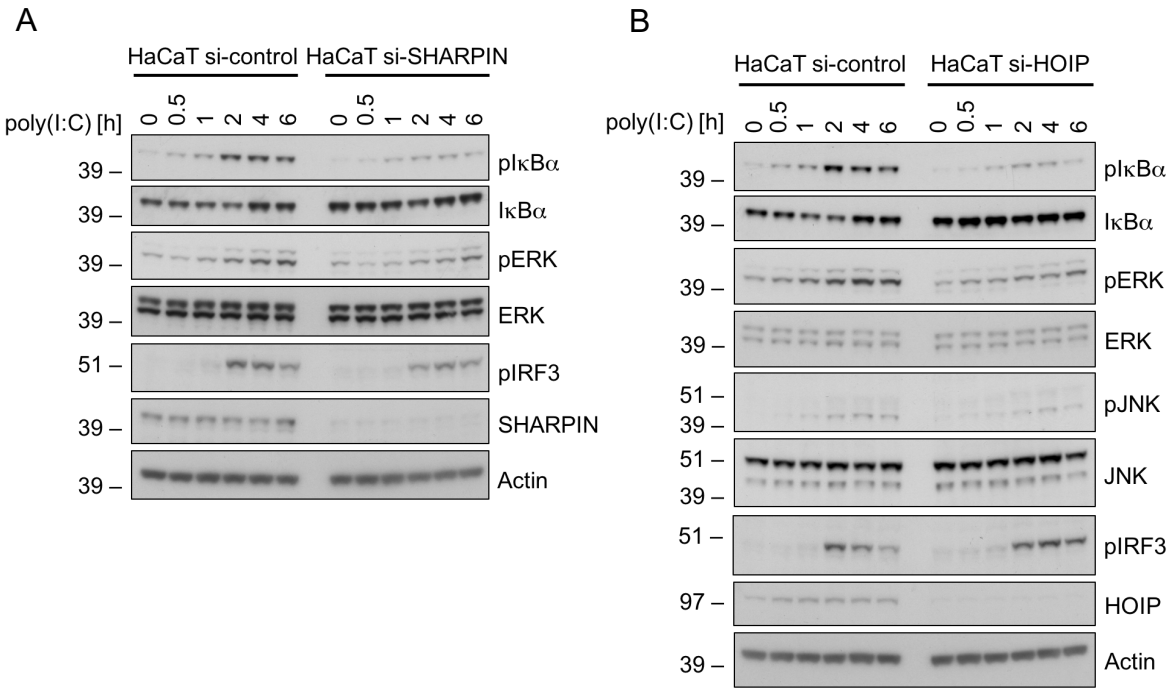

Figure 10. Presence of SHARPIN or HOIP is required to maintain TLR3-mediated gene activation.

HaCaT cells were transfected with control siRNA or siRNA targeting SHARPIN (A) or HOIP (B). 72h after transfection cells were stimulated with 5µg/ml poly(I:C) for the indicated times, lysed and subjected to analysis by western blot. Membranes were probed for phosphorylated forms of IκBα, ERK and IRF3 and total amounts of IκBα and ERK (A) or for phosphorylated forms of IκBα, ERK, JNK and IRF3 and total amounts of IκBα, ERK and JNK (B). SHARPIN (A) or HOIP (B) protein expression was determined for knockdown efficiency. Actin serves as loading control. Images are representative of at least three independent experiments. Molecular weight markers are indicated on the left.

SHARPIN, SHANK-associated RBCK1 homology domain-interacting protein; HOIP, HOIL-1-interacting protein; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; h, hour; poly(I:C), polyriboinosinic:polyribocytidylic acid; p, phospho; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinases; IRF, interferon regulatory factor.

Since poly(I:C)-induced gene activatory signalling pathways were affected by absence of either SHARPIN or HOIP (Figure 10), next, poly(I:C)-induced cytokine and chemokine secretion was evaluated in presence versus absence of the three different LUBAC components. Assessment of poly(I:C)-induced secretion of the cytokines IL-6 and TNF (Figure 11A) and of the chemokines IL-8 and CCL5 (Figure 11B) revealed that secretion of these cytokines and chemokines into the supernatant following poly(I:C) stimulation was reduced in the absence of either SHARPIN, HOIL-1 or HOIP. HOIP as the central and catalytically active LUBAC component was required the most for an adequate cytokine and chemokine response following stimulation with poly(I:C) (Figure 11).

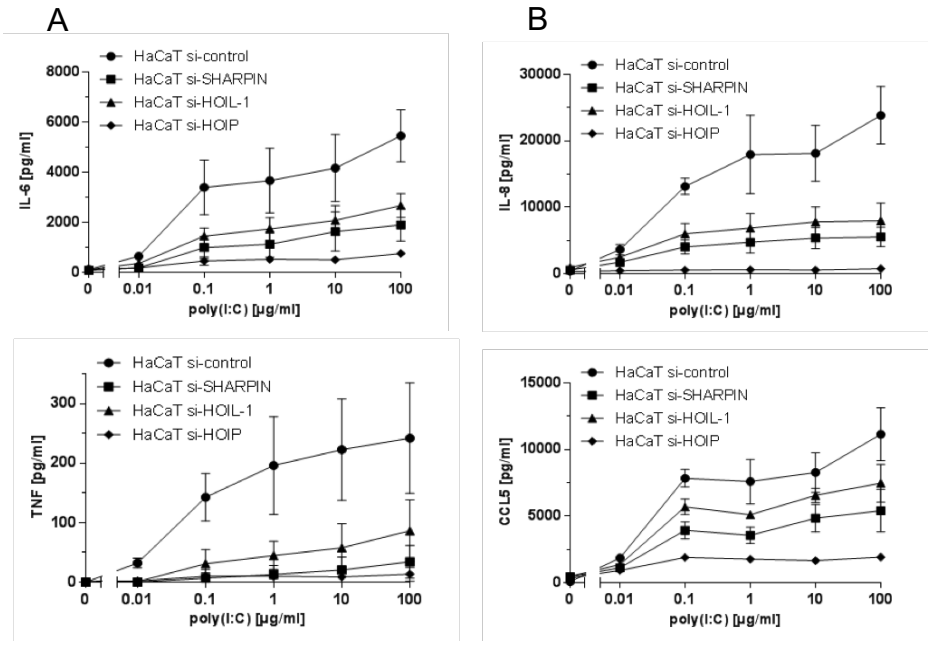

Figure 11. LUBAC maintains TLR3-induced cytokine and chemokine secretion.

HaCaT cells were transfected with control siRNA or siRNA targeting SHARPIN, HOIL-1 or HOIP. Cells were stimulated with the indicated concentrations of poly(I:C) 72h after transfection. Cell supernatants were collected 24h later and concentration of IL-6 and TNF (A), and of IL-8 and CCL5 (B) were determined by ELISA. Values are presented as mean ± SD of at least three independent experiments performed in triplicates.

LUBAC, linear ubiquitin chain assembly complex; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; poly(I:C), polyriboinosinic:polyribocytidylic acid; h, hours; IL, interleukin; TNF, tumour necrosis factor; CCL, chemokine (C-C motif) ligand; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation.

To further confirm the results obtained by RNAi-mediated knockdown of the different LUBAC components, and since HOIP as the central component was most crucial for maintaining poly(I:C)-induced gene activation, HOIP-deficient HaCaT and HeLa cell lines were generated (Figure 12).

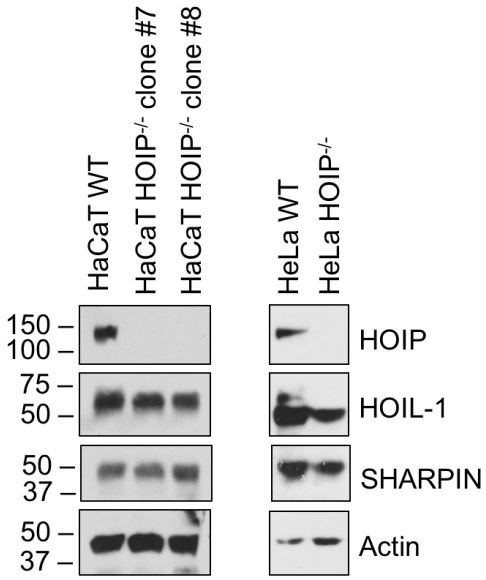

Figure 12. Generation of HOIP-deficient HaCaT and HeLa cell lines.

HaCaT and HeLa WT and HOIP-deficient cells were lysed and subjected to analysis by western blot. SHARPIN, HOIL-1 and HOIP protein amount was determined by immunoblotting. Actin serves as loading control. Molecular weight markers are indicated on the left.

HOIP, HOIL-1-interacting protein; HaCaT, human adult low calcium high temperature; HeLa, Henrietta Lacks; WT, wildtype; SHARPIN, SHANK-associated RBCK1 homology domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1.

HaCaT WT and HOIP-deficient cells were further analysed for downstream signalling events following stimulation of TLR3 by poly(I:C). These experiments revealed that absence of HOIP disabled activation of NF-κB signalling in HOIP-deficient clones #7 and #8 (Figure 13) as compared to cells expressing WT HOIP. Phosphorylation of IRF3 was not affected by absence of HOIP in HaCaT cells in line with results obtained by RNAi-mediated knockdown (Figure 13, for comparison see Figure 10B). In addition, phosphorylation of TBK1, a factor that is required for TLR3-mediated induction of IFNs, was assessed. Interestingly, phosphorylation of TBK1 required presence of HOIP in HaCaT cells (Figure 13).

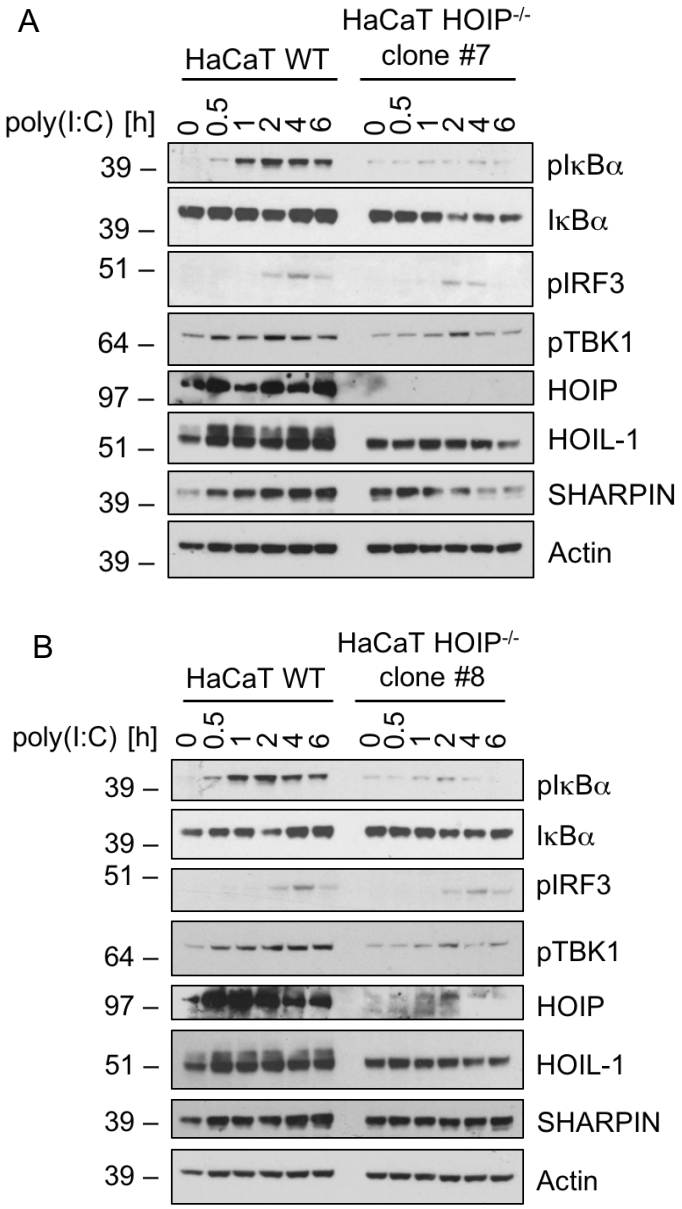

Figure 13. HOIP-deficient cells show diminished TLR3-mediated gene activation.

HaCaT WT and two different HOIP-deficient clones (#7 in A and #8 in B) were stimulated with 5µg/ml poly(I:C) for the indicated times, lysed and subjected to analysis by western blot. Membranes were probed for phosphorylated forms of IκBα, IRF3 and TBK1. Total amounts of IκBα were determined. Immunoblotting for SHARPIN, HOIL-1 and HOIP was performed. Actin serves as loading control. Images are representative of at least two independent experiments.

HOIP, HOIL-1-interacting protein; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; WT, wildtype; poly(I:C), polyriboinosinic:polyribocytidylic acid; p, phospho; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IRF, interferon regulatory factor; TBK1, TANK-binding kinase 1; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; h, hours.

Next, HOIP-deficient HeLa cells were analysed for activation of NF-κB and MAPK signalling. Results show that HOIP was also required for phosphorylation of IκBα and ERK in HeLa cells (Figure 14). In addition, phosphorylation of TBK1 was diminished in the absence of HOIP. Interestingly, whereas phosphorylation of IRF3 occurred irrespective of HOIP being absent or present in HaCaT cells, in HeLa cells phosphorylation of IRF3 was dependent on presence of HOIP (Figure 14).

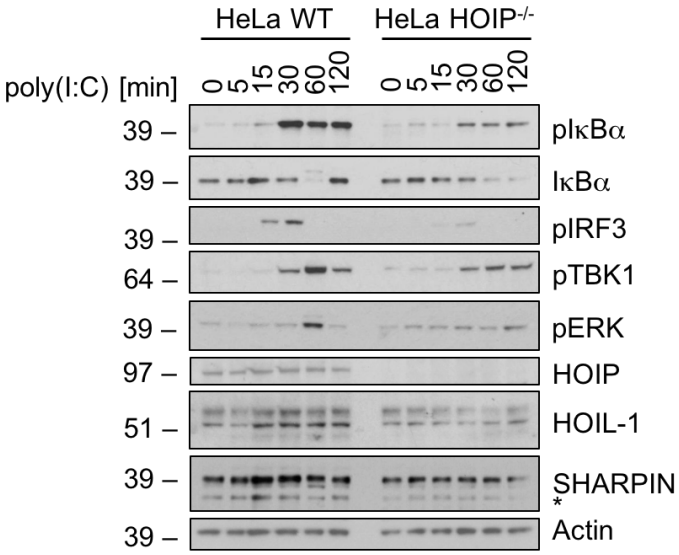

Figure 14. HOIP-deficient HeLa cells show diminished TLR3-mediated gene activation.

HeLa cells expressing HOIP WT or deficient in HOIP were stimulated with 5µg/ml poly(I:C) for the indicated times, lysed and subjected to analysis by western blot. Membranes were probed for phosphorylation of IκBα, ERK, IRF3 and TBK1. Total amounts of IκBα were determined. Immunoblotting for SHARPIN, HOIL-1 and HOIP was performed. Actin serves as loading control. Images are representative of at least two independent experiments. Non-specific bands are indicated by asterisk, molecular weight markers are indicated on the left.

HOIP, HOIL-1-interacting protein; HeLa, Henrietta Lacks; TLR, toll-like receptor; WT, wildtype; poly(I:C), polyriboinosinic:polyribocytidylic acid; p, phospho; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IRF, interferon regulatory factor; TBK1, TANK binding kinase 1; ERK, extracellular signal-regulated kinase; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1.

Also chemokine secretion was assessed in HaCaT and HeLa WT and HOIP-deficient cells following stimulation with poly(I:C) (Figures 15 and 16). Secretion of CCL5 and IL-8 in HaCaT cells required presence of HOIP (Figure 15), in line with results obtained by RNAi-mediated knockdown of HOIP (Figure 11).

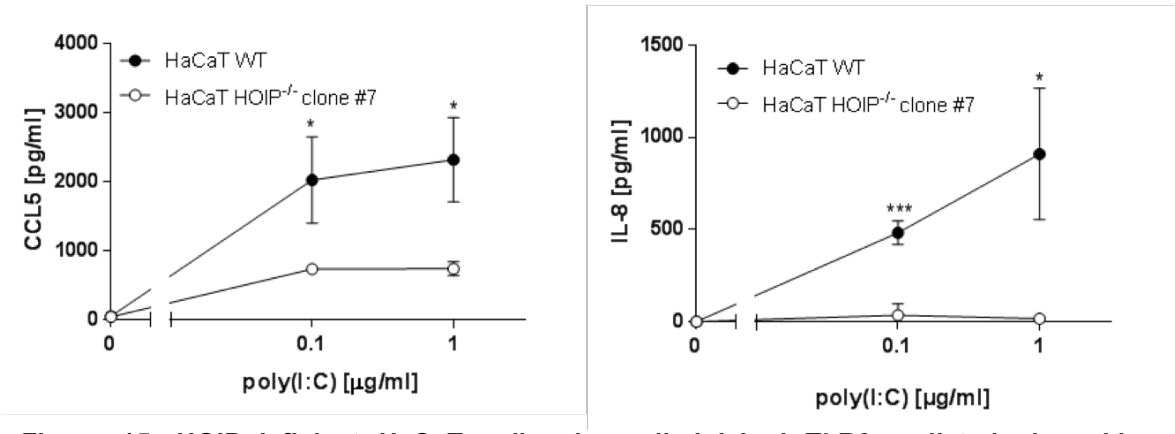

Figure 15. HOIP-deficient HaCaT cells show diminished TLR3-mediated chemokine secretion.

HaCaT WT and HOIP-deficient cells (clone #7) were stimulated with the indicated concentrations of poly(I:C) and levels of the chemokines CCL5 and IL-8 were determined in the supernatant by ELISA 24h later. Values are presented as mean ± SD of at least two independent experiments. * $p < 0.05$; *** $p < 0.001$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; HaCaT, human adult low calcium high temperature; TLR, toll-like receptor; WT, wildtype; poly(I:C), polyriboinosinic:polyribocytidylic acid; CCL, chemokine (C-C motif) ligand; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; h, hours.

Moreover, poly(I:C)-induced secretion of CCL5 in HeLa cells was critically dependent on presence of HOIP (Figure 16).

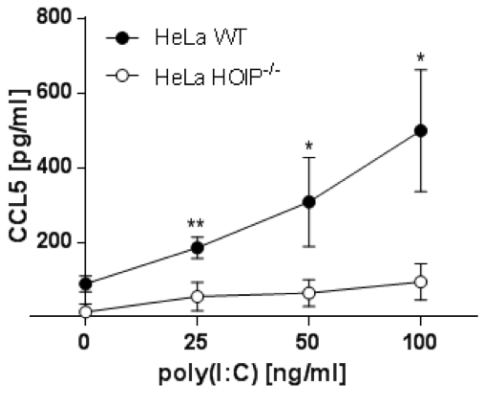

Figure 16. HOIP-deficient HeLa cells show diminished TLR3-mediated chemokine secretion.

HeLa WT and HOIP-deficient cells were stimulated with the indicated concentrations of poly(I:C), and levels of the chemokine CCL5 were determined by ELISA 24h later. Values are presented as mean ± SD of at least two independent experiments performed in triplicates. * $p < 0.05$; ** $p < 0.01$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; HeLa, Henrietta Lacks; TLR, toll-like receptor; WT, wildtype; poly(I:C), polyriboinosinic:polyribocytidylic acid; CCL, chemokine (C-C motif) ligand; ELISA, enzyme-linked immunosorbent assay; h, hours; SD, standard deviation.

In pursuance of identifying whether linear ubiquitination is required for the induction of IFNs, IFN-β secretion following stimulation with poly(I:C) in presence versus absence of HOIP was assessed in HaCaT cells. Interestingly, IFN-β secretion following TLR3 stimulation was greatly reduced in HaCaT cells in which expression of HOIP protein was downregulated by RNAi (Figure 17), even though phosphorylation of IRF3, a prerequisite of IFN induction, remained almost unaffected by knockdown of HOIP in the same cell line (Figure 10B).

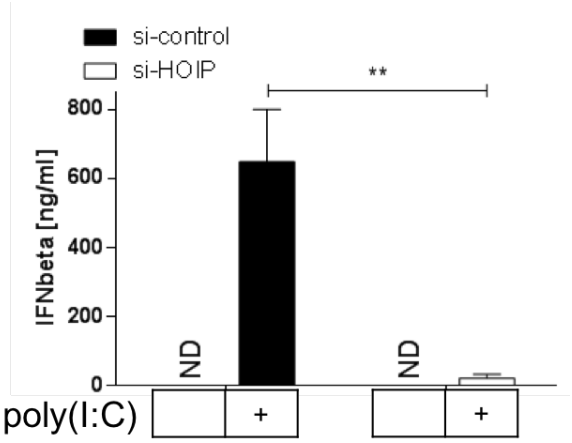

Figure 17. Presence of HOIP is essential for IFN-β secretion.

HaCaT cells were transfected with non-targeting siRNA or siRNA targeting HOIP. After 72h cells were stimulated with 10µg/ml poly(I:C). Cell supernatants were collected after 4h of stimulation and analysed for IFN-β concentration by ELISA. Values are presented as mean ± SD of at least three independent experiments performed in triplicates. ** $p < 0.01$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; IFN, interferon; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; h, hours; poly(I:C), polyriboinosinic:polyribocytidylic acid; ELISA, enzyme-linked immuno sorbent assay; SD, standard deviation; ND, not detectable.

In summary, these results demonstrate that TLR3-mediated gene activation critically depends on presence of all three LUBAC components.

5.2.2 LUBAC CONTROLS TLR3-MEDIATED CELL DEATH

Next, TLR3-mediated cell death was evaluated in absence versus presence of linear ubiquitination. Silencing of each of the three LUBAC components by RNAi-mediated knockdown sensitised cells to TLR3-mediated death as assessed by loss of cell viability (Figure 18A). In addition, knockdown of HOIP by RNAi rendered HaCaT cells more sensitive to cell death induction by poly(I:C) as assessed by PI positivity (Figure 18B).

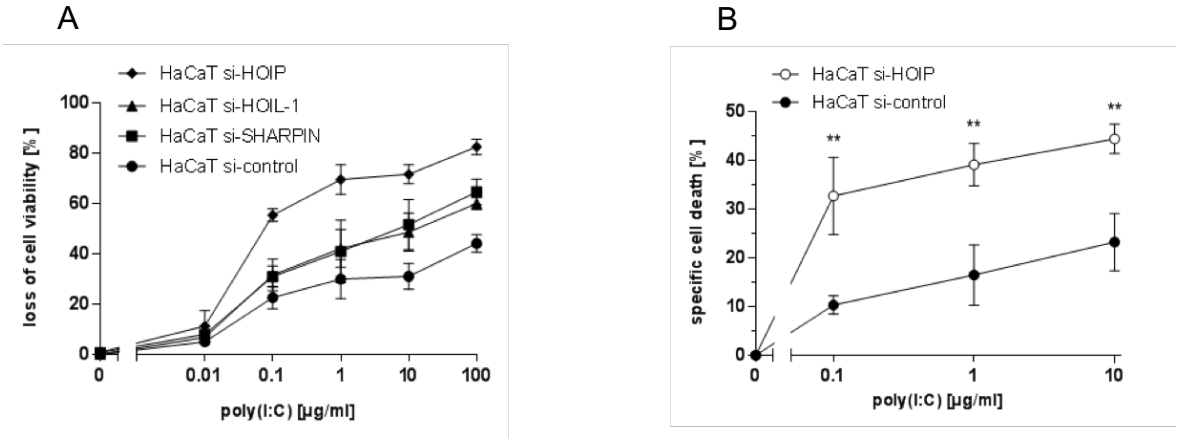

Figure 18. LUBAC controls TLR3-mediated cell death.

(A) HaCaT cells were transfected with control siRNA or siRNA targeting SHARPIN, HOIL-1 or HOIP. After 72h cells were stimulated with the indicated concentrations of poly(I:C). Loss of cell viability was assessed 24h later by CellTiter-Glo® assay. (B) HaCaT cells were transfected with non-targeting siRNA or siRNA targeting HOIP. After 72h cells were stimulated with the indicated concentrations of poly(I:C) and subjected to analysis for PI positivity by FACS 24h later. Spontaneous background cell death of less than 20% was subtracted from all values (specific cell death). Values are presented as mean ± SD of at least three independent experiments performed in triplicates. ** $p < 0.01$, Student's t-test.

LUBAC, linear ubiquitin chain assembly complex; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; HOIP, HOIL-1-interacting protein; h, hours; poly(I:C), polyriboinosinic:polyribocytidylic acid; PI, propidium iodide; FACS, fluorescence activated cell sorting; SD, standard deviation.

Furthermore, cell viability of HaCaT (Figure 19A) and HeLa (Figure 19B) WT and HOIP-deficient cell lines was assessed following stimulation with poly(I:C). These experiments revealed that both cell lines were sensitised to poly(I:C)-induced cell death in the absence of HOIP (Figure 19).

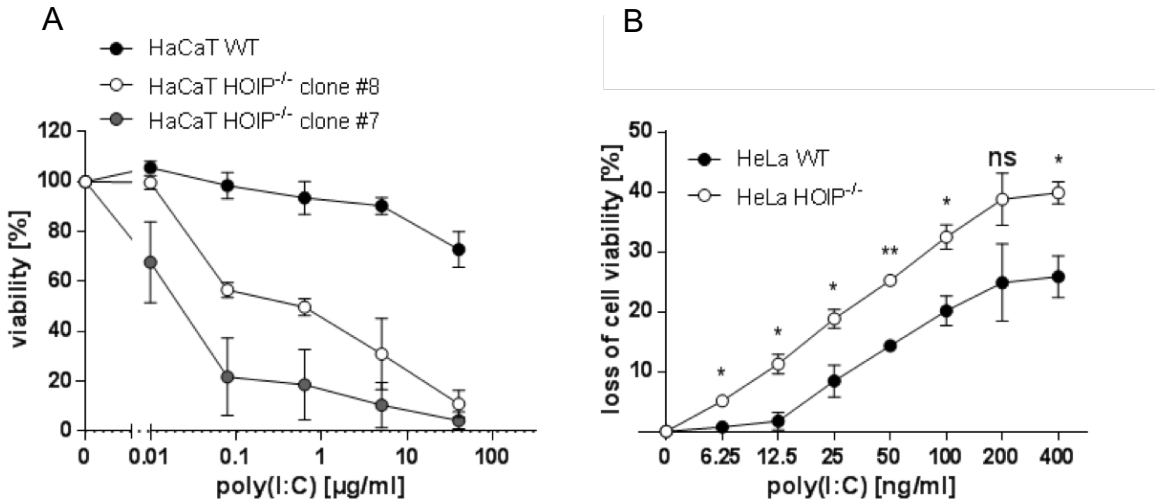

Figure 19. HOIP deficiency sensitises cells to poly(I:C)-induced death.

(A) HaCaT WT and HOIP-deficient cells (clone #7 and clone #8) were stimulated with the indicated concentrations of poly(I:C), and cell viability was determined 24h later. All values are presented as mean ± SD of at least three independent experiments performed in triplicates. (B) HeLa WT and HOIP-deficient cells were stimulated with the indicated concentrations of poly(I:C), and loss of cell viability was determined 24h later. Values are presented as mean ± SD of at least two independent experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; poly(I:C), polyriboinosinic:polyribocytidylic acid; HaCaT, human adult low calcium high temperature; WT, wildtype; h, hours; SD, standard deviation; HeLa, Henrietta Lacks.

Thus, presence of LUBAC is required to prevent excessive TLR3-induced cell death.

Next, the impact of linear ubiquitination on the type of TLR3-induced cell death was assessed. HaCaT cells were transfected with either control siRNA or siRNA targeting HOIP. Subsequently, either Nec-1, zVAD or a combination thereof was applied prior to stimulation with poly(I:C). Cell viability was assessed following stimulation. Interestingly, and although absence of HOIP sensitised cells to poly(I:C)-induced cell death, in line with the results shown in Figure 18 and 19, absence of HOIP did not alter the type of poly(I:C)-induced cell death (Figure 20). Poly(I:C) stimulation resulted in induction of a cell death that was mainly apoptotic, independently of HOIP being absent or present, as the pan-caspase inhibitor zVAD efficiently blocked poly(I:C)-induced cell death. The remaining cell death could further be inhibited by simultaneous application of the RIP1 kinase inhibitor Nec-1 together with zVAD indicating that caspase activity was required to prevent poly(I:C)-induced necroptosis (Figure 20).

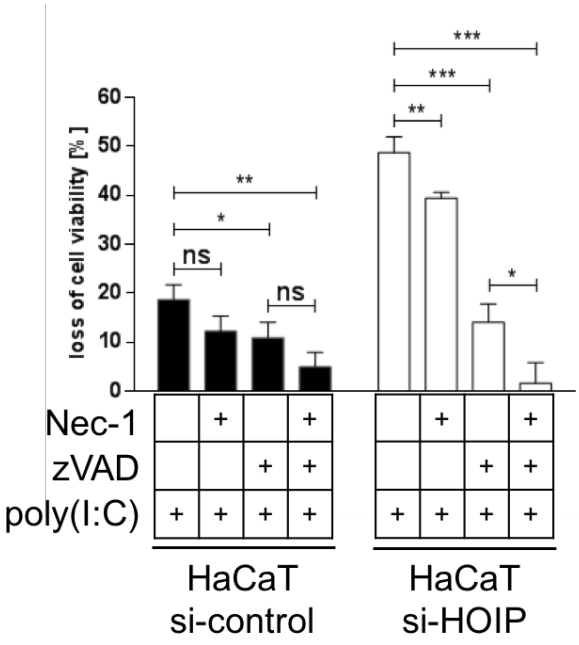

Figure 20. LUBAC does not alter the type of poly(I:C)-induced cell death.

HaCaT cells were transfected with non-targeting siRNA or siRNA targeting HOIP. After 72h cells were incubated with 10µM Nec-1, 20µM zVAD or a combination thereof 1h prior to stimulation with 1µg/ml poly(I:C). Loss of cell viability was assessed 24h later. Values are presented as mean ± SD of at least three independent experiments performed in triplicates. *p < 0.05; **p < 0.01, ***p < 0.001, unpaired Student's t-test.

LUBAC, linear ubiquitin chain assembly complex; poly(I:C), polyriboinosinic:polyribocytidylic acid; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; HOIP, HOIL-1-interacting protein; Nec-1, 7-Cl-O-Nec-1; zVAD, Z-VAD(OMe)-FMK; SD, standard deviation; h, hours; ns, not significant.

Having shown that HaCaT and HeLa HOIP-deficient cell lines are sensitised to poly(I:C)-induced cell death (Figure 19), these cell lines were further analysed for induction of typical hallmarks of apoptosis, i.e. cleavage of Caspase-3 and -8 and Poly-(ADP-ribose) polymerase (PARP) following stimulation with poly(I:C) (Figure 21). Analysis by western blot revealed that HOIP deficiency in HaCaT (Figure 21A) and HeLa (Figure 21B) cells indeed resulted in an increased cleavage of Caspase-3 and -8 and PARP following stimulation with poly(I:C).

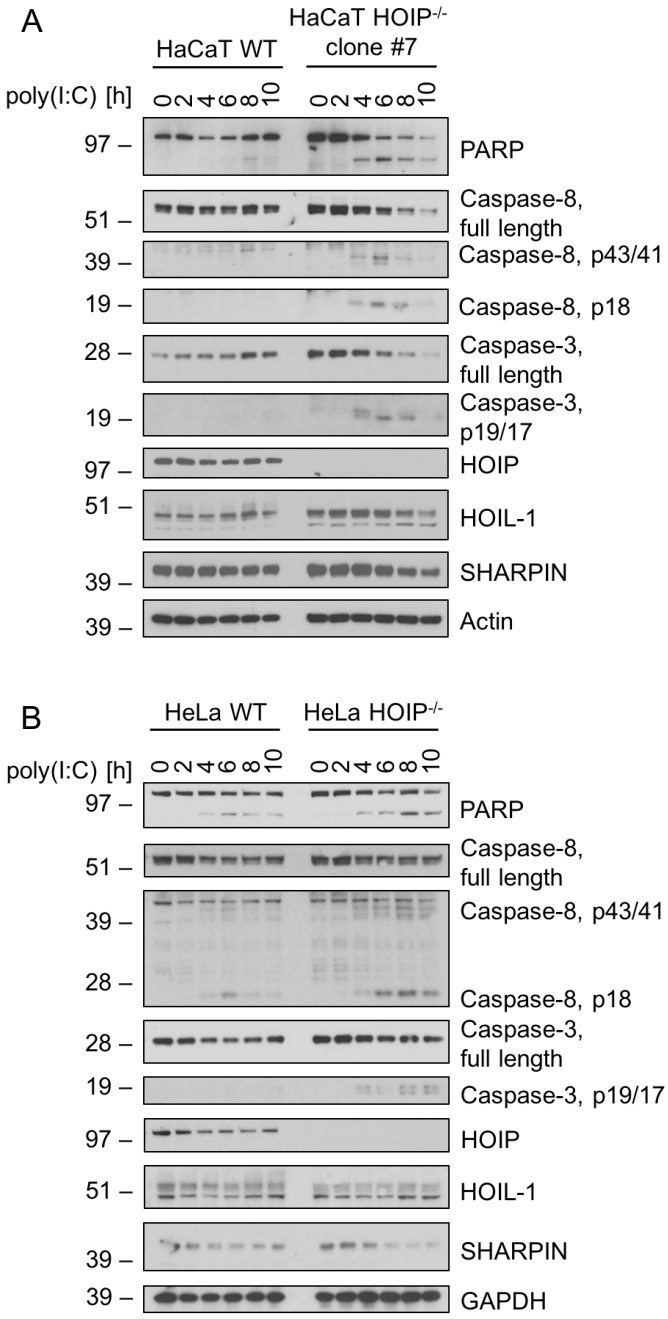

Figure 21. HOIP deficiency results in increased hallmarks of apoptosis following stimulation with poly(I:C).

HaCaT cells (A) or HeLa cells (B) WT or deficient in HOIP were stimulated with 5µg/ml poly(I:C) for the indicated times and subjected to further analysis by western blot. Membranes were probed with antibodies specific to Caspase-3 and -8 and PARP and their respective cleavage fragments. Protein levels of SHARPIN, HOIL-1 and HOIP were determined. Actin or GAPDH served as loading control. Western blots are representatives of at least three independent experiments.

HOIP, HOIL-1-interacting protein; HaCaT, human adult low calcium high temperature; HeLa, Henrietta Lacks; WT, wildtype; PARP; Poly-(ADP-ribose) polymerase; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Poly(I:C) stimulation resulted in TNF secretion (Figure 5A). To assess whether cell death and sensitisation to cell death in the absence of LUBAC upon poly(I:C) stimulation were mediated by autocrine TNF and residual TNF secretion (Figure 11A), Etanercept, a soluble TNFR2-fragment crystallisable (Fc) fusion protein (also known as Enbrel®), was applied as TNF blocker. HaCaT cells transfected with control siRNA or siRNA targeting HOIP were stimulated with poly(I:C) following pre-incubation with Enbrel® (Figure 22B). In order to control for efficacy of the soluble TNFR2-Fc Enbrel®, HT29 cells were pre-incubated with Enbrel® in parallel prior to stimulation with a combination of Smac Mimetic (SM) and zVAD (Figure 22A). Interestingly, autocrine TNF was not responsible for TLR3 stimulation-induced cell death as incubation with Enbrel® did not affect poly(I:C)-induced death of HaCaT cells, independently of HOIP being present or absent (Figure 22B), whereas Enbrel® efficiently prevented autocrine TNF-induced necroptosis of HT29 cells (Figure 22A).

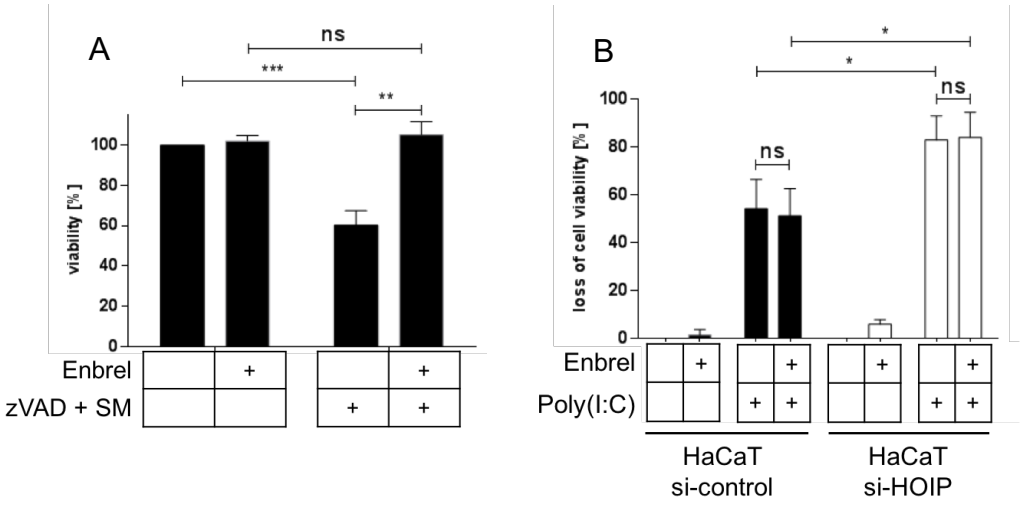

Figure 22. Sensitisation to TLR3-mediated cell death in the absence of HOIP is independent of autocrine TNF.

(A) HT29 cells were incubated or not with 50µg/ml Enbrel® 2h prior to stimulation with 100nM SM and 20µM zVAD. Cell viability was assessed after 48h. (B) In parallel, HaCaT cells, transfected with control or HOIP siRNA 72h earlier, were incubated or not with 50µg/ml Enbrel® 2h prior to stimulation with 100µg/ml poly(I:C). Loss of cell viability was determined 24h later. Values are presented as means ± SD of at least three independent experiments performed in triplicates. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test.

TLR, toll-like receptor; HOIP, HOIL-1-interacting protein; TNF, tumour necrosis factor; h, hours; SM, Smac Mimetic; zVAD, Z-VAD(OMe)-FMK; HaCaT, human adult low calcium high temperature; siRNA, small interfering RNA; poly(I:C), polyriboinosinic:polyribocytidylic acid; SD, standard deviation; ns, not significant.

To address the question whether the ligase activity of HOIP was required for the regulation of TLR3-mediated signalling outputs, HaCaT cells stably expressing a control vector, WT HOIP (HOIP) or a catalytically inactive mutant form of HOIP (HOIPmut) were generated.

Next, poly(I:C) stimulation-induced secretion of the chemokine CCL5 was determined in the supernatant of these cells (Figure 23A). In parallel, loss of cell viability was assessed (Figure 23B). Analysis revealed that expression of the mutant form of HOIP, but not expression of WT HOIP, resulted in diminished secretion of the chemokine CCL5 and sensitised HaCaT cells to poly(I:C)-induced death. Thus, HOIP's ability to form linear linkages is required for the maintenance of TLR3-mediated gene activation and for the prevention of TLR3-induced cell death.

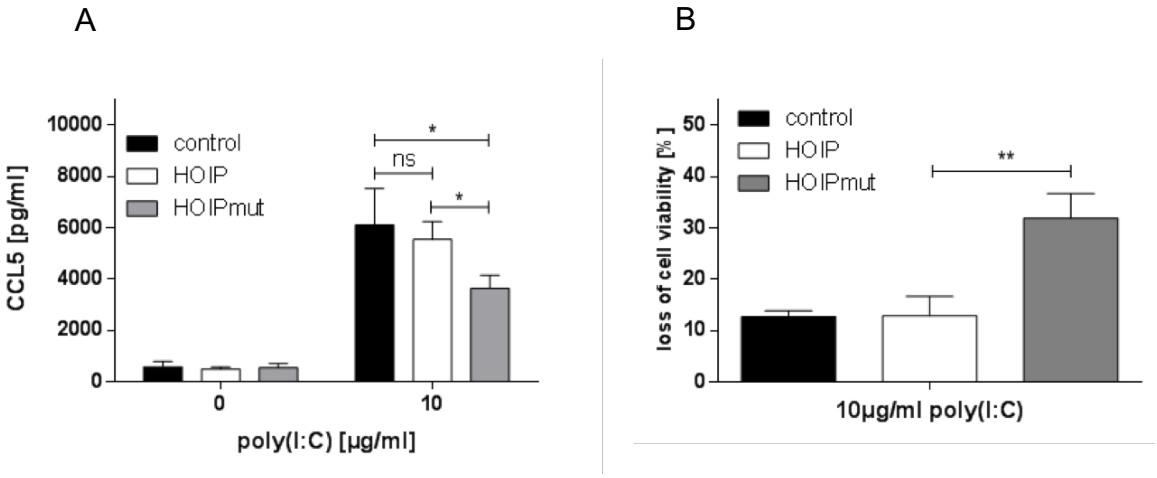

Figure 23. HOIP's ligase activity is required for maintenance of TLR3-mediated gene activation and prevention of TLR3-induced cell death.

HaCaT cells stably expressing WT HOIP (HOIP), a catalytically inactive mutant form of HOIP (HOIPmut) or empty vector (control) were stimulated with 10µg/ml poly(I:C). Concentration of CCL5 was determined in the supernatant 24h later by ELISA (A) and loss of cell viability was assessed in parallel (B). Values are presented as mean ± SD of at least three independent experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; WT, wildtype; poly(I:C), polyriboinosinic:polyribocytidylic acid; CCL, chemokine (C-C motif) ligand; h, hours; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; ns, non-significant.

In summary, these results show that all three LUBAC components are required to maintain TLR3-mediated gene activation and to prevent excessive TLR3-mediated cell death, that LUBAC does not influence the type of poly(I:C)-induced cell death, that cell death upon stimulation of TLR3 neither in presence nor absence of LUBAC is mediated by autocrine TNF, and that HOIP's ability to form linear linkages is required to mediate these effects.

5.3 LUBAC FORMS PART OF TLR3-INDUCED SIGNALLING COMPLEXES

LUBAC components crucially determine all signalling outputs of TLR3. Thus, the question arose whether LUBAC controls TLR3 signalling directly by forming part of the TLR3-signalling complex (TLR3-SC). As IP of TLR3 is complicated due to lack of specific and suitable TLR3 antibodies, HaCaT and HeLa cell lines stably expressing Flag-tagged TLR3 were generated (HaCaT-TLR3 and HeLa-TLR3).

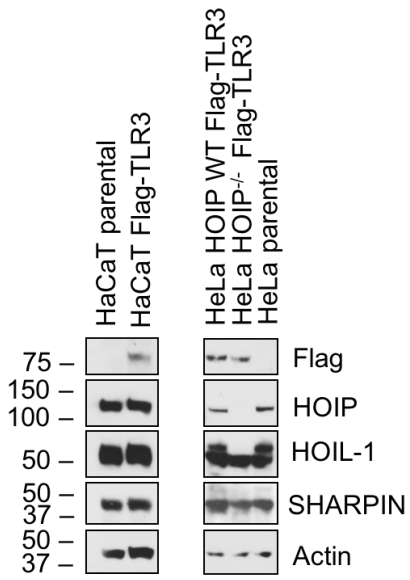

Figure 24. Cell lines stably expressing Flag-TLR3.

HaCaT cells and HeLa WT and HOIP-deficient cells stably expressing Flag-tagged TLR3 were lysed and subjected to analysis by western blot. Expression levels of Flag-tagged TLR3 were evaluated by staining membranes for Flag. Expression of LUBAC components was determined. Actin serves as loading control.

TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; HeLa; Henrietta Lacks; WT, wildtype; HOIP, HOIL-1-interacting protein; LUBAC, linear ubiquitin chain assembly complex.

Next, HaCaT-TLR3 cells were stimulated with poly(I:C) and the TLR3-SC was isolated via IP of Flag-TLR3 following stimulation (Figure 25). Analysis of co-immunoprecipitated proteins revealed stimulation-dependent recruitment of all three LUBAC components to TLR3 (Figure 25). In addition, linear ubiquitin linkages were present in the complex upon stimulation with poly(I:C). Moreover, TRIF, RIP1, NF-κB essential modulator (NEMO), pTBK1 and Caspase-8 were recruited along with them (Figure 25).

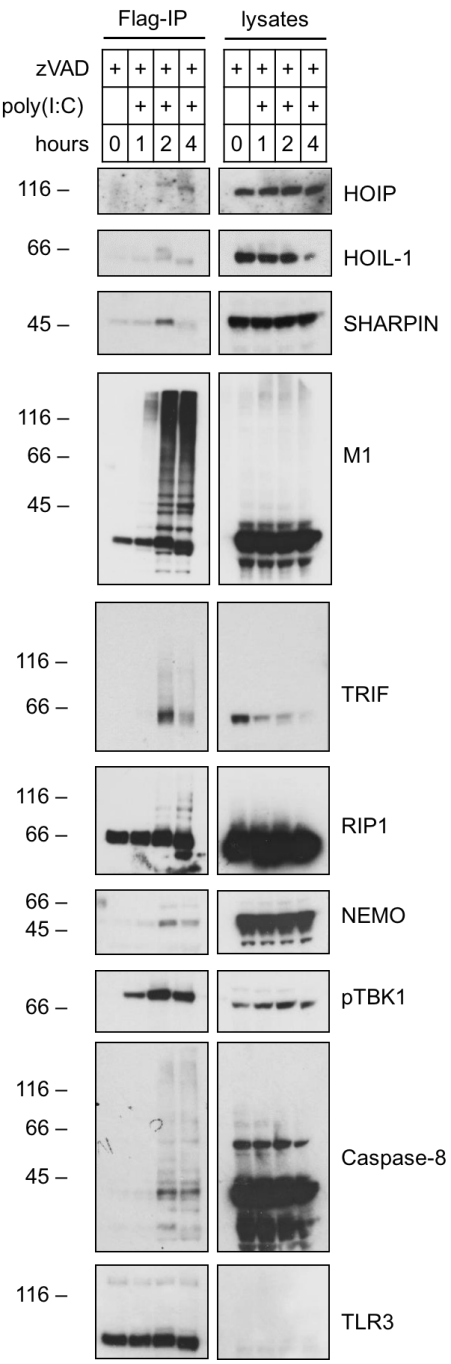

Figure 25. LUBAC forms part of the TLR3-SC.

HaCaT-TLR3 cells were stimulated with 25µg/ml poly(I:C) in presence of 20µM zVAD for the indicated times. TLR3 was subsequently immunoprecipitated via its Flag-tag. Analysis of co-immunoprecipitated proteins by immunoblotting was performed as indicated. Images are representatives of at least two independent experiments, molecular weight markers are indicated on the left.

LUBAC, linear ubiquitin chain assembly complex; TLR, toll-like receptor; SC, signalling complex; HaCaT, human adult low calcium high temperature; zVAD, Z-VAD(OMe)-FMK; poly(I:C), polyriboinosinic:polyribocytidylic acid; HOIP, HOIL-1-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; M1, linear ubiquitin linkages; TRIF, TIR domain-containing adapter protein inducing IFN-β; RIP, receptor interacting protein; NEMO, NF-κB essential modulator; p, phospho; TBK, TANK-binding kinase; IP, immunoprecipitation.

Thus, LUBAC forms part of the TLR3-SC. Next, Caspase-8 IP was performed in order to isolate the endogenous TLR3-SC from parental HaCaT cells. Analysis of co-immunoprecipitated proteins by immunoblotting revealed that, strikingly, all three LUBAC components were associated with Caspase-8 in a poly(I:C) stimulation-dependent manner (Figure 26). In addition, TRAF6, cIAP1/2, RIP1, FADD and cFLIP associated with Caspase-8 upon poly(I:C) stimulation (Figure 26). Interestingly, TLR3 was hardly detectable in the Caspase-8 IP, and only formed part of this native complex when Caspase activity was inhibited by zVAD (Figure 26).

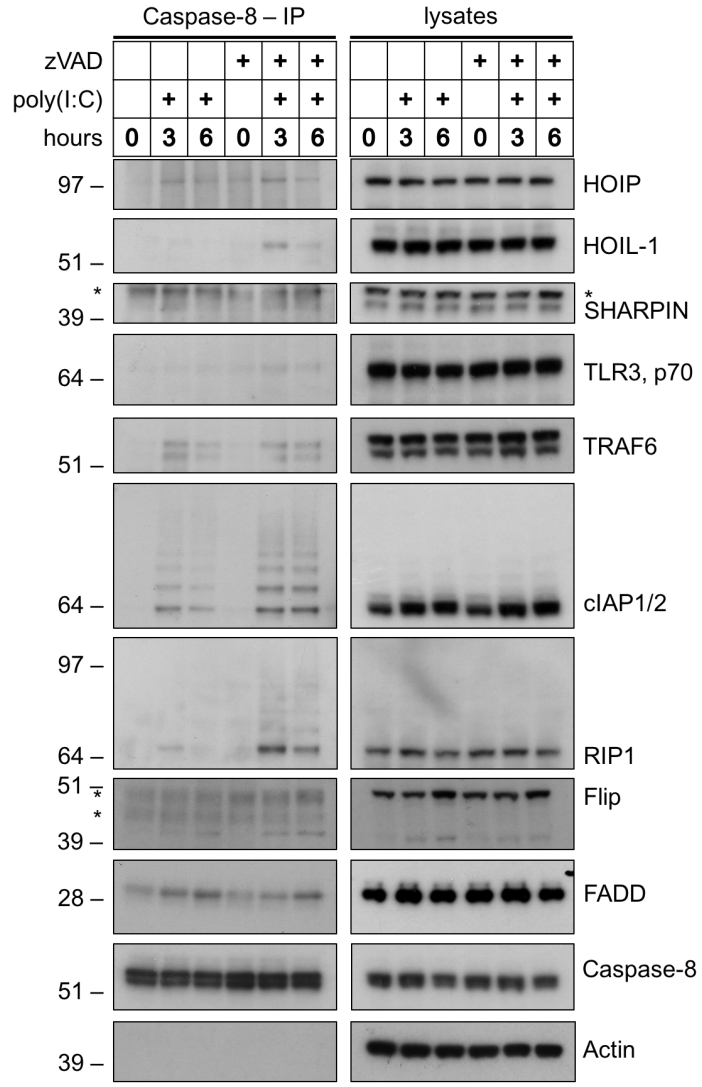

Figure 26. LUBAC is recruited to Caspase-8 upon poly(I:C) stimulation.

HaCaT cells were incubated with 20µM zVAD or not prior to stimulation with 25µg/ml poly(I:C) for the indicated times. Caspase-8 was immunoprecipitated and analysis of co-immunoprecipitated proteins was performed as indicated. Images are representatives of at least two independent experiments. Non-specific bands are indicated by asterisk.

LUBAC, linear ubiquitin chain assembly complex; TLR, toll-like receptor; HaCaT, human adult low calcium high temperature; zVAD, Z-VAD(OMe)-FMK; poly(I:C), polyriboinosinic:polyribocytidylic acid; HOIP, HOIL-1-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; TRAF, TNF receptor associated factor; cIAP, cellular inhibitor of apoptosis protein; RIP, receptor interacting protein; Flip, FLICE-like inhibitory protein; FADD, Fas-associated protein with death domain.

IP of endogenous SHARPIN confirmed these results by showing that TLR3, RIP1, TRAF6, cIAP1/2 and Caspase-8 associate with LUBAC components upon poly(I:C) stimulation (Figure 27).

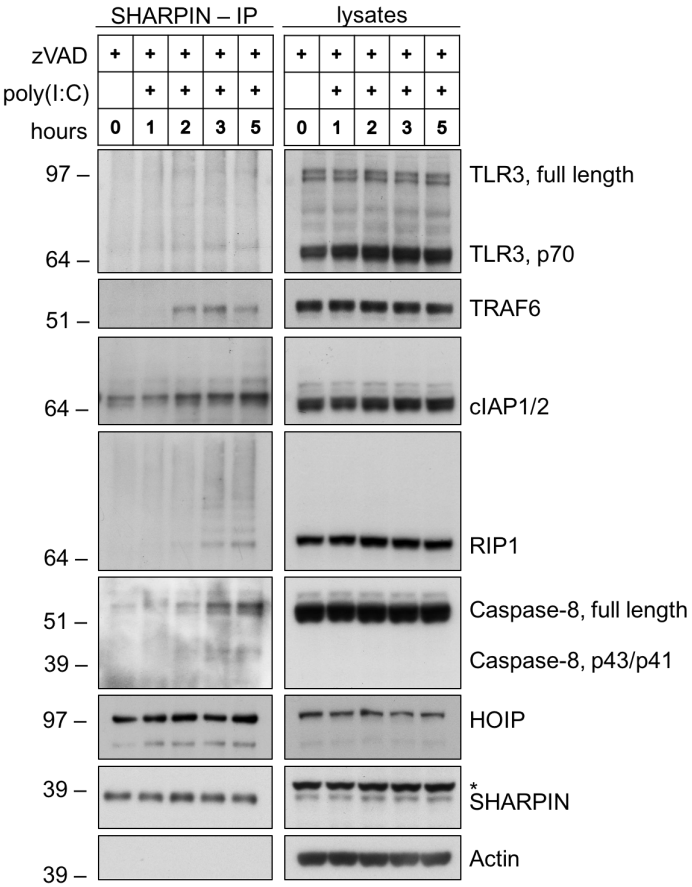

Figure 27. LUBAC components and Caspase-8 co-immunoprecipitate upon poly(I:C) stimulation.

HaCaT cells were incubated in presence of 20µM zVAD prior to stimulation with 25µg/ml poly(I:C) for the indicated times. SHARPIN-IP was performed and co-immunoprecipitated proteins were analysed by immunoblotting as indicated. Images are representatives of at least two independent experiments. Non-specific bands are indicated by asterisk.

LUBAC, linear ubiquitin chain assembly complex; poly(I:C), polyriboinosinic:polyribocytidylic acid; HaCaT, human adult low calcium high temperature; zVAD, Z-VAD(OMe)-FMK; TLR, toll-like receptor; TRAF, TNF receptor associated factor; cIAP, cellular inhibitor of apoptosis protein; RIP, receptor interacting protein; HOIP, HOIL-1-interacting protein; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; IP, immunoprecipitation.

Given that TLR3 was hardly detectable in the Caspase-8 IP (Figure 26), the hypothesis was addressed of whether there is a transition from a TLR3-associated SC to a second complex which is devoid of the receptor. To address this hypothesis further, TLR3 was immunoprecipitated from HeLa-TLR3 cells via Flag (Figure 28A) and the same lysates were then subjected to a second IP of Caspase-8 (re-IP) (Figure 28B).

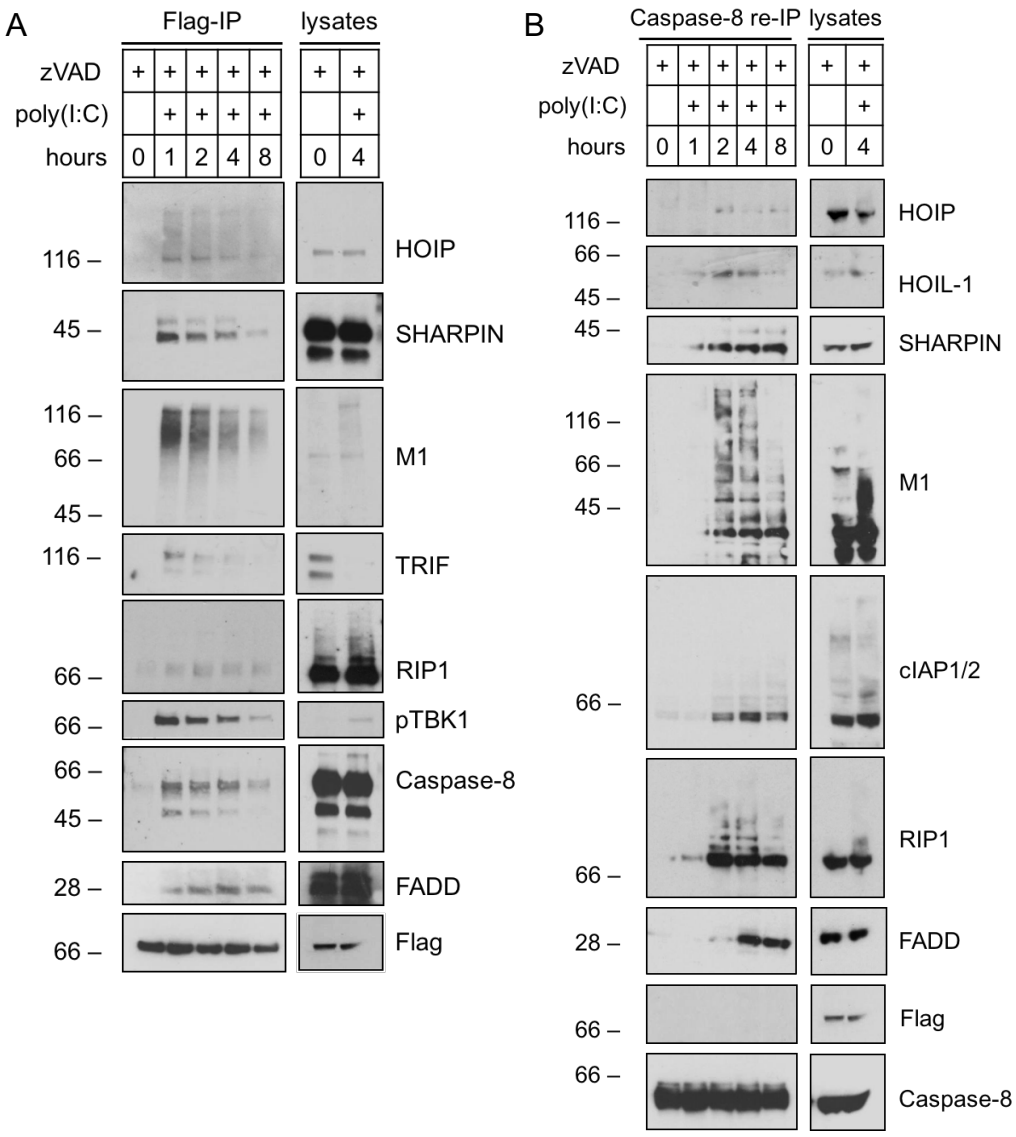

Figure 28. Identification of the TLR3-induced DISC.

(A) HeLa-TLR3 cells were stimulated with 25μg/ml poly(I:C) in presence of 20μM zVAD for the indicated times, and TLR3 was immunoprecipitated via its Flag-tag and subjected to analysis for co-immunoprecipitated proteins by immunoblotting. (B) Lysates from (A) were subjected to Caspase-8 re-IP following immunoprecipiation of TLR3 via Flag and co-immunoprecipitated proteins were analysed by immunoblotting. Experiments are representative of at least two independent experiments.

TLR, toll-like receptor; DISC, death-inducing signalling complex; HeLa, Henrietta Lacks; poly(I:C), polyriboinosinic:polyribocytidylic acid; zVAD, Z-VAD(OMe)-FMK; HOIP, HOIL-1-interacting protein; HOIL-1, Heme-oxidised IRP2 ubiquitin ligase-1; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; M1, linear ubiquitin linkages; cIAP, cellular inhibitor of apoptosis protein; TRIF, TIR domain-containing adapter protein inducing IFN-β; RIP, receptor interacting protein; TBK, TANK-binding kinase; FADD, Fas-associated protein with death domain; IP, immunoprecipitation.

This analysis revealed that also in HeLa-TLR3 cells LUBAC is recruited to TLR3 in a poly(I:C) stimulation-dependent manner and that LUBAC assembles linear ubiquitin linkages upon stimulation (Figure 28A). TRIF, RIP1, pTBK1, FADD and Caspase-8 are recruited to this complex in a stimulation-dependent manner (Figure 28A). Caspase-8 re-IP shows that RIP1, FADD, cIAPs and LUBAC, but not TLR3, associate with Caspase-8 following stimulation suggesting that there is formation of a second complex upon stimulation with poly(I:C) which is devoid of the receptor but induced by its stimulation (Figure 28B). Since this complex is composed of components involved in induction of TLR3-mediated cell death, i.e. Caspase-8, FADD, RIP1, LUBAC and cIAPs, it was termed TLR3-induced death-inducing signalling complex (DISC).

Having identified LUBAC as new component of the TLR3-SC and of the previously unknown TLR3-induced DISC, the question was addressed whether absence of LUBAC results in an altered composition of either of the two complexes. Thus, IP of TLR3 was performed following stimulation with poly(I:C) in HeLa-TLR3 WT and HOIP-deficient cells. Analysis of co-immunoprecipitated proteins revealed that absence of HOIP prevented recruitment of the other LUBAC components, SHARPIN and HOIL-1, to the TLR3-SC and that no linear ubiquitin linkages were detectable in the TLR3-SC in the absence of HOIP. However, recruitment of the other components was not majorly affected by absence of HOIP (Figure 29A). Next, Caspase-8 re-IP following IP of TLR3 from the same lysates was performed and co-immunoprecipitated proteins were analysed by immunoblotting (Figure 29B). This analysis revealed that HOIP prevents formation of the TLR3-induced DISC as association of RIP1, FADD and cIAPs with Caspase-8 was majorly increased in the absence of HOIP.

Thus, HOIP prevents formation of the TLR3-induced DISC.

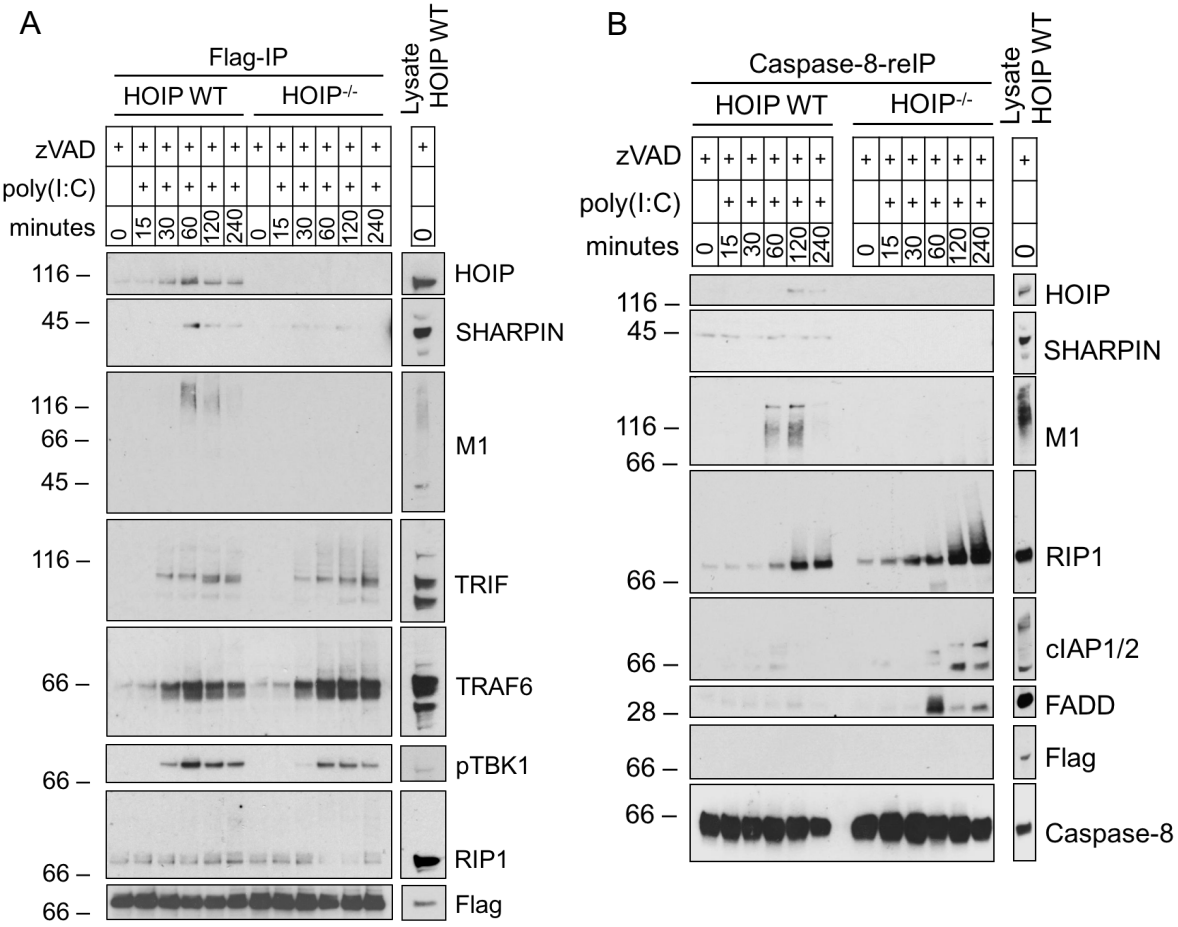

Figure 29. HOIP prevents formation of the TLR3-induced DISC.

(A) HeLa-TLR3 cells WT or deficient in HOIP were stimulated with 25μg/ml poly(I:C) in presence of 20μM zVAD for the indicated times and TLR3 was immunoprecipitated via its Flag-tag and subjected to analysis by immunoblotting for co-immunoprecipitated proteins. (B) Lysates from experiment in (A) were subjected to Caspase-8 re-IP following IP of TLR3 via Flag and co-immunoprecipitated proteins were analysed by immunoblotting. Experiments are representatives of at least two independent experiments.

HOIP, HOIL-1-interacting protein; TLR, toll-like receptor; DISC, death-inducing signalling complex; HeLa, Henrietta Lacks; WT, wildtype; poly(I:C), polyriboinosinic:polyribocytidylic acid; zVAD, Z-VAD(OMe)-FMK; SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; M1, linear ubiquitin linkages; TRIF, TIR domain-containing adapter protein inducing IFN-β; TRAF, TNF receptor associated factor; p, phospho; TBK, TANK-binding kinase; RIP, receptor interacting protein; cIAP, cellular inhibitor of apoptosis protein; FADD, Fas-associated protein with death domain; IP, immunoprecipitation.

5.4 LUBAC REGULATES TLR3 SIGNALLING *EX VIVO*

To examine whether absence of LUBAC components also influences TLR3 signalling in a setting resembling the in-vivo situation more closely, TLR3 signalling outputs were assessed in PMKs deficient in one of the LUBAC components.

First, the role of linear ubiquitination in TLR3 signalling was evaluated in PMKs isolated from WT and SHARPIN-deficient *cpdm* mice. PMKs were stimulated with poly(I:C) and secretion of the chemokines CCL5 and CCL20 was assessed in *cpdm* as compared to WT PMKs (Figure 30A). Poly(I:C) induced secretion of the chemokines CCL5 and CCL20 in WT PMKs in a concentration-dependent manner. Upon stimulation with poly(I:C) *cpdm* PMKs were found to secrete significantly less of these chemokines than WT PMKs (Figure 30A).

Analysis of viability of *cpdm* PMKs following stimulation with poly(I:C) cultured in absence versus presence of cycloheximide (CHX) revealed that SHARPIN-deficient PMKs were sensitised to poly(I:C)-induced cell death when protein synthesis was concomitantly blocked by CHX (Figure 30B). Analysis of SHARPIN protein expression confirmed that WT PMKs expressed SHARPIN protein whereas it was absent from *cpdm* PMKs (Figure 30C).

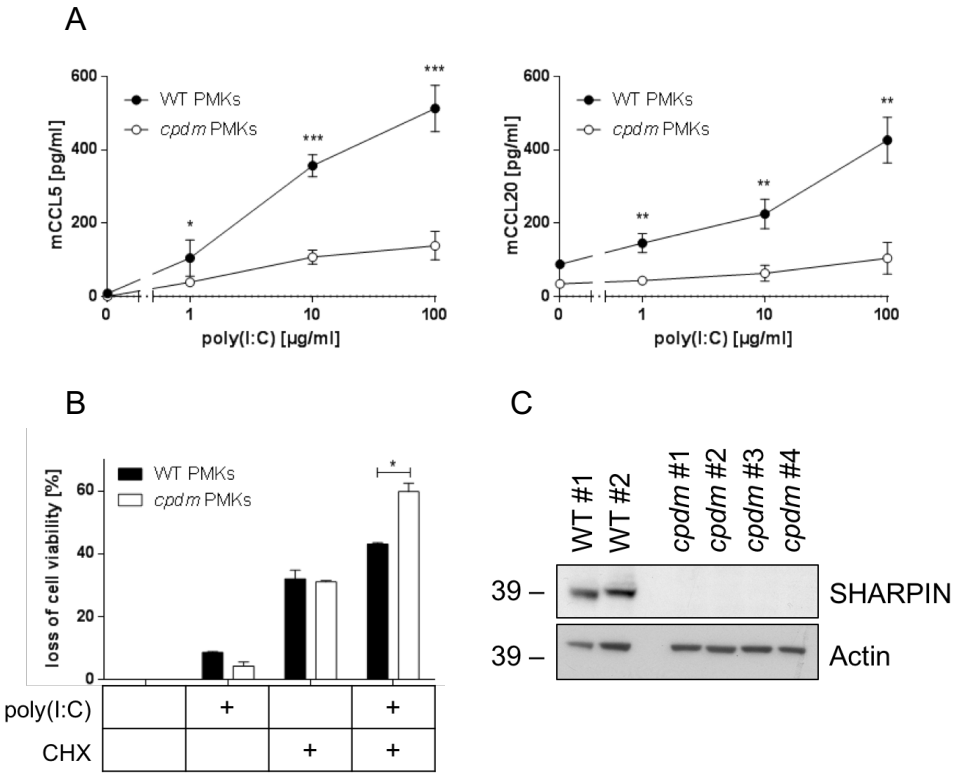

Figure 30. SHARPIN maintains poly(I:C)-induced chemokine secretion and prevents poly(I:C)-induced cell death *ex vivo*.

(A) PMKs of newborn WT (n=2) and *cpdm* (n=4) mice were stimulated with poly(I:C) at different concentrations as indicated. After 24h cell supernatants were collected and concentrations of murine CCL5 (mCCL5) and murine CCL20 (mCCL20) were determined by ELISA. (B) Cell viability was determined in PMKs from adult mice in presence versus absence of CHX upon stimulation with poly(I:C). (C) SHARPIN protein expression of PMKs from newborn WT (n=2) and *cpdm* (n=4) mice was analysed by western blot. Values are presented as mean ± SD for the indicated number of animals. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test.

SHARPIN, SHANK-associated RBCK1 homology-domain-interacting protein; poly(I:C), polyriboinosinic:polyribocytidylic acid; PMKs, primary murine keratinocytes; WT, wildtype; cpdm, chronic proliferative dermatitis mice; h, hours; CCL, chemokine (C-C motif) ligand; ELISA, enzyme-linked immune sorbent assay; CHX, cycloheximide; SD, standard deviation.

To study TLR3 signalling in absence versus presence of the central and catalytically active LUBAC component HOIP *ex vivo*, *Hoip*$^{fl/fl}$ mice were interbred with Tamoxifen-inducible Cre^{+} mice. By intercrossing *Hoip*$^{fl/fl}$ Cre^{+} males with *Hoip*$^{fl/fl}$ Cre^{-} females an offspring was generated leading to both *Hoip*$^{fl/fl}$ Cre^{+} as well as *Hoip*$^{fl/fl}$ Cre^{-} animals. PMKs of both, *Hoip*$^{fl/fl}$ Cre^{+} and *Hoip*$^{fl/fl}$ Cre^{-} mice were isolated. HOIP protein expression was blunted in PMKs of *Hoip*$^{fl/fl}$ Cre^{+} mice treated with 4-OHT whereas incubation of PMKs with 4-OHT isolated from *Hoip*$^{fl/fl}$ Cre^{-} mice did not affect HOIP protein expression (Figures 31A and B). Analysis of chemokine secretion following stimulation with poly(I:C) revealed that presence of HOIP is essential for TLR3-mediated chemokine secretion *ex vivo* as HOIP-deficient PMKs secreted substantially less CCL5 and CCL20 following stimulation as compared to PMKs expressing HOIP protein (Figure 31C). PMKs of *Hoip*$^{fl/fl}$ Cre^{-} mice stimulated with poly(I:C) showed no difference in chemokine secretion irrespective of whether the cells were cultured in absence or presence of 4-OHT (Figure 31D).

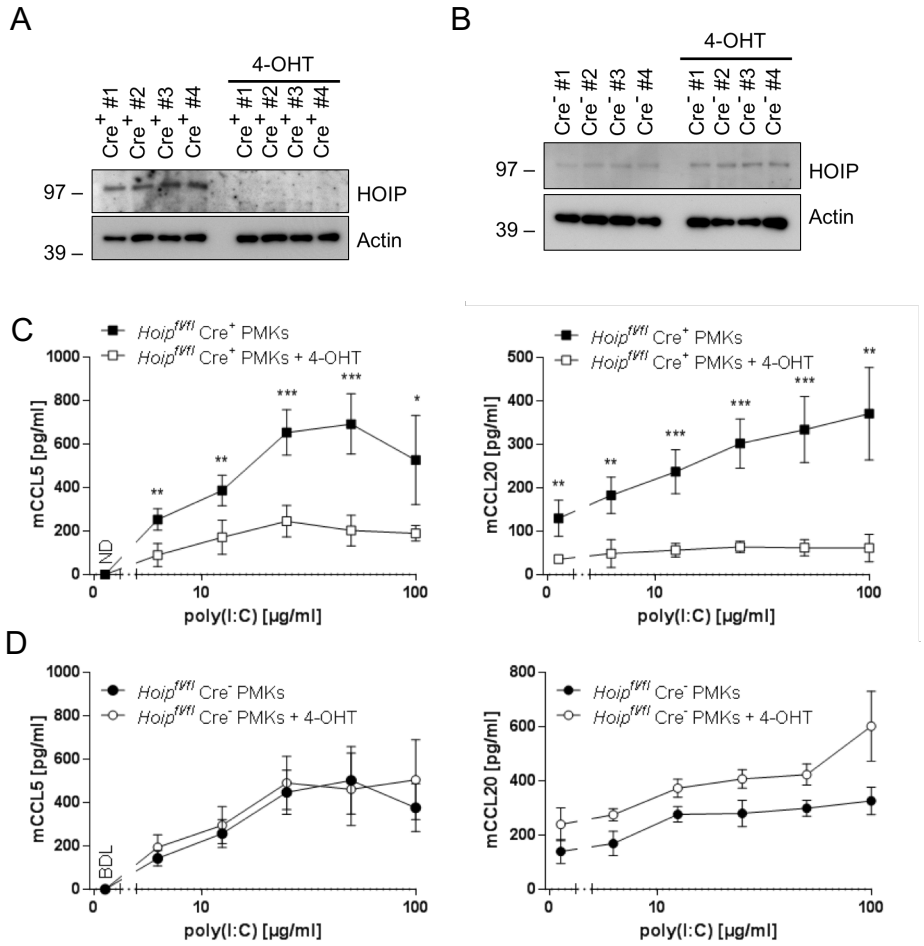

Figure 31. HOIP is essential for poly(I:C)-induced chemokine secretion *ex vivo*.

HOIP protein expression of PMKs isolated from *Hoip*$^{fl/fl}$ Cre^{+} (n=4) (A) or of PMKs isolated from *Hoip*$^{fl/fl}$ Cre^{-} (n=4) (B) newborn mice cultured in absence versus presence of 1µM 4-OHT for 72h was analysed by western blot. (C) PMKs of *Hoip*$^{fl/fl}$ Cre^{+} (n=4) newborn mice were cultured in presence of 1µM 4-OHT or not for 72h. Cells were washed and subsequently stimulated with different concentrations of poly(I:C). Cell supernatants were collected 24h later and concentrations of murine CCL5 (mCCL5) and murine CCL20 (mCCL20) were determined by ELISA. (D) PMKs of *Hoip*$^{fl/fl}$ Cre^{-} (n=4) newborn mice were cultured in absence or presence of 1µM 4-OHT for 72h. Cells were washed and subsequently stimulated with different concentrations of poly(I:C). Cell supernatants were collected 24h later and concentrations of murine CCL5 (mCCL5) and murine CCL20 (mCCL20) were determined by ELISA. Values are presented as mean ± SD for the indicated number of animals. * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; poly(I:C), polyriboinosinic:polyribocytidylic acid; PMKs, primary murine keratinocytes; fl, floxed; 4-OHT, 4-hydroxy tamoxifen; h, hours; CCL, chemokine (C-C motif) ligand; ELISA, enzyme-linked immune sorbent assay; SD, standard deviation; ND, not detectable, BDL, below detection limit.

In addition, loss of cell viability upon stimulation with poly(I:C) was assessed in both, PMKs isolated from *Hoip*$^{fl/fl}$ Cre^{+} (Figure 32A) and *Hoip*$^{fl/fl}$ Cre^{-} (Figure 32B) mice cultured in absence versus presence of 4-OHT. PMKs with inducible deletion of HOIP exhibited a significant increase in poly(I:C)-induced cell death (Figure 32A) whereas control cells neither in presence nor in absence of 4-OHT showed sensitisation to poly(I:C)-induced cell death, demonstrating that presence of HOIP is required to prevent TLR3-mediated cell death in PMKs *ex vivo*.

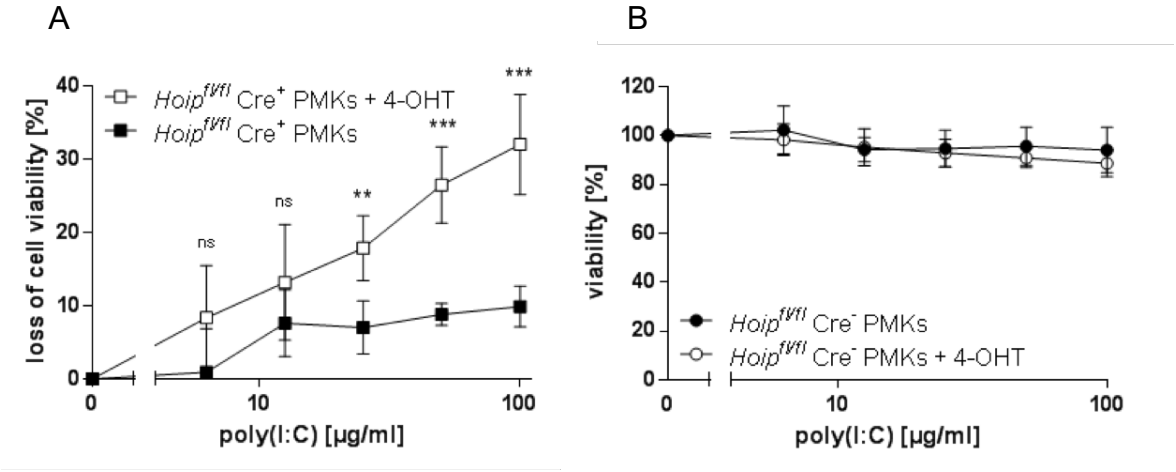

Figure 32. HOIP prevents poly(I:C)-induced cell death *ex vivo*.

(A) PMKs of *Hoip*$^{fl/fl}$ Cre^{+} newborn mice (n=4) were cultured in presence versus absence of 1µM 4-OHT for 72h. Cells were washed and subsequently stimulated with different concentrations of poly(I:C). Loss of cell viability was determined 24h later. (B) PMKs of *Hoip*$^{fl/fl}$ Cre^{-} newborn mice (n=4) were cultured in presence versus absence of 1µM 4-OHT for 72h. Cells were washed and subsequently stimulated with different concentrations of poly(I:C). Cell viability was assessed after 24h. Values are presented as mean ± SD for the indicated number of animals. ** $p < 0.01$, *** $p < 0.001$, unpaired Student's t-test.

HOIP, HOIL-1-interacting protein; poly(I:C), polyriboinosinic:polyribocytidylic acid; PMKs, primary murine keratinocytes; fl, floxed; 4-OHT, 4-hydroxy tamoxifen; h, hours; SD, standard deviation; ns, not significant.

In summary, these results indicate that presence of both, SHARPIN and HOIP are essential for the ex-vivo response of PMKs following poly(I:C) stimulation.

5.5 TLR3 DEFICIENCY AMELIORATES *CPDM* DERMATITIS

LUBAC is crucial for maintaining a balanced TLR3 signalling output by ensuring gene activation whilst controlling cell death. Since TLR3 is a known sensor of tissue damage, it was addressed whether deregulated TLR3 signalling in the absence of SHARPIN, and, thus, a fully functional LUBAC, might contribute to the chronic skin inflammation characteristic for *cpdm* mice.

In order to generate equal numbers of WT, *cpdm*, *Tlr3*$^{-/-}$ and *Tlr3*$^{-/-}$*.cpdm* littermates *Tlr3*$^{+/-}$*.cpdm*$^{+/-}$ males were interbred with *Tlr3*$^{+/-}$*.cpdm*$^{+/-}$ females. Animals were genotyped by PCR (Figure 33).

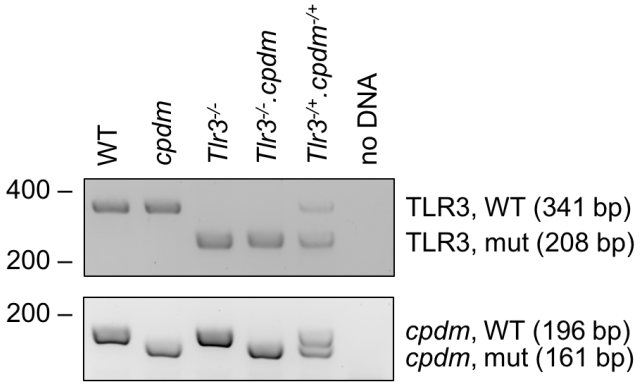

Figure 33. Generation of *Tlr3*$^{-/-}$*.cpdm* mice.

Representative PCR genotyping results of WT, *cpdm*, *Tlr3*$^{-/-}$, *Tlr3*$^{-/-}$*.cpdm* and *Tlr3*$^{+/-}$*.cpdm*$^{+/-}$ mice are shown.

TLR3 WT (341 bp) and mut (208 bp) bands and *cpdm* WT (196 bp) and mut (161 bp) bands are indicated.

TLR, toll-like receptor; cpdm, chronic proliferative dermatitis mice; PCR, polymerase chain reaction; WT, wildtype; mut, mutant; bp, base pairs.

The offspring of *Tlr3*$^{+/-}$*.cpdm*$^{+/-}$ males and females was closely monitored for signs of dermatitis development. When mice from experimental groups reached the age of 8 weeks, they were humanely killed and severity of dermatitis was assessed on 6 different body parts, namely head, neck, anterior trunk, posterior trunk, forelegs and hind legs, in a blinded manner. The score was obtained by grading each of the six above-named body parts with either lesion absent, 0 points, or lesion detectable, 1 point, accounting to a maximum of 6 points per mouse.

Strikingly, TLR3 deficiency substantially ameliorated skin inflammation of *cpdm* mice (Figure 34).

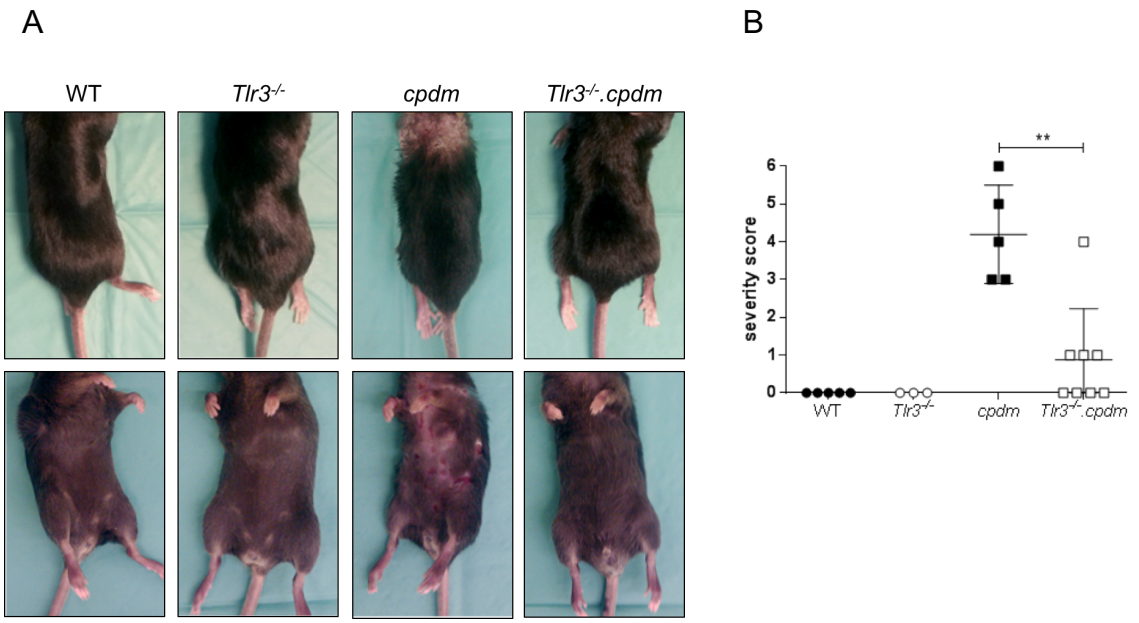

Figure 34. TLR3 deficiency ameliorates *cpdm* dermatitis.

(A) Representative pictures of 8-week-old mice of indicated genotypes are shown. (B) Severity scoring of dermatitis was performed (WT (n=5), *Tlr3*$^{-/-}$ (n=3), *cpdm* (n=5) and *Tlr3*$^{-/-}$*.cpdm* (n=8)) at the age of 8 weeks. Six different body parts (head, neck, anterior trunk, posterior trunk, forelegs and hind legs) were scored in a blinded manner. The score was obtained by grading each of the six above-named body parts with lesion absent (0 points) or lesion detectable (1 point), accounting to a maximum of 6 points/mouse. Dots represent scoring of individual mice, ± SD. ** $p < 0.01$.

TLR, toll-like receptor; cpdm, chronic proliferative dermatitis mice; WT, wildtype; SD, standard deviation.

Comparison of skin sections at the age of 8 weeks revealed no obvious differences in skin organisation between WT and *Tlr3*$^{-/-}$ mice. Interestingly, *Tlr3*$^{-/-}$*.cpdm* mice showed reduced signs of skin inflammation with less hyperkeratosis, parakeratosis and cell death as compared to *cpdm* littermates (Figure 35).

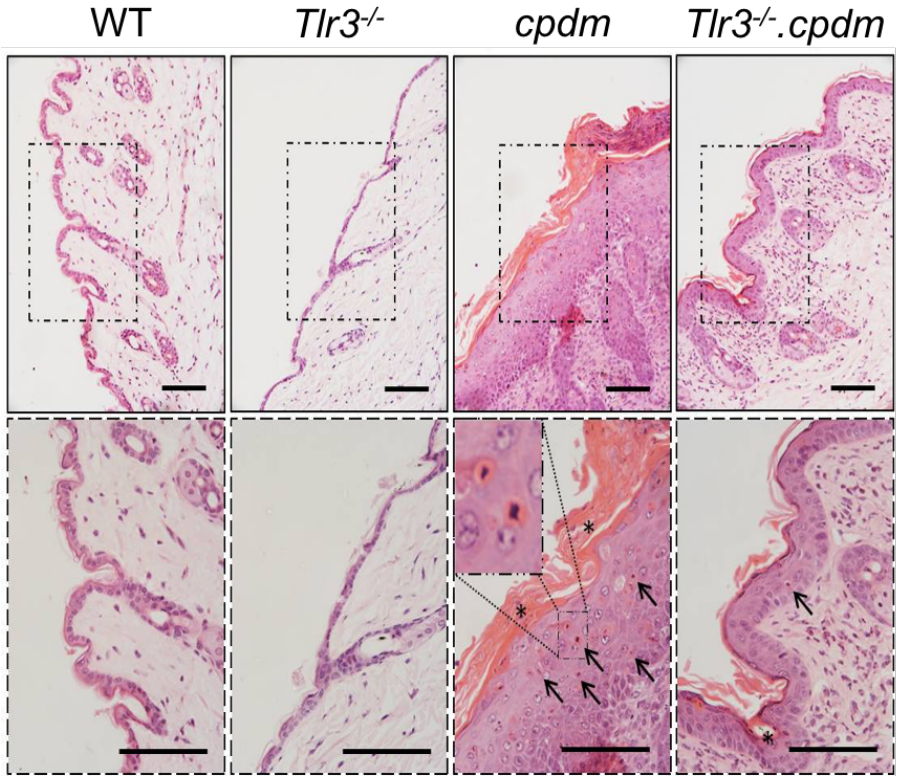

Figure 35. *Tlr3*$^{-/-}$*.cpdm* mice show reduced signs of skin inflammation.

Representative H&E stainings of skin sections of 8-week-old mice of the indicated genotypes are shown (scale bars equate to 100 µm). Parakeratosis and dead cells are indicated by asterisk and arrow, respectively.

TLR, toll-like receptor; cpdm, chronic proliferative dermatitis mice; H&E, haematoxylin & eosin.

Thus, TLR3 signalling plays a role in the pathogenesis of *cpdm* dermatitis.

In addition, spleens and livers of all four experimental groups were analysed (Figure 36). Analysis of spleens at the age of 8 weeks revealed that WT and *Tlr3*$^{-/-}$ mice have comparable spleen sizes whereas *cpdm* mice present with splenomegaly. Interestingly, *Tlr3*$^{-/-}$*.cpdm* also present with an enlarged spleen comparable in size to those of *cpdm* mice (Figures 36A).

In addition, microscopic analysis of tissue sections of spleens and livers revealed that whilst WT and *Tlr3*$^{-/-}$ mice have normal spleen architecture and no liver inflammation, *cpdm* and *Tlr3*$^{-/-}$*.cpdm* mice suffer from comparable inflammatory cell infiltrates in their livers and show the same defect in lymphoid tissue organisation.

Thus, the immunological phenotype of *cpdm* mice is not corrected by genetic co-ablation of TLR3.

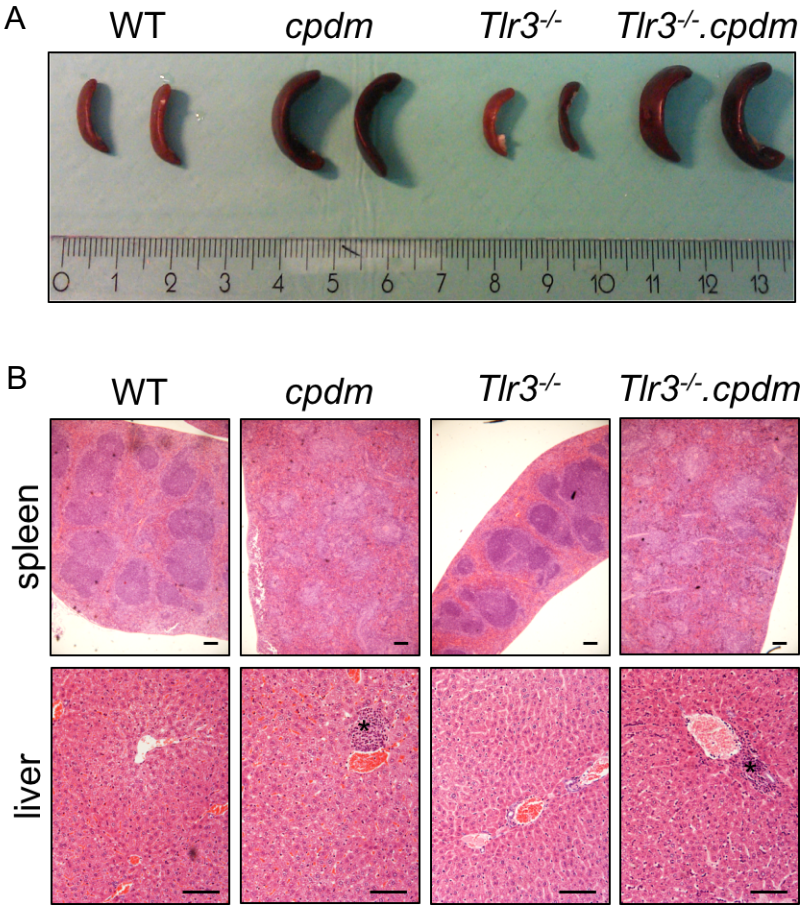

Figure 36. TLR3 deficiency does not correct the immunological phenotype of *cpdm* mice.

(A) Spleens of animals of the indicated genotype at the age of 8 weeks are shown. (B) Representative H&E stainings of spleen and liver sections (scale bars equate to 100 µm) of 8-week-old mice of the indicated genotypes are shown. Immune cell infiltrates are indicated by asterisk.

TLR, toll-like receptor; cpdm, chronic proliferative dermatitis mice; H&E, haematoxylin & eosin.

6 DISCUSSION

6.1 LUBAC BALANCES TLR3-MEDIATED SIGNALLING OUTPUTS

TLR3 is known to mediate activation of a number of different gene activatory signalling pathways, amongst them NF-κB, MAPK and IRF3 signalling [6], leading to the transcription of target genes ultimately culminating in the secretion of cytokines, chemokines and IFNs. Activation of TLR3 by dsRNA or its synthetic ligand, poly(I:C), has not only been described to lead to gene activation but, in addition, to result in TLR3-mediated cell death [27, 28]. Apart from TLR3 also the cytosolic receptors retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) are known to sense dsRNA [92]. Poly(I:C)-induced gene activation and subsequent secretion of cytokines and chemokines, and cell death critically depend on presence of TLR3 in the keratinocyte line HaCaT (Figures 4 – 6) ruling out the possibility that poly(I:C)-induced effects are mediated by stimulation of RIG-I or MDA5 in this cellular setting. Cell death induced by poly(I:C) in HaCaT cells was found to be mainly apoptotic (Figure 7) in line with previous reports [27, 107]. In addition, poly(I:C) stimulation resulted in TLR3-dependent formation of a Caspase-8-containing signalling platform (Figure 8). Altogether, this data shows that HaCaT cells are a suitable cellular system to further assess the role of LUBAC in TLR3 signalling.

To address the question whether presence of LUBAC is crucial for TLR3 signalling, expression of the different LUBAC components was either downregulated using RNAi or HOIP knocked out using CRISPR/Cas9 [85]. Analysis of protein expression of LUBAC components revealed that transient downregulation of one LUBAC component affected protein expression of the other two (Figure 9) but that HOIP-deficient cells generated using CRISPR/Cas9 did not show any difference in expression levels of SHARPIN or HOIL-1 as compared to control (Figure 12). This difference is likely due to compensation of the cells for the permanent loss of HOIP. Analysis of TLR3 signalling outputs upon stimulation with poly(I:C) demonstrated that presence of LUBAC is essential for maintaining TLR3-mediated NF-κB and MAPK activation, and that LUBAC is required for sufficient cytokine and chemokine secretion following stimulation with poly(I:C) (Figures 10, 11 and 13 – 16). Interestingly, absence of HOIP as the central and catalytically active LUBAC component had the most striking negative effect on gene activation.

In addition to controlling NF-κB and MAPK signalling, presence of LUBAC was also required for secretion of IFN-β (Figure 17). Controversially, IRF3 phosphorylation, a prerequisite of IFN production, was not markedly affected in HaCaT cells in which HOIP protein expression was either inhibited by RNAi or completely absent due to deletion by CRISPR/Cas9 (Figure 10 and 13). This observation is in line with reports showing that IFN-β secretion requires both, activation of NF-κB and IRF3 to initiate IFN-β gene expression [95, 94]. Thus, LUBAC is required for TLR3-mediated IFN-β secretion by enabling NF-κB activation upon poly(I:C) stimulation in HaCaT cells.

Noteworthy and on the contrary, analysis of gene activatory signalling pathways in HeLa cells showed that in these cells presence of HOIP is required for induction of phosphorylation of IRF3 (Figure 14) indicating that LUBAC differentially regulates IRF3 phosphorylation in different cell lines.

In line with the above, recent observations have pointed to a potential role of LUBAC components in the regulation of poly(I:C)-induced signalling, as it was reported that absence of SHARPIN in bone marrow-derived dendritic cells negatively affected poly(I:C)-induced cytokine and IFN induction [106] and that fibroblasts isolated from human HOIL-1-deficient patients secreted less IFN-β in response to poly(I:C) [7].

That IFN production is seemingly differentially regulated by LUBAC in different pathways is highlighted by two recent studies in which a negative role for LUBAC in IFN induction upon activation of the RIG-I signalling pathway was reported [4, 46]. Belgnaoui *et al.* showed, contrary to the results obtained here which identified LUBAC as positive regulator of IFN production, that in the RIG-I signalling pathway, LUBAC downregulates IFN secretion by attaching linear ubiquitin linkages to NEMO thereby leading to enhanced NF-κB signalling whilst negatively affecting IFN production [4]. As a consequence thereof, viral replication was diminished in SHARPIN-deficient mouse embryonic fibroblasts [4]. In the second study, Inn *et al.* proposed that LUBAC targets tripartite motif containing 25 leading to its degradation thereby restricting IFN production [46].

Moreover, the LUBAC component HOIL-1 has previously been identified to directly attach ubiquitin molecules to IRF3 leading to its degradation and, thus, to negatively affect IFN production following Sendai virus infection [111]. It is unclear from this study, however, what kind of linkages were attached to IRF3 by HOIL-1 and whether HOIL-1 acts together with SHARPIN and HOIP in this scenario or independently of the other LUBAC components.

Interestingly, linear ubiquitination of IRF3 has been identified to be required for RIG-I-mediated induction of apoptosis after viral infection [17], suggesting opposing roles for LUBAC in RIG-I- versus TLR3-mediated apoptosis, as LUBAC plays a crucial role in prevention of aberrant TLR3-induced cell death (Figures 18 and 19).

It has recently been shown that absence of the two E3 ubiquitin ligases cIAP1 and 2 sensitises cells to poly(I:C)-induced cell death [28, 107]. This cell death in the absence of cIAPs was described to be mainly necroptotic [28]. TNF-induced cell death was identified to be increased and switched from apoptosis to necroptosis in the absence of SHARPIN [32]. This suggests that ubiquitin linkages do not only control for the amount of cell death but also for its type. Interestingly, and although absence of linear linkages renders cells more sensitive to TLR3-mediated cell death (Figures 18 and 19), absence of HOIP as the central and catalytically active LUBAC component did not alter the type of cell death induced by poly(I:C) stimulation which remained mainly apoptotic (Figures 20 and 21).

Given the fact that it has recently been shown that TNF-induced cell death is increased in the absence of LUBAC components [32], and that poly(I:C) stimulation resulted in secretion of TNF (Figures 5 and 11), it was evaluated whether autocrine TNF was responsible for cell death induction in HaCaT cells following stimulation with poly(I:C) in absence of linear ubiquitination (Figure 22B). HT29 cells have recently been shown to die by autocrine TNF-induced necroptosis in presence of the pan-caspase inhibitor zVAD and SM [37], small molecule inhibitors known to counteract IAPs [60, 99, 101], and, thus, served as internal control for the efficacy of Enbrel (Figure 22A). These experiments demonstrate that poly(I:C)-induced cell death occurs independently of TNF irrespective of whether HOIP is present or absent (Figure 22B).

A recent publication identified LUBAC to be required for antigen receptor-mediated NF-κB activation essential for survival of activated B-cell diffuse large B-cell lymphoma [23]. The authors showed that expression of a catalytically inactive form of LUBAC was sufficient to maintain antigen receptor-induced NF-κB signalling indicating that LUBAC's ligase activity was not required to mediate NF-κB activation [23].

Interestingly, expression of a catalytically inactive form of HOIP resulted in diminished chemokine secretion following stimulation with poly(I:C) (Figure 23A) and rendered cells more sensitive to poly(I:C)-induced cell death (Figure 23B)

suggesting that HOIP maintains TLR3-mediated gene activation and prevents TLR3-induced cell death not by its mere presence but via the assembly of linear ubiquitin linkages.

6.2 LUBAC FORMS PART OF THE TLR3-SC & THE TLR3-INDUCED DISC

Given that IP of TLR3 is complicated due to lack of specific TLR3-directed antibodies, HaCaT and HeLa cell lines stably expressing a Flag-tagged version of TLR3 were generated (Figure 24). Via this approach the question whether LUBAC directly regulates TLR3 signalling outputs by forming part of the TLR3-SC was addressed. LUBAC was identified as new component of the TLR3-SC in HaCaT cells (Figure 25). Upon poly(I:C) stimulation, LUBAC components were recruited to the TLR3-SC along with other components implicated in TLR3 signalling, amongst them TRIF, RIP1, NEMO, TBK1, TRAF6, FADD and Caspase-8 [27, 30, 41, 47, 66, 107]. LUBAC generates linear ubiquitin linkages in the TLR3-SC as demonstrated by stimulation-dependent presence of M1 linkages (Figure 25). Next, IP of Caspase-8 was performed to isolate the endogenous TLR3-SC (Figure 26). In line with the above, LUBAC components associated with Caspase-8 in a stimulation-dependent manner (Figure 26). Interestingly, TLR3 was hardly detectable in the Caspase-8 IP. Next, SHARPIN was immunoprecipitated following stimulation with poly(I:C) (Figure 27). Analysis of co-immunoprecipitated proteins revealed that apart from the other LUBAC components, also proteins that have been implicated to play a role in TLR3 signalling were recruited to SHARPIN. Of note, also TLR3 was clearly detectable. This led to the hypothesis of whether Caspase-8 only transiently associates with TLR3, and whether TLR3-induced cell death is executed by a second signalling platform not associated to TLR3 but induced by its stimulation, similar to complex II in TNF signalling [67]. Thus, the TLR3-SC was isolated from HeLa cells following stimulation with poly(I:C) via Flag-tagged TLR3. Subsequently, IP of Caspase-8 from the same lysate (re-IP of Caspase-8) was performed indeed revealing the presence of a second signalling platform comprising LUBAC, RIP1, Caspase-8, FADD and cIAPs (Figure 28). All components of this second signalling platform have been described to be involved in the induction of TLR3-mediated cell death [27, 28]. Thus, this complex was termed TLR3-induced DISC. Next, it was analysed whether absence of the central LUBAC component HOIP alters complex composition of the TLR3-SC thereby explaining how LUBAC controls TLR3-induced

signalling outputs (Figure 29). HOIP absence prevented recruitment of SHARPIN and HOIL-1, and, consequently, no linear ubiquitin linkage formation was detectable in the TLR3-SC (Figure 29). Surprisingly, the other components were recruited to TLR3 with the same kinetics and efficiency. Comparing the TLR3-induced DISC in absence and presence of HOIP, however, revealed that formation of this second complex was strongly enhanced in the absence of LUBAC, thereby providing a possible explanation how LUBAC limits cell death induction by TLR3 (Figure 29).
Previously, poly(I:C)-induced cell death was suggested to be mediated by a platform consisting of Caspase-8, RIP1, cIAPs, TRIF and TLR3 [27]. In addition, cIAPs have been identified to prevent formation of a poly(I:C)-induced signalling platform termed 'Ripoptosome' consisting of RIP1 as its core component, Capase-8 and TRIF [28].
In these studies the poly(I:C)-induced SC was isolated by IP of Caspase-8 making a discrimination between the TLR3-SC and the TLR3-induced DISC, which both contain Caspase-8, impossible. Given that the TLR3-induced DISC is induced by TLR3 stimulation, but devoid of TLR3, it is likely that ligation of TLR3 results in recruitment of Caspase-8 which only transiently associates with TLR3 to subsequently form part of the TLR3-induced DISC. This sequence of events parallels TNFR1 signalling in which complex II, originating from the TNFR1-SC known to induce gene activation, was identified to induce cell death [67].
The question of whether the TLR3-induced DISC emerges from the TLR3-SC over time or forms independently in parallel cannot yet be definitely answered. Thus, future work will be required to provide the ultimate proof for this hypothesis.

For TNF signalling it has been demonstrated that recruitment of LUBAC is strictly dependent on presence of TRAF2, TNFR1-Associated Death Domain Protein and cIAP1/2 [32, 35]. How LUBAC is recruited to the TLR3-SC remains to be determined.
The reversal of ubiquitination, deubiquitination, is carried out by so-called deubiquitinating enzymes (DUBs). Linear ubiquitin linkages formed by LUBAC have recently been identified to be specifically cleaved by a DUB called OTU deubiquitinase with linear linkage specificity (OTULIN) [50, 82]. Recently, it has been shown that LUBAC activity in the TNFR1-SC is tightly controlled by the DUBs CYLD and A20 [22]. Which DUBs control LUBAC activity in the TLR3-SC and the TLR3-induced DISC remains to be identified. Moreover, which component/s is/are targeted by linear ubiquitination in the TLR3-SC or the TLR3-induced DISC is

currently unknown. Targets of linear ubiquitination have previously been identified for components in other innate immune signalling pathways [21, 25, 29, 32, 83]. Within these signalling pathways, NEMO, RIP1, RIP2, interleukin-1 receptor-associated kinase 1 and apoptosis-associated speck-like protein containing a carboxy-terminal CARD have been identified as substrates for linear ubiquitination [25, 29, 32, 83], reviewed in [114]. As TLR3 signalling seems to parallel TNF signalling in many aspects, it is, of course, tempting to speculate that in TLR3 signalling LUBAC is recruited and controlled in a similar way to TNF signalling, and that these two signalling pathways also share the same LUBAC targets.

6.3 LUBAC IS CRUCIAL FOR TLR3 SIGNALLING *EX VIVO* AND *IN VIVO*

Having identified LUBAC as new component of the TLR3-SC and of the TLR3-induced DISC, it was next evaluated how absence of LUBAC components influences signalling in primary cells *ex vivo*. Given that absence of SHARPIN results in dermatitis development, PMKs were chosen for further analysis. Primary cells deficient in SHARPIN showed defects in poly(I:C)-induced chemokine production as compared to WT PMKs (Figure 30). SHARPIN deficiency alone, however, did not sensitise to poly(I:C)-induced cell death unless protein translation was blocked by co-application of CHX, in line with previous reports in which TNF-induced cell death in presence and absence of SHARPIN was evaluated [53]. This suggests that gene activation in the absence of SHARPIN is still sufficient to produce anti-apoptotic factors preventing cell death of SHARPIN-deficient PMKs *ex vivo*. HOIP deficiency was shown to result in embryonic lethality [74]. Thus, in order to study the role of HOIP absence *ex vivo*, PMKs of Tamoxifen-inducible *Hoip*$^{fl/fl}$ Cre^{+} and *Hoip*$^{fl/fl}$ Cre^{-} mice were isolated, incubated or not with 4-OHT and subsequently stimulated with poly(I:C). HOIP-deficient PMKs showed diminished chemokine secretion following stimulation with poly(I:C) as compared to WT cells (Figure 31). Absence of HOIP also resulted in increased susceptibility to poly(I:C)-induced cell death, even in the absence of CHX, indicating that HOIP presence is even more crucial than presence of SHARPIN for prevention of TLR3-induced cell death (Figure 32). Important controls from *Hoip*$^{fl/fl}$ Cre^{-} mice showed that 4-OHT treatment neither affected HOIP protein expression nor chemokine production, or cell death (Figures 31 and 32).

TLR3's role in the pathogenesis of autoimmune and inflammatory disorders such as rheumatoid arthritis, tissue necrosis or solar injury is well established [5, 12, 16]. The exact role of TLR3, however, remains controversial [54, 55, 104, 112]. Whereas wound healing was shown to critically depend on TLR3-mediated inflammation [54], deleterious effects of acute and chronic tissue damage have also been identified to be caused by TLR3 activation [5, 12, 16].

Mice deficient in TLR3 show normal appearance, growth, size, fertility and lifespan, and show no obvious behavioural abnormalities [2]. In order to evaluate whether TLR3 served as sensor of tissue damage in *cpdm* mice, and whether deregulated TLR3 signalling in the absence of SHARPIN contributed to *cpdm* pathogenesis, mice deficient in TLR3 were interbred with SHARPIN-deficient mice (Figure 33). Interestingly, co-ablation of *Tlr3* ameliorated *cpdm* dermatitis, identifying TLR3 signalling as contributing factor to *cpdm* pathogenesis (Figures 34 and 35). Further investigation recently revealed that disrupted skin architecture of *cpdm* mice was rescued by genetic co-ablation of *Tlr3* [115]. In addition, it was demonstrated that dsRNA is highly abundant in inflamed skin of *cpdm* mice whereas there is significantly less dsRNA in *Tlr3*$^{-/-}$*.cpdm* skin suggesting that presence of TLR3 contributes to release of DAMPs during the course of *cpdm* dermatitis [115]. This result further supports the role of TLR3 as a damage sensor and the concept of DAMP-induced inflammation as a contributor to disease pathogenesis. Interestingly, TNF deficiency completely prevented inflammation in *cpdm* mice [32] whilst genetic ablation of TLR3, though exerting a substantial effect, only ameliorated it.

Thus, it is likely that absence of SHARPIN results in TNF-induced death of keratinocytes [32], inducing an initial release of different DAMPs, including dsRNA, in turn resulting in activation of TLR3, which, in the absence of SHARPIN triggers further release of DAMPs, including more dsRNA, because of a further increase in aberrant cell death [115].

As TNF-induced cell death results in release of all different kinds of DAMPs it may well be that apart from TLR3 also other damage sensors play a role in fueling the inflammation in SHARPIN-deficient mice explaining why absence of TLR3, though substantially ameliorating *cpdm* skin inflammation, fails to completely rescue the skin phenotype in contrast to TNF deficiency [115]. Interestingly, whilst TNF deficiency also protected from liver inflammation induced by absence of SHARPIN, TLR3 deficiency did not, suggesting that the mechanism resulting in chronic liver

inflammation in *cpdm* mice is different from the one resulting in dermatitis development and does not involve stimulation of TLR3 (Figure 36). Future work will be required to elucidate the precise sequence of events in the pathogenesis of dermatitis and organ inflammation of SHARPIN-deficient mice which will allow a better understanding of how perturbed linear ubiquitination results in chronic inflammation by affecting different signalling pathways in different tissues.

As TLR3 senses dsRNA, an intermediate replicate of most viruses, the role of TLR3 in the pathogenesis of viral infections by all different kinds of viruses has been evaluated in the past [15, 55, 78, 104, 112]. The precise role of TLR3 in innate immunity to viral infection remains controversial and only partially resolved [75]. On the one hand, TLR3 has been implicated in host protection against certain viruses: Patients deficient in TLR3 and downstream signaling molecules, i.e. TRIF, TRAF3, TBK1 or IRF3 have been identified to be highly susceptible to HSV 1 encephalitis [3, 38, 76, 84, 112, 113]. In addition, deficiency in unc-93 homolog B1, a protein that is essential for endosomal TLR signalling, also results in increased susceptibility to HSV 1-induced encephalitis [15]. Furthermore, infantile susceptibility to rotavirus infection was suggested to be due to low expression of TLR3 during infancy [78]. Controversially, presence of TLR3 in the context of infection with West Nile Virus was reported to be detrimental causing lethal encephalitis due to TLR3-mediated disruption of the blood-brain-barrier integrity thereby allowing viral entrance to the brain [104]. A missense mutation in the *Tlr3* gene was identified in a patient with IAV-associated encephalopathy [40], and children with TLR3 polymorphisms have an increased risk of pneumonia when infected by the pandemic A/H1N1/2009 influenza virus [26]. In addition, TLR3 deficiency was shown to protect mice from inflammation-induced lethality by IAV [55]. This data was later supported by the finding of Leung *et al.* who showed that different strains of IAV show differential dependency on TLR3 [59].

Patients with LUBAC deficiency due to mutations in either *HOIL1* or *HOIP* genes have previously been shown to present with a controversial phenotype consisting of amylopectinosis, autoinflammation and immunodeficiency [7, 8]. Two independent studies identified truncating mutations in *HOIL1* to be causative for a phenotype encompassing muscular weakness, progressive cardiomyopathy and signs of amylopectinosis, however none of the patients suffered from severe immunodeficiency or overt hyperinflammation [71, 103]. The authors speculated

that this discrepancy to the LUBAC-deficient patients described by Boisson *et al.* may be due to different sites of the mutations. Variability seen in the phenotype of patients with the same genetic disorder may also come from the human virome as suggested by MacDuff *et al.* who showed that chronic viral infection results in activation of the innate immune system thereby complementing immunodeficiency of mice deficient in e.g. HOIL-1 to bacterial infections [63]. Of note, human patients with deficiency in SHARPIN have not yet been described. Given the above, it is tempting to speculate that presence of Sharpin is also required for host protection during the course of an infection. Based on the results obtained in this thesis establishing the crucial role of LUBAC in balancing TLR3 signalling outputs, and in skin homeostasis, it was further evaluated whether presence of SHARPIN plays a role during the course of infection with IAV [115]. Of note, both, TLR3 and SHARPIN are required to initiate an adequate host response to IAV infection [115]. It was suggested that TLR3-deficient mice die due to increased viral load whereas SHARPIN-deficient mice show diminished disease tolerance due to increased TLR3-mediated cell death in their lungs [115]. Proof for the hypothesis that cell death is the cause of death of SHARPIN-deficient mice when infected with IAV remains to be provided. Interestingly, SHARPIN deficient mice show signs of autoinflammation and immunodeficiency, and, thus, present with a similar phenotype as HOIL-1- and HOIP-deficient patients. The autoinflammatory phenotype of SHARPIN-deficient mice is, however, characterized by severe skin inflammation, whereas the described patients deficient in HOIL-1 or HOIP appear not to suffer from inflammatory skin symptoms. To answer the question whether the immunodeficient and autoinflammatory phenotypes of HOIL-1- or HOIP-deficient patients are related to, or completely independent of, the immunodeficient and autoinflammatory symptoms of SHARPIN-deficient mice will require further analysis. It is tempting to speculate that deregulated TLR3 signalling may contribute to the immunodeficiency and autoinflammation observed in patients with LUBAC deficiency, and, although none of the patients with LUBAC deficiency identified so far suffered from IAV infection, the outcome of TLR3-triggering viral infections may be worse in patients with deficiency in LUBAC components.

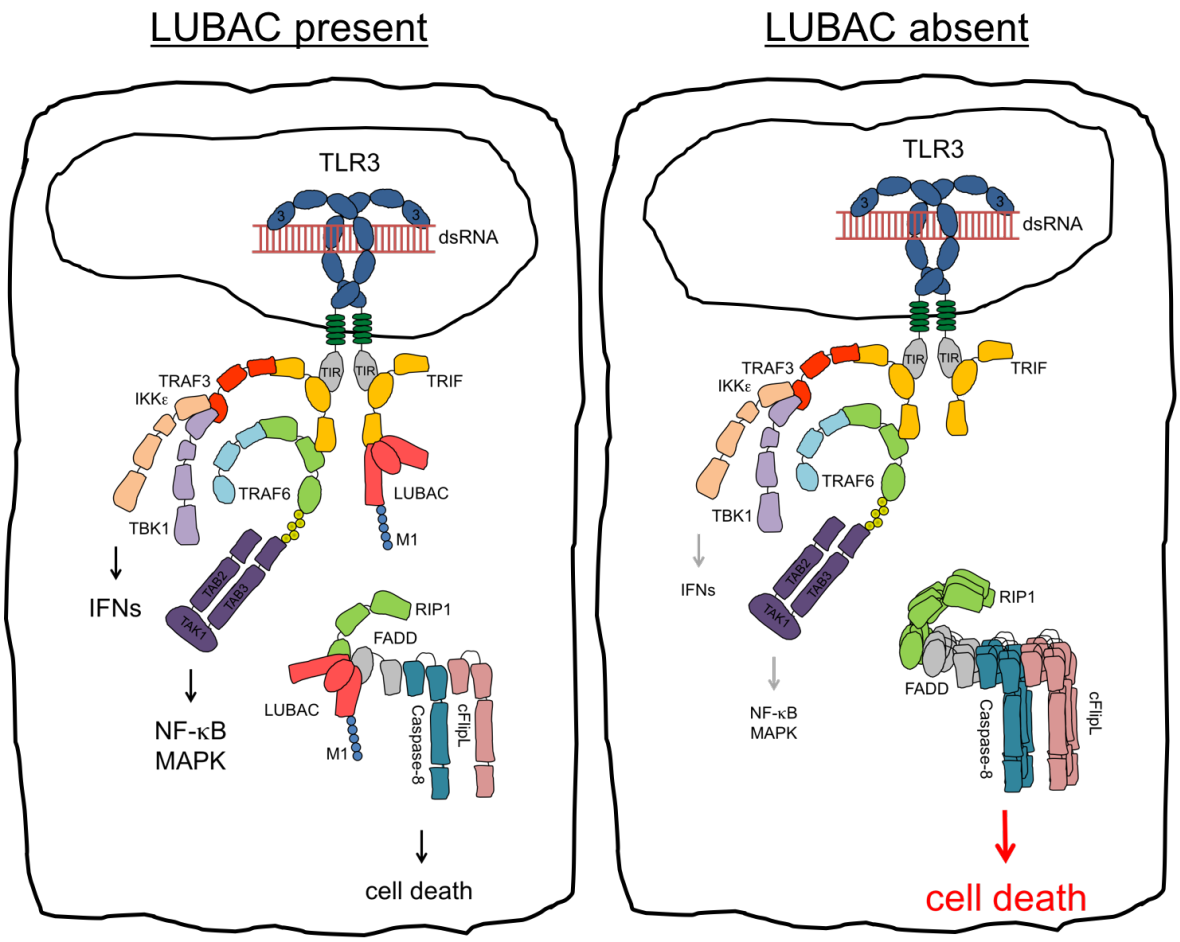

Figure 37. The TLR3 signalling pathway is tightly controlled by LUBAC.

The TLR3 signalling pathway is activated by ligation of TLR3 with its ligand dsRNA. In presence of LUBAC, TLR3 induces gene activation via formation of the TLR3-SC as well as cell death via formation of a secondary cytosolic platform termed TLR3-induced DISC. In the absence of LUBAC, composition of the TLR3-SC is mostly unaltered. However, gene activation is reduced whereas cell death is increased due to enhanced formation of the TLR3-induced DISC.

TLR, toll-like receptor; LUBAC, linear ubiquitin chain assembly complex; ds, double-stranded; TLR3-SC, TLR3-signalling complex; DISC, death-inducing signalling complex; TRIF, TIR domain-containing adapter protein inducing IFN-β; TRAF, TNF receptor associated factor; IKK, IκB kinase; TBK, TANK-binding kinase; TAK, TGF β-activated kinase; TAB, TAK binding protein; M1, linear ubiquitin linkages; RIP, receptor interacting protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK, mitogen-activated protein kinase; IFNs, interferons; cFlip, cellular FLICE-like inhibitory protein; FADD, Fas-associated protein with death domain.

6.4 Conclusion & Outlook

This thesis establishes linear ubiquitination mediated by the tripartite complex LUBAC comprising HOIP, HOIL-1 and SHARPIN as an important regulator of TLR3 signalling. LUBAC is crucial for maintaining TLR3-mediated gene activation whilst limiting cell death induction. Furthermore, LUBAC is identified as novel component of the TLR3-SC. In addition, this thesis provides evidence that TLR3-induced cell death is executed by a second signalling platform, termed TLR3-induced DISC, which is not associated to TLR3, but whose formation is induced by TLR3 stimulation. Formation of the TLR3-induced DISC is tightly controlled by linear ubiquitination as absence of HOIP results in enhanced formation of this complex thereby providing a possible explanation why TLR3-induced cell death is enhanced in the absence of LUBAC. Since TLR3 has been implicated in the detection of sterile tissue damage [5, 12, 16, 48, 70], it was further evaluated whether deregulated TLR3 signalling in the absence of the LUBAC component SHARPIN contributes to *cpdm* skin pathogenesis. Indeed, TLR3 deficiency ameliorated the chronic inflammatory skin phenotype of *cpdm* mice suggesting that LUBAC is crucial for maintaining tissue homoeostasis by balancing TLR3 signalling outputs.

We are only beginning to understand the role of linear ubiquitination in human disease pathogenesis. Very recently, some of the patients with mutations in the *HOIL1* or *HOIP* genes were identified to suffer from immunodeficiency and autoinflammation [7, 8, 71, 103]. Given the crucial role of LUBAC in controlling TLR3 signalling outputs it is tempting to speculate that the outcome of TLR3-triggering infections may be worse in these patients, and that deregulated TLR3 signalling may also contribute to their autoinflammatory symptoms. Causal therapy is not yet available for these patients. Understanding the exact underlying molecular mechanisms causing the controversial phenotype of these patients may help to identify possible therapeutic targets. Whether stabilisation of linear ubiquitin linkages via inhibition of linear ubiquitin linkage-specific DUBs may help to reduce autoinflammation and immunodeficiency in LUBAC-deficient patients needs to be evaluated in the future.

7 SUMMARY

Central question: Recently, linear ubiquitination mediated by the linear ubiquitin chain assembly complex (LUBAC), comprising SHANK-associated RBCK1 homology-domain-interacting protein (SHARPIN), Heme-oxidised IRP2 ubiquitin ligase-1 (HOIL-1) and HOIL-1-interacting protein (HOIP), was identified as critical regulator of inflammation. Toll-like receptor (TLR) 3 is activated by double-stranded RNA (dsRNA) and plays a crucial role in the detection of both, viral infection and tissue damage. TLR3 signalling can be both, beneficial and detrimental for the host, depending on the respective setting, for yet unknown reasons. The central question was whether linear ubiquitination, a crucial regulator of inflammation, might be a central regulator of TLR3 signalling thereby providing a possible explanation why TLR3 signalling, if not accurately balanced, results in host-harming signalling outputs.

Material & Methods: cell lines: human adult low calcium high temperature (HaCaT), HT29, Phoenix-AMPHO, HEK293FT, Henrietta Lacks (HeLa); mice: *Tlr3*$^{-/-}$ (9675, B6N.129S1-Tlr3^{tm1Flv}/J), *cpdm* (7599, C57BL/KaLawRij-*Sharpin*^{cpdm}/RijSunJ) and Tamoxifen-inducible Cre (4682, B6.Cg-Tg(CAG-cre/Esr1*)5Amc/J) mice; cell culture, isolation and culture of primary murine keratinocytes (PMKs); generation of cell lines stably expressing exogenous proteins; generation of knockout cell lines; cell viability and cell death assays; western blotting; immunoprecipitation; enzyme-linked immunosorbent assay; RNA interference; histology.

Results: LUBAC was identified as crucial regulator of TLR3 signalling enabling TLR3-mediated gene activation whilst preventing TLR3-induced cell death. In addition, all three LUBAC components were identified as new components of the TLR3-signalling complex (TLR3-SC). Moreover, LUBAC formed also part of the previously unrecognised TLR3-induced death-inducing signalling complex (DISC) which is devoid of TLR3 but whose formation is induced by TLR3 stimulation. In the absence of HOIP, TLR3-induced DISC formation is enhanced thereby providing the molecular explanation how LUBAC prevents TLR3-mediated cell death. In addition, further analysis of PMKs deficient in either SHARPIN or HOIP revealed decreased gene activation upon stimulation of TLR3 and sensitisation to TLR3-mediated cell death as compared to wildtype (WT) cells, in line with the results obtained *in vitro*. Mice with perturbed linear ubiquitination due to absence of the LUBAC component

SHARPIN suffer from severe chronic skin inflammation and are therefore called *chronic proliferative dermatitis mice* (*cpdm*). Co-ablation of *Tlr3* ameliorated dermatitis caused by SHARPIN deficiency identifying deregulated TLR3 signalling in the absence of SHARPIN as contributing factor to *cpdm* pathogenesis.

Discussion: LUBAC is a central regulator of TLR3-induced signalling outputs *in vitro* and *ex vivo*, and forms part of both, the TLR3-SC and the previously unrecognized TLR3-induced DISC. LUBAC prevents TLR3-induced cell death by restricting TLR3-induced DISC formation. Whether the TLR3-induced DISC emerges from the TLR3-SC or forms independently of it in parallel remains to be identified. Moreover, it is still unclear how LUBAC is recruited to the TLR3-SC and to the TLR3-induced DISC, how LUBAC activity is controlled in these two signalling complexes, and which component/s is/are targeted by LUBAC in the TLR3 signalling pathway. During the course of *cpdm* dermatitis, TLR3 is probably activated by dsRNA from dying keratinocytes shown to be highly abundant in *cpdm* skin. Consequently, genetic co-ablation of *Tlr3* ameliorates dermatitis in SHARPIN-deficient mice. Given that TNF deficiency completely rescued the *cpdm* skin phenotype, whereas TLR3 deficiency only resulted in its amelioration, it is tempting to speculate that TNF acts upstream of TLR3 in *cpdm* dermatitis. Interestingly, SHARPIN-deficient mice were shown to succumb more rapidly to infection with TLR3-triggering influenza A virus suggesting that presence of LUBAC is essential to balance TLR3 signalling outputs during both, acute and chronic inflammatory events.

Conclusion: Linear ubiquitination is an important regulator of TLR3 signalling. Thus, perturbed linear ubiquitination may be the reason why activation of TLR3 is detrimental, and not beneficial, under certain circumstances. Some of the patients with mutations in the *HOIL1* or *HOIP* genes, resulting in defective linear ubiquitination, suffer from autoinflammation and immunodeficiency. Given the crucial role of LUBAC in TLR3 signalling identified in this thesis, disease outcome of TLR3-triggering viral infections may be worse in these patients. In-depth understanding of the underlying molecular mechanisms may help to identify possible therapeutic targets to be able to provide patients with defective innate immune signalling due to perturbed linear ubiquitination with causal treatment strategies.

8 LITERATURE

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9 ACKNOWLEDGEMENTS

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10 CURRICULUM VITAE

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