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# TLRs Go Linear – on the Ubiquitin Edge

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Abstract

Toll-like receptors (TLRs) are crucial to protect the host from pathogens, their exact role in disease, however, remains incompletely understood. TLR signaling needs to be tightly controlled as too little or too much activation of TLRs can result in immunodeficiency or autoinflammation, respectively. There is increasing evidence that linear ubiquitination, mediated by the linear ubiquitin chain assembly complex (LUBAC), plays a pivotal role in the regulation of TLR signaling. Recent advances have identified an intricate interaction between LUBAC and TLRs with immunological consequences to infection and the development of autoinflammation in the host. We propose that defective linear ubiquitination contributes to TLR-mediated disease pathogenesis and that perturbed TLR signaling adds to the phenotype of humans and mice with inherited LUBAC deficiency.

Keywords: linear ubiquitin, LUBAC, TLRs, immunodeficiency, autoinflammation, cell death

## TLRs Meet Ubiquitin

The superfamily of **pattern-recognition receptors (PRRs)** (see Glossary) can be divided into different subfamilies, including the **Toll-like receptors (TLRs)**, the **C-type lectin receptors (CLRs)**, the **nucleotide oligomerization domain (NOD)-like receptors (NLRs),** the **RIG-I-like receptors (RLRs)**, and cytosolic DNA sensors [1, 2]**.** PRRs orchestrate innate immunity against a wide variety of different microorganisms and represent the first line of defense against pathogens [3]. PRRs recognize so-called **pathogen-associated molecular patterns (PAMPs)** – conserved microbial motifs specific to different groups of pathogens – or **damage-associated molecular patterns (DAMPs)** – host biomolecules released in response to tissue damage [3]. The TLR subfamily consists of 10 and 12 different TLRs in human and mouse, respectively [1, 2]. According to their cellular localization on the plasma versus the endosomal membrane, TLRs can be divided into two groups. Whereas TLRs on the cellular surface mainly recognize microbial membrane components, the endosomal TLRs sense nucleic acids [1]. Their involvement in host protection against microbial infection is well established, the exact role of TLRs, however, is insufficiently understood as their presence can both, improve or worsen disease outcome, as in the case of viral infection, bacterial infection, or even during infection with protozoa [4-6]. What determines whether ligation of a certain TLR is protective or harmful to the host appears to depend on the respective disease pattern and possibly on additional, yet unidentified factors. There is increasing evidence that one important regulator of TLR signaling is linear **ubiquitination** mediated by the **linear ubiquitin chain assembly complex (LUBAC)**. Ubiquitination, also known as ubiquitylation, describes the energy-dependent process in which a ubiquitin molecule is either directly covalently attached to a substrate protein, or indirectly by being covalently added onto an already substrate-associated ubiquitin. In the latter case,

this results in the formation of inter-ubiquitin linkages and, if repeated, the formation of ubiquitin chains.

The post-translational modification of proteins by ubiquitin molecules has been shown to play a crucial role in the regulation of a variety of cellular processes, e.g. the degradation of proteins via the proteasome or the coordination of cell signaling events [7, 8]. Ubiquitination is achieved via cooperation of three different enzymes, called ubiquitin-activating enzyme, E1, ubiquitin-conjugating enzyme, E2, and ubiquitin ligase, E3 [9, 10]. Ubiquitin is highly conserved across species and contains seven internal lysine (K) residues (K6, K11, K27, K29, K33, K48 and K63). Inter-ubiquitin linkages can be formed between the ε-amino group of any of these lysine residues of the acceptor ubiquitin and the carboxyl group of the C-terminus of the incoming, or donor, ubiquitin. In addition, an eighth type of ubiquitin linkage, the so called linear ubiquitin linkage, was identified to be formed between the C-terminus of the donor ubiquitin and the α-amino group of the N-terminal methionine (M1) of the acceptor ubiquitin [11]. Different inter-ubiquitin linkage types have been identified to have specific functions [7, 8, 12-20]. The only E3 in mammals known to date to be capable of generating linear ubiquitin linkages *de novo* under endogenous conditions is LUBAC. LUBAC consists of **heme-oxidized IRP2 ubiquitin ligase-1 (HOIL-1), HOIL-1-interacting protein (HOIP)**, and **SHANK-associated RH-domain-interacting protein (SHARPIN)** [11, 16, 21, 22]. The last few years have seen a plethora of studies reporting on the involvement of LUBAC in signaling induced by a number of different innate and adaptive immune receptors [16, 21-25], including different TLRs [26-34].

Here, we discuss recent advances in the fast-developing field of linear ubiquitination in regards to LUBAC as a crucial regulator of different TLR signaling pathways and how LUBAC as a crucial regulator of TLR signaling might impact on the phenotype of

mice and humans with inherited LUBAC deficiency. We propose that deregulated linear ubiquitination might contribute to TLR-mediated immunodeficiency and autoinflammation and that deregulated TLR signaling, in turn, contributes to the phenotype of mice and humans with inherited defects in linear ubiquitination.

## Crosstalk of LUBAC and TLRs on Inflammation and Cell Death

### *Regulation of TLR Signaling by LUBAC*

In the last few years we have witnessed the establishment of LUBAC as a crucial regulator of various TLR-induced signaling pathways in mouse and man (Key Figure, Figure 1).

*LUBAC in TLR1/2 signaling*

TLR1 and TLR2 are localized on the cellular surface and form a heterodimer which recognizes lipoproteins of gram-negative bacteria [1]. LUBAC has been implicated in the regulation of TLR1/2 signaling by a study showing that K63/M1-hybrid ubiquitin chains are present on interleukin-1 receptor-associated kinase 1 (IRAK1), a signaling molecule which acts downstream of TLR1/2 [27]. LUBAC's role in regulating TLR1/2 signaling is substantiated by the fact that SHARPIN-deficient **bone marrow-derived macrophages (BMDMs)** show diminished production of the cytokines interleukin (IL)-12p40, IL-12B, tumor necrosis factor (TNF), and IL-18 when stimulated by **tripalmityl-cysteine-tetralysine ($Pam_3CysK_4$)**, a synthetic bacterial lipopeptide mimic known to activate TLR1/2 [34].

*LUBAC in TLR3 signaling*

TLR3 is localized in the endosomal compartment of the cell and senses dsRNA which is either generated by viruses during their replication cycle as PAMP or

released from damaged cells as a DAMP [1]. We recently identified LUBAC as a crucial regulator of TLR3 signaling [31]. LUBAC maintains TLR3-mediated NF-κB and MAPK activation as shown by RNAi-mediated knockdown of the different LUBAC components and CRISPR/Cas9-mediated knockout of *HOIP* in human HaCaT and HeLa cells [31]. We could further show that LUBAC controls TLR3-induced cell death [31]. We found that LUBAC forms part of the **TLR3-signaling complex (SC)** [31]**.** Interestingly, LUBAC also forms part of a previously unidentified cytosolic signaling platform [31]. This signaling platform comprises of components implicated in TLR3-mediated cell death induction, namely cellular inhibitor of apoptosis proteins (cIAP) 1/2, Fas-associated protein with death domain (FADD), receptor-interacting protein (RIP) 1 and Caspase-8, and we therefore dubbed it the TLR3-induced **death-inducing signaling complex (DISC)** [31]. Identification of the TLR3-induced DISC revises the hitherto known model of TLR3 signaling according to which TLR3-induced cell death was thought to be induced directly at the TLR3-SC on the endosomal membrane [31]. In addition, we found that SHARPIN- and HOIP-deficient primary murine keratinocytes were sensitized to TLR3-mediated cell death and showed defects in secretion of C-C motif chemokine ligand (CCL) 5 and CCL20 [31]. This is in line with a report in which bone marrow-derived dendritic cells (BMDCs) from SHARPIN-deficient mice were shown to exhibited reduced NF-κB activation and diminished production of IL-12p70, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and interferons (IFNs) following activation of TLR3 [30]. In addition, K63/M1-hybrid ubiquitin chains were shown to be present in murine BMDMs upon activation of TLR3 with poly(I:C), a synthetic double stranded (ds) RNA [26]. It remains to be determined, however, to which signaling complex component(s) these K63/M1-hybrid ubiquitin chains are attached to upon activation of TLR3.

*LUBAC in TLR4 and TLR9 signaling*

TLR4 is localized on the cellular surface and recognizes lipopolysaccharide (LPS) [1]. Like TLR3, TLR9 is localized in the endosomal compartment of the cell but in contrast to TLR3, TLR9 senses DNA [1]. A role for LUBAC in TLR4 signaling was first suggested by a study showing that BMDMs from SHARPIN-deficient mice exhibited defects in NF-κB activation and in secretion of the cytokine TNF and the chemokine monocyte chemotactic protein (MCP) 1 upon stimulation with LPS ex vivo [21]. In addition, other work demonstrated that bone marrow-derived dendritic cells (BMDCs) from SHARPIN-deficient mice exhibited reduced NF-κB activation and diminished production of IL-12p70, IL-6, GM-CSF and IFNs upon TLR4 ligation [30]. Moreover, murine B cells carrying a point mutation in the *Hoip* gene which disrupts the ubiquitin ligase activity of HOIP were found to present defects in NF-κB and MAPK signaling upon activation of TLR4 and TLR9, demonstrating that these TLR signaling pathways require LUBAC activity [35].

*LUBAC in regulation of the NLRP3 inflammasome*

Another pathway of inflammatory activation occurs through the **inflammasome.** Assembly of the inflammasome is triggered by deubiquitination and upregulation of the **NLR family member Pyrin Domain Containing 3 (NLRP3)** which is induced by prior activation of TLRs [36]. TLR4 is the best characterized TLR family member with regard to this process known as **"priming"** (an external stimulus resulting in deubiquitination of NLRP3 [37] and, additionally, in upregulation of NLRP3 via NF-κB activation allowing inflammasome assembly and activation [38, 39]) [36]. Activation of the NLRP3 inflammasome is important for the initiation of an adequate host response to infection [40]. The NLRP3 inflammasome is a multiprotein platform consisting of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a

CARD (ASC) and Caspase-1. Its main function is to process and thereby activate the cytokines IL-1β and IL-18, and to induce an inflammatory type of cell death known as **pyroptosis** [40]. Pyroptosis is induced in order to clear bacterial infections with e.g. *Salmonella typhimurium*, *Shigella flexneri* and others but has also been shown to be responsible for CD4 T cell depletion upon infection with HIV-1 [41-43]. LUBAC has been implicated in the control of inflammasome activation [28, 29, 33, 44]. HOIL-1-deficient mice were protected from lethal challenge with LPS in vivo suggesting that inflammasome activity may require presence of HOIL-1 [33]. This notion was further supported by the finding that Caspase-1 activation could be markedly reduced in murine SHARPIN-deficient BMDMs upon activation of the NLRP3 inflammasome via priming of TLR2 or TLR4 with $Pam_3CysK_4$ or LPS, respectively [28]. Concomitantly, following NLRP3 inflammasome activation, the secretion of IL-1β and IL-18 in SHARPIN-deficient BMDMs was diminished relative to that of WT BMDMs implicating that SHARPIN-deficient mice may have difficulties in clearing NLRP3-activating infections [28]. Contrary to these in-vitro results, SHARPIN-deficient mice displayed enhanced IL-1β and IL-18 levels when challenged with LPS in vivo [29]. A role for inflammasome activation in the pathogenesis of *cpdm* dermatitis has recently been demonstrated as dermatitis induced by SHARPIN deficiency is partially corrected by genetic ablation of *Caspases-1* and *-11* or *Nlrp3* in vivo [44]. Recently, it was shown that LUBAC forms part of the NLRP3 inflammasome modifying its core component ASC with linear ubiquitin linkages, as shown by co-expression of ASC and HOIP/HOIL-1 in human 293T cells, as well as in murine BMDMs under endogenous conditions, thereby directly controlling NLRP3 inflammasome activation [33].

Thus, to date LUBAC components have been identified as regulators of TLR1/2,

TLR3, TLR4 and TLR9 signaling outputs, as well as of NLRP3 inflammasome activation (Key Figure, Figure 1). We are only beginning to understand the complex role of linear ubiquitination in these pathways. Thus far, we know that LUBAC is crucial, on the one hand, for maintaining TLR-induced gene activation and subsequent production of cytokines, chemokines and IFNs and, on the other, for controlling TLR-induced cell death. Little is known about LUBAC recruitment to TLR-SCs and its substrates in them.

**LUBAC in TLR Biology: Lessons from Human and Murine Genetic Deficiencies**

Evidence that LUBAC deregulation contributes to TLR-mediated autoinflammation and immunodeficiency has recently emerged. Understanding the underlying molecular mechanisms of linear ubiquitination in the regulation of the different TLR signaling pathways will help to provide new insights into TLR-mediated autoinflammation and immunodeficiency and to identify possible therapeutic targets to treat or even prevent these disorders. Absence of the different LUBAC components in mice and humans in vivo, and the consequences for the host resulting therefrom, will be summarized and discussed in light of the recent advances in the field of TLR regulation by linear ubiquitin.

*HOIP Deficiency*

Homozygous deletion of *Hoip* in mice has been shown to result in death of mouse embryos at day 10.5 of embryonic development [45]. Aberrant TNFR1-mediated endothelial cell death – which results in defective vascularization of the yolk sac – causes lethality in HOIP-deficient mice at this stage of embryonic development [45]. Ablation of *Tnfr1* prolongs survival of mouse embryos up to day 17.5 of embryonic development [45]. What causes lethality at this stage remains to be identified. Given

the facts that (i) TNF induces cell death in the absence of HOIP during embryonic development, (ii) TLRs are involved in the detection of **sterile tissue damage**, and (iii) LUBAC is a crucial regulator of TLR signaling, it is tempting to speculate that deregulated signaling by TLRs (and/or other damage-sensing receptors) might mediate lethality of HOIP-deficient embryos at this later stage of gestation. Mice with catalytically inactive HOIP have been reported to also die before day 12 of embryonic development; whether these mice also die from aberrant TNFR1-mediated endothelial cell death with resulting defects in vascularization of the yolk sac, similar to mice with constitutive deficiency in the *Hoip* gene [45], is currently unknown [27, 35].

HOIP deficiency in humans has been described for a single case of a 19-year-old woman suffering from multiorgan inflammation, repeated fever episodes, recurrent viral and bacterial infections, spleno- and hepatomegaly, generalized **lymphadenopathy**, growth retardation, chronic diarrhea, oral ulcers, intestinal **lymphangiectasia** and muscular weakness [46]. Specifically, at the molecular level, fibroblasts of this patient showed impaired responses to TNF and IL-1β stimulation ex vivo whereas the patient's monocytes were hyperresponsive to IL-1β stimulation [32, 46]. This differential response of the different cell types to different innate immune stimuli was suggested to account for the seemingly paradoxical symptoms with autoinflammation, a clinical disorder defined by abnormally increased inflammation, and immunodeficiency with recurrent viral and bacterial infections [46]. Many questions remain and it is unclear whether cell type-specific signaling outputs are causative for the disease observed in this HOIP-deficient patient remains to be seen.

*HOIL-1 Deficiency*

Mice deficient in the *Hoil1* gene do not exhibit any overt phenotype [47]. Moreover, these mice are protected when challenged with a lethal dose of LPS due to diminished formation of the inflammasome and, consequently, less IL-1β secretion [33]. By contrast, HOIL-1 deficiency results in increased lethality of mice to infection with *Listeria*, *Citrobacter rodentium* and *Toxoplasma gondii* [48]. Interestingly, increased susceptibility to infection with *Listeria* could be offset by concomitant chronic viral infection with murine γ-herpesvirus 68 (MHV68) [48]. Thus, chronic viral infection – which resulted in hyperactivation of the innate immune system – allowed HOIL-1-deficient mice to mount an immune response to infection with *Listeria* which they otherwise were not able to achieve [48].

Three patients were identified with mutations in the *HOIL1* gene suffering from a syndrome encompassing autoinflammation, immunodeficiency and **muscular amylopectinosis** [32], similar to the syndrome observed in the HOIP-deficient patient [46]. Similar to patient-derived HOIP-deficient cells, HOIL-1-deficient fibroblasts isolated from these patients were hyporesponsive to stimulation with IL-1β and TNF, but patient-derived HOIL-1-deficient monocytes showed an increased response to these innate immune stimuli [32]. Whether this divergence in response to innate immune stimulation by these different cell types accounts for the pleiotropic and paradoxical symptoms in these patients remains to be shown [32, 46].

In two additional independent studies, truncating mutations in the *HOIL1* gene were suggested to cause a phenotype consisting of muscular weakness, progressive cardiomyopathy and signs of amylopectinosis; however, none of these patients suffered from severe immunodeficiency or overt hyperinflammation [49, 50]. This phenotypic discrepancy in LUBAC-component-deficient patients [32, 49, 50] may be due to different mutation sites in the *HOIL1* gene as the three patients described by

Boisson *et al.* with immune dysfunction were loss-of-function mutations located in the N-terminal region of *HOIL1* [32] whereas most of the other patients were homozygous for mutations in the central or C-terminal part of *HOIL1* [49, 50]. The exact underlying mechanism, however, remains to be determined. Symptomatic variability in patients harboring the same genetic disorder may also be the result of changes in human viromes, as previously suggested based on results obtained in HOIL-1-deficient mice which, only when suffering from chronic MHV68 infection, were able to fight an otherwise lethal infection with *Listeria* [48]. Thus, variability observed in the phenotype of HOIL-1-deficient patients may also be caused by concomitant chronic viral infections. This is, however, only speculative as a complete infection status of HOIL-1-deficient patients has not been provided. Given the drastic consequences of HOIL-1 deficiency in humans, it seems surprising that HOIL-1-deficient mice would not exhibit a more prominent phenotype; yet, this may also be due to the mice being housed under near sterile conditions. Thus, we are only beginning to understand the complex phenotype caused by absence of HOIL-1 in humans and, to date, it is unclear what we can extrapolate from the studies performed in mice.

*SHARPIN Deficiency*

Long before the discovery of SHARPIN as a component of LUBAC [16, 21, 22], a spontaneous mutation in the *Sharpin* gene in mice, which resulted in the near complete loss of the SHARPIN protein, was identified; this deficiency led to the development of severe dermatitis at three-to-five weeks of age in the animals [51]. Due to their overt skin phenotype, SHARPIN-deficient mice are known as ***chronic proliferative dermatitis mice*** (***cpdm***). These mice suffer from several additional organ dysfunctions including inflammation of joints, spleen, liver, gut and lungs, as

well as lymphoid tissue abnormalities with absence of Peyer's patches, as well as of B cell follicles, follicular dendritic cells and germinal centers in the remaining secondary lymphoid organs [52, 53]. Spleen organization is disrupted and *cpdm* mice show defects in B cell isotype switching and immunoglobulin production [52, 53]. *Cpdm* mice have a shortened life span due to their severe dermatitis [51]. Dead cells are detectable in the epidermis of SHARPIN-deficient mice, even prior to onset of macroscopic skin inflammation [21, 54-56]. Importantly, this aberrant cell death in the epidermis has been reported to be induced by TNF and, indeed, to be causative for an inflammatory state as SHARPIN-deficient mice with genetic co-ablation of either *Tnf* [16], *Tnfr1* [56, 57] or essential components of cell death pathways, i.e. ablation of the kinase activity of RIP1 (*Ripk1(K45A)*) [58], or ablation of *Rip3* together with epidermis-specific deletion of *Fadd* [57] or with heterozygous constitutive deletion of Caspase-8 [56], were protected from dermatitis development.

Of note, the deletion of only one allele of the *Tnf* gene is sufficient to rescue *cpdm* mice from developing dermatitis indicating that TNF expressed above a certain threshold triggers dermatitis in the absence of SHARPIN protein expression [16]. Recently, we identified TLR3 as a contributing factor to *cpdm* skin disease pathogenesis as genetic co-ablation of *Tlr3* ameliorated, but in contrast to *Tnf* deficiency – or indeed *Tnf* heterozygosity – did not prevent, SHARPIN deficiency-induced dermatitis in vivo [31]. We found that dsRNA in the inflamed skin of *cpdm* mice is increased in comparison with WT mice and that this increase occurs in a TLR3-dependent manner [31] (Figure 2). We could further show that disrupted skin architecture induced by SHARPIN deficiency [59] was partially restored by co-ablation of *Tlr3* [31]. In addition, characteristic features of *cpdm* skin, including immune cell infiltration, **hyperkeratosis**, **acanthosis**, and cell death were markedly

reduced in the absence of TLR3 [31, 51]. Importantly, ablation of *Tnf*, or prevention of cell death induced by TNF via genetic ablation of essential components of cell death pathways completely prevented *cpdm* dermatitis [16, 56-58] whilst genetic ablation of *Tlr3* only ameliorated *cpdm* skin disease. Consequently, this implies that TNF is responsible for the initial damage by inducing the death of keratinocytes, thereby providing the trigger of inflammation in the skin of *cpdm* mice [16, 31, 56, 57]. We propose a model according to which the pathogenesis of *cpdm* dermatitis is initiated by TNF-mediated keratinocyte death [16, 56] which, in turn, results in the release of DAMPs, including of dsRNA which results in the activation of TLR3 (Figure 2). As TLR3 signaling requires LUBAC for prevention of cell death, the activation of TLR3 in *cpdm* skin results in additional aberrant cell death leading to the further release of DAMPs, including of dsRNA). TLR3 and dsRNA might subsequently engage in a vicious circle fueling the full-blown dermatitis that is characteristic of *cpdm* mice [31] (Figure 2). Importantly, this model would explain the presence of increased levels of dsRNA in the skin of *cpdm* mice, the amelioration of *cpdm* dermatitis by *Tlr3* deficiency and the reduced presence of dsRNA in the skin of Tlr3-deficient *cpdm* mice [31]. As TNF-induced cell death probably results in the release of several DAMPs in addition to dsRNA, it is likely that apart from TLR3 activation, other damage sensors might also play a role in exacerbating skin inflammation in SHARPIN-deficient mice. This might help explain why the absence of TLR3, despite substantially ameliorating the skin inflammatory phenotype, fails to prevent it [16, 31].

There is increasing evidence that *cpdm* mice, apart from suffering from an autoinflammatory skin syndrome, are also more susceptible to infections. SHARPIN-deficient mice – by contrast to WT mice – were found to be more susceptible to challenge with LPS in a **sepsis model** indicating that SHARPIN deficiency also

affects priming of the inflammasome via TLR4 [29]. In this scenario, SHARPIN was suggested to directly bind and inhibit Caspase-1 as identified by co-immunoprecipitation experiments [29]. HOIL-1 and HOIP were not detectable in this Caspase-1-containing complex suggesting that SHARPIN may have a LUBAC-independent function in this scenario [29], in addition to its role in regulating the NLRP3 inflammasome together with HOIL-1 and HOIP [33].

We recently showed that both, TLR3 and SHARPIN are essential in fighting intranasal infection with H1N1 influenza A virus (IAV) in vivo [31] (Figure 3). We showed that absence of SHARPIN resulted in diminished disease tolerance to IAV infection as SHARPIN-deficient mice lost more weight than WT littermates [31]. We found that following IAV infection the production of (C-X-C motif) ligand (CXCL) 10 and IFN-stimulated gene (ISG) 15 was diminished in lungs of SHARPIN-deficient mice as compared to WT mice [31]. Moreover, whereas viral titers in the lungs of SHARPIN-deficient mice were comparable to WT mice, cell death in their lungs, however, was markedly increased upon IAV infection in this model [31]. The implication of TLR3 in sensing IAV is supported by various lines of evidence; the data however, is conflicting. On the one hand, a missense mutation in the *TLR3* gene has been identified in a patient with IAV-associated encephalopathy [60]. Moreover, children with *TLR3* polymorphisms present an increased risk of developing pneumonia when infected by the pandemic A/H1N1/2009 influenza virus [61]. On the other hand, TLR3 deficiency has been reported to protect mice against IAV-induced lethality despite the fact that TLR3-deficient mice presented elevated lung IAV viral titers and diminished CCL5 and IL-6 production during infection [62]. Of note, the outcome of IAV infection is dependent on both, presence of TLR3 and the IAV strain, as TLR3-deficient mice showed better survival than WT when infected with the IAV strain H5N1 but worse survival when infected with H1N1 IAV [63]. Hence, the role of

TLR3 in sensing and fighting IAV remains incompletely resolved. We found that loss of TLR3 resulted in uncontrolled viral replication and a reduced inflammatory response with diminished production of CXCL10 and ISG15 in the lungs culminating in diminished disease tolerance as evidenced by increased weight loss of the mice following infection with H1N1 IAV as compared to WT [31]. Importantly, no increase in cell death was observed in TLR3-deficient mice as compared to WT [31]. TLR3/SHARPIN double-knockout (KO) mice also present diminished disease tolerance, and viral replication is increased when compared to TLR3-deficient mice [31]. In contrast to SHARPIN-deficient mice, TLR3/SHARPIN-double-deficient mice, however, do not present increased cell death in their lungs, demonstrating that the increased cell death observed in the lungs of *cpdm* mice upon IAV infection is indeed mediated by TLR3 [31] (Figure 3). Disease tolerance during IAV infection depends on the delicate balance between virus- and host-induced tissue damage and the clearance of infection [64]. We propose that the enhanced cell death observed in SHARPIN-deficient lungs may restrict the viruses' ability of replication, providing a possible explanation why viral load in SHARPIN-deficient mice is equal to WT despite the fact that SHARPIN deficiency impairs the inflammatory response during IAV infection [31]. If the initial viral dose is too high it might cause lung damage to SHARPIN-deficient mice beyond repair, resulting in diminished disease tolerance [31]. When infected cells die, they release viral particles but also DAMPs, including dsRNA. Recently, it was shown that mice require presence of the cytoplasmic DNA sensor **DNA-dependent activator of interferon-regulatory factors (DAI)** to fight IAV infection and that infection of mouse embryonic fibroblasts (MEFs) with IAV in vitro results in activation of DAI, leading to RIPK3-dependent necroptosis, importantly, independently of TLR activation [65, 66]. That said, the increase in TLR3-mediated cell death observed in the lungs of SHARPIN-deficient mice [31] is

likely to not occur in the IAV-infected cells shown to die independently of TLR activation [66], but rather in uninfected bystander cells where TLR3 is activated by dsRNA released by dying cells. This hypothesis, and whether increased TLR3-mediated cell death in the lungs of SHARPPIN-deficient mice is indeed causative of diminished disease tolerance, still lack experimental evidence.

Thus, presence of SHARPIN is required to fight TLR3- and TLR4-triggering infections in vivo as its absence results in increased lethality in response to such infections. Although humans with mutations in the *SHARPIN* gene have not yet been identified, our lessons from SHARPIN-deficient mice suggest that linear ubiquitination needs to be considered in the prevention of TLR-mediated autoinflammation and immunity.

*OTULIN Deficiency*

Not only too little but also too much linear ubiquitination was identified to be harmful in patients deficient for the **OTU dub with linear linkage specificity (OTULIN)** [67, 68] – a deubiquitinating enzyme (DUB) known to specifically hydrolyze linear ubiquitin linkages [69, 70]. OTULIN has been shown to form part of LUBAC, thereby controlling its basal activity [71]. In addition, even though OTULIN has been suggested to be recruited to the TNFR1-SC [72, 73], we and others subsequently found it to be neither present in the TNFR1- nor in the NOD2-SC [71, 74, 75].

OTULIN deficiency in humans results in a syndrome consisting of neonatal-onset fever, skin rash and failure to thrive, and has been termed OTULIN-related autoinflammatory syndrome (ORAS) [67] or otulipenia [68]. Patient-derived fibroblasts and peripheral blood mononuclear cells showed increased activation of NF-$\kappa$B signaling and elevated levels of inflammatory cytokines, e.g. IL-1$\alpha$, IL-1$\beta$, IL-6, IL-12p40, IL-12, IL-16, IL-17, IL-18, TNF and IFN$\gamma$ [68]. Three out of six patients

identified to date showed intermediate to good response to therapy with a TNF inhibitor [67, 68] and one patient presented with improved symptoms when treated with an IL-1β inhibitor [68]. OTULIN-deficient patients are described to suffer from recurrent bacterial and viral infections [67, 68]. However, they do not show any clear evidence for primary immunodeficiency as levels of immunoglobulin and of B, T and NK cells are normal [68] so that the recurrent infections observed in these patients are more likely to be a side-effect of immunosuppressive therapy rather than caused by absence of OTULIN [67]. OTULIN deficiency in mice also results in systemic autoinflammation and autoimmunity [67]. Interestingly, OTULIN deficiency specifically in myeloid cells was identified to be causative for a TNF-dependent “cytokine storm” via enhanced NF-κB activity leading to autoinflammation [67].

In summary, we are only beginning to understand the underlying molecular mechanisms of the human and murine phenotypes caused by deficiency in the different LUBAC components SHARPIN, HOIL-1 and HOIP. Deficiency in OTULIN resulting in an autoinflammatory syndrome only adds to the complexity and underlines once again that the level of linear ubiquitin in the host is decisive for proper control of signal transduction. More work is required to identify drivers of autoinflammation induced by deregulated linear ubiquitination, and to elucidate the apparent species-specific and LUBAC-component-specific controversies.

## Concluding Remarks:

### Preventing Disease: Regulation of TLR Signaling by LUBAC

We propose that deregulation of linear ubiquitination contributes to TLR-mediated immunodeficiency and autoinflammation. We further propose that deregulation of TLR signaling significantly contributes to autoinflammation and immunodeficiency in LUBAC-deficient mice and humans because LUBAC has been identified as crucial regulator of different TLR signaling pathways. The amount of linear ubiquitination needs to be precisely controlled; evidence suggests that an aberrant surplus or deficit of linear ubiquitination can have profound consequences for the host resulting in autoinflammation. Given the wide variety of different microorganisms detected by different TLRs, in addition to increasing evidence that LUBAC tightly controls TLR signaling, we suggest that the paradoxical phenotype characterized by co-occurrence of autoinflammation and immunodeficiency observed in LUBAC-component-deficient mice and humans may be partially explained by the fact that TLR signaling requires LUBAC for proper signal transduction. We are only beginning to understand the complex interplay between LUBAC and TLRs. Various microbes have been shown to inhibit LUBAC function and thereby evade host immunity [76, 77]. Thus, we propose that the paradox on whether the presence of certain TLRs is beneficial in one scenario, whilst being detrimental in another, may be due to deregulated linear ubiquitination. Thus, in light of recent findings indicating that LUBAC is a crucial regulator of TLR signaling, a re-evaluation of the role of LUBAC in different scenarios of inflammation and immunodeficiency, in the absence versus presence of infection, is warranted (see Box 1 and Outstanding Questions). Such investigations in this exciting and emerging field may indeed increase our understanding of TLR-mediated disease pathogenesis.

Further insight on the complex biochemical, functional and pathophysiological interplay between LUBAC and TLRs will hopefully help to identify therapeutic targets to provide patients with inherited defects in linear ubiquitination chain formation, but perhaps also patients with other etiologies who suffer from autoinflammation and/or immunodeficiency, with rational treatment options.

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Glossary

**Acanthosis:** hyperplasia of the epidermis with thickening of the basal (stratum basale) and granular layer (stratum spinosum) of the epidermis.

**BMDMs**: bone marrow-derived macrophages; primary macrophages derived from bone marrow cells in presence of specific growth factors.

**CLRs:** CLRs belong to the family of PRRs, possess a carbohydrate recognition domain (CRD) whereby they sense carbohydrates and are well known for their role in recognizing fungal infection.

***cpdm***: *chronic proliferative dermatitis mice*; mice bearing a spontaneous mutation in the *Sharpin* gene resulting in abrogated SHARPIN protein expression. The name *cpdm* derives from their severe dermatitis but these mice suffer from additional organ dysfunctions.

**DAI:** DAI belongs to the family of PRRs and is a cytosolic DNA sensor involved in innate immune recognition.

**DAMPs**: damage-associated molecular patterns; endogenous, cell-derived factors released in response to injury of any kind known to initiate an inflammatory response via stimulation of, e.g. PRRs.

**DISC:** death-inducing signaling complex; a signaling platform that induces cell death, first identified to be formed upon ligation of death receptor family members. Today, the term DISC is no longer exclusively used for signaling complexes induced by death receptors as other receptors that do not belong to the death receptor family were identified to be capable of inducing cell death by assembling a signaling platform similar to the conventional DISC.

**HOIL-1**: heme-oxidized IRP2 ubiquitin ligase-1; HOIL-1 is an E3 ubiquitin ligase forming part of LUBAC.

**HOIP**: HOIL-1-interacting protein; HOIP is an E3 ubiquitin ligase identified to be the central and catalytically active component of LUBAC.

**Hyperkeratosis:** describes thickening of corneal layer (stratum corneum), the outer layer of the epidermis.

**inflammasome:** A protein complex consisting of an inflammasome sensor, the adaptor protein ASC and Caspase-1. Its main function is to process and thereby activate the cytokines IL-1β and IL-18, and to induce an inflammatory type of cell death, termed pyroptosis.

**LUBAC**: linear ubiquitin chain assembly complex; a complex consisting of SHARPIN, HOIL-1 and HOIP that assembles linear ubiquitin linkages in response to a wide variety of stimuli.

**Lymphadenopathy:** literal translation for disease of the lymph nodes, often used as term for enlarged lymph nodes.

**Lymphangiectasia:** also known as lymphangiectasis; improperly formed lymph vessels or blockage of lymph flow, usually in the intestine; symptoms that occur are

chronic diarrhea and hypoproteinemia (low protein levels in the blood) resulting in ascites, pleural effusion and edema.

**Muscular amylopectinosis:** also known as glycogen storage disease type IV, a rare hereditary disorder due to a mutation in the glycogen branching enzyme (GBE) 1 with abnormal glycogen molecules accumulating in the body. The consequences are muscular weakness, cardiomyopathy, and liver failure.

**Necroptosis:** regulated necrosis, a form of programmed cell death dependent on RIP3 and the pseudokinase mixed lineage kinase domain-like protein (MLKL), and the kinase activity of RIP1.

**NLRs:** NLRs belong to the family of PRRs, are intracellular proteins that trigger the activation of NF-κB and MAPK signaling, and regulate the activation of inflammatory caspases. Mutations in NLRs have been linked to disease, e. g. mutations in NOD2 are associated with Crohn's disease.

**OTULIN:** OTU dub with linear linkage specificity; OTULIN was identified to form a complex with LUBAC, and to constitutively and specifically cleave linear ubiquitin linkages formed by LUBAC thereby restricting its basal activity. OTULIN has also been shown to be recruited to the TNF-RSC I.

**$Pam_3CysK_4$:** ligand for TLR1/2, a synthetic analog of the triacylated N-terminal part of bacterial lipoproteins.

**PAMPs**: pathogen-associated molecular patterns; conserved microbial motifs specific for different groups of pathogens recognized by different PRRs.

**Priming:** Activation of the NLRP3 inflammasome occurs in two steps: the first step, called priming, is induced via activation of e.g. TLRs resulting in deubiquitination of

NLRP3 and its upregulation via activation of NF-κB. During the second step, the inflammasome is assembled resulting in its activation.

**PRRs**: pattern-recognition receptors; the superfamily of PRRs comprises different subfamilies of receptors including the TLRs. PRRs are part of the innate immune system and represent the first line of defense during an immune response.

**Pyroptosis:** is a form of programmed cell death initiated upon microbial infection which is sensed by NLRs leading to the formation of the inflammasome and processing of Caspase-1.

**RLRs:** RLRs belong to the family of PRRs and are involved in the detection of viral infection.

**Sepsis model:** experimental sepsis in mice usually induced by intraperitoneal or intravenous injection of LPS or E.coli.

**SHARPIN**: SHANK-associated RH-domain-interacting protein; SHARPIN was identified as the third component of LUBAC.

**Sterile tissue damage:** Injury in response to trauma, ischemia-reperfusion injury or chemically-induced injury typically occurs in the absence of microorganisms and is therefore termed sterile tissue damage. DAMPs released from injured cells activate the innate immune system via PRRs inducing an inflammatory response.

**TLR3-SC:** the TLR3-SC is formed upon ligation of TLR3 with dsRNA on the endosomal membrane. The adaptor protein TRIF is recruited to TLR3 initiating further recruitment of downstream signaling, i.e. production of type I IFNs and activation of NF-κB and MAPK signaling.

**TLRs**: Toll-like receptors; TLRs belong to the superfamily of PRRs and have been implicated in the recognition of host invasion by a wide range of pathogens. To date, 10 different human TLRs have been identified.

**Ubiquitination:** An energy-dependent, post-translational modification process in which one ubiquitin moiety is attached to a target protein via cooperation of three different enzymes, called E1, E2, and E3. When ubiquitin is the target protein, this results in the formation or an inter-ubiquitin linkage. When this process is repeated, the result is a ubiquitin chain. Ubiquitination has been implicated in the regulation of many cellular processes.

Box 1. Clinician's Corner

- Linear ubiquitination is a post-translational modification mediated by a complex called LUBAC. Many innate and adaptive immune signaling pathways have been shown to depend on linear ubiquitination for signal transduction. In the absence of LUBAC, signaling outputs are perturbed.
- LUBAC-deficient patients suffer from a paradoxical syndrome with signs of autoinflammation and immunodeficiency. We are only beginning to understand the molecular basis for the co-occurrence of these supposedly opposing symptoms.
- TLR signaling has been implicated in the pathogenesis of several diseases as well as the prevention of others. The exact roles played by different TLRs, however, remain controversial and incompletely understood. It is unclear why the presence of a given TLR can either be beneficial or harmful for the host, depending on the physiological or pathological setting.
- Understanding the complex biochemical and functional interplay between LUBAC and TLRs will hopefully help identify possible therapeutic strategies to provide patients harboring defects in linear ubiquitination pathways with rational treatment options.

Trends

- Linear ubiquitination, mediated by a tripartite protein complex consisting of HOIP, HOIL-1 and SHARPIN, in both, humans and mice is crucial for signal transduction in a wide variety of innate and adaptive immune cells.
- Humans bearing mutations in the *HOIL1* or *HOIP* genes are deficient in the respective proteins which in turn leads to diminished expression of the other two LUBAC components resulting in instability of the entire LUBAC, and suffer from syndromes encompassing both, immunodeficiency and autoinflammation.
- TLRs play a crucial role in detection of both, infection and sterile tissue damage. Their precise function in disease etiology is not entirely understood as, depending on physiological context, their presence can either be detrimental or beneficial to the host.
- There is increasing evidence that linear ubiquitination mediated by LUBAC plays a central role in the regulation of different TLR signaling pathways.

Outstanding Questions

- What is the underlying cause for the paradoxical phenotype of LUBAC-component-deficient patients encompassing, on the one hand, autoinflammation and, on the other, immunodeficiency? The differential response of different cell types to stimulation with TNF and IL-1β was suggested to cause these opposing symptoms. Proof for this hypothesis, however, remains to be provided.
- All patients with deficiency in HOIL-1 suffer from muscular weakness, cardiomyopathy and amylopectinosis. A small subset of patients in addition suffers from immunodeficiency and multiorgan inflammation. Why do some patients deficient in HOIL-1 suffer from a more severe phenotype than others? Different mutation sites were suggested to have an impact on the manifestation of the characteristics. A comprehensive analysis of patient samples is, however, missing to date.
- Do mutations in the human *SHARPIN* gene cause pathology? Humans with mutations or deficiency in SHARPIN have not yet been described.
- SHARPIN-deficient mice suffer from autoinflammation and immunodeficiency as do humans with deficiency in HOIL-1 or HOIP. Is this striking similarity a mere coincidence or is there a biological explanation for observation?
- Humans with deficiency in HOIP suffer from immunodeficiency and autoinflammation whereas HOIP deficiency in mice results in embryonic lethality. What is the reason for these species-specific characteristics? Why do mice deficient in HOIL-1 not suffer from any overt phenotype whilst humans with deficiency in HOIL-1 do?

- LUBAC has been identified to regulate TLR1/2, TLR3, TLR4 and TLR9 signaling. Does LUBAC also play a role in signaling mediated by the remaining TLRs?
- Recruitment of LUBAC to any TLR-associated SC has only been demonstrated for the TLR3-SC and the TLR3-induced DISC. Does LUBAC also form part of other TLR-induced SCs? And if so, how is LUBAC recruited and which components of these signaling complexes are modified with M1-linked ubiquitin? So far, IRAK1 acting downstream of TLR1/2 is the only identified LUBAC target in TLR signaling. Other targets of LUBAC in TLR signaling are currently unknown.
- The role of TLR signaling in disease pathogenesis is controversial as the presence of a certain TLR, depending on the respective disease pattern and additional, yet unidentified factors, can either be protective for or harmful to the host. Could perturbed TLR signaling due to deregulation of LUBAC account for these disparities? Or, in other words, could deregulated LUBAC and/or linear ubiquitination be the missing piece in the picture?

Figure Legends

**Key Figure, Figure 1. LUBAC Regulates TLR Signaling Pathways.**

(A) Toll-like receptor (TLR) 1/2 and TLR4 on the cellular surface use myeloid differentiation primary response gene (MYD) 88 as adaptor protein for downstream signaling. Activation of TLR1/2 or TLR4 results in recruitment of interleukin (IL)-1 receptor-associated kinase (IRAK) 2/4 and, subsequently, IRAK1 leading to activation of NF-κB and MAPK signaling via recruitment of TNF receptor-associated factor (TRAF6) and the TGF-β–activated kinase (TAK)/ TAK1-binding protein (TAB) complex. LUBAC attaches K63-/M1-hybrid ubiquitin chains to IRAK1. The mechanism of LUBAC recruitment is unresolved. In the absence of LUBAC, TLR1/2- and TLR4-induced gene activation and cytokine secretion are diminished.

(B) TLR3 belongs to the endosomal TLRs. TIR-domain-containing adapter-inducing interferon-β (TRIF) serves as adaptor protein and leads to activation of downstream signaling: (i) induction of interferons (IFNs) via TRAF3, IκB kinase (IKK) ε and TANK binding kinase (TBK) 1, (ii) activation of NF-κB and MAPK signaling via receptor-interacting protein (RIP) 1, TRAF6 and the TAK/TAB complex, and (iii) induction of cell death via a cytosolic platform called TLR3-induced death-inducing signaling complex (DISC) comprising RIP1, Fas-associated protein with death domain (FADD), Caspase-8, cellular FLICE-like inhibitory protein (cFlip) and cellular inhibitor of apoptosis proteins (cIAP) 1 and 2. LUBAC forms part of both TLR3-induced signaling complexes. In the absence of LUBAC, gene activation and induction of IFNs are diminished whilst cell death is enhanced. M1 linkages were identified to be present in the TLR3-signaling complex (SC) and in the TLR3-induced DISC. To which component they are attached and how LUBAC is recruited to the TLR3-SC and the TLR3-induced DISC, however, remains to be established.

(C) Activation of the NLR family member Pyrin Domain Containing 3 (NLRP3) inflammasome is dependent on presence of TLRs. It consists of NLRP3, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1. Activation of the inflammasome results in activation of IL-1β (and IL-18, not depicted). In the absence of LUBAC, processing of IL-1β is reduced. LUBAC was identified to modify ASC with M1 linkages. How LUBAC is recruited to the NLRP3 inflammasome remains to be identified.

**Figure 2. Aberrant TLR3-Induced Cell Death Contributes to *Cpdm* Dermatitis.**

Tumor necrosis factor (TNF)-induced cell death is causative for dermatitis development in SHANK-associated RH-domain-interacting protein (SHARPIN)-deficient mice.

Wildtype (WT) / *Toll-like receptor* (*Tlr) 3*$^{-/-}$: The healthy epidermis is organized as follows: basal layer, spinous layer, granular layer and cornified layer.

*Chronic-proliferative dermatitis mice (cpdm)*: Absence of SHARPIN results in destruction of this upper skin layer organization. Chronic inflammation, hyperkeratosis (thickening of the cornified layer), acanthosis (perturbed differentiation with a thickening of the spinous layer), and, importantly, cell death are characteristics of the *cpdm* skin. Dead cells lead to attraction of immune cells with subsequent release of cytokines and chemokines. Dying cells release damage-associated molecular patterns (DAMPs), including double-stranded (ds) RNA. TLR3 is activated by dsRNA. TLR3 signaling requires the linear ubiquitin chain assembly complex (LUBAC) for a balanced signaling output. In the absence of SHARPIN, the signaling output of TLR3 is tipped in favor of cell death. This results in the additional aberrant release of dsRNA (as well as of other DAMPs), resulting in a vicious circle that was

initiated by TNF but is subsequently fueled by dsRNA and TLR3 without the need for further contribution by TNF.

*Tlr3*$^{-/-}$*cpdm*: Absence of TLR3 interrupts the vicious circle of cell death induction by dsRNA thereby ameliorating the disease.

**Figure 3. Absence of SHARPIN Impacts Disease Outcome of IAV Infection.**

Wildtype (WT): Influenza A virus (IAV) infection results in death of infected cells thereby allowing spread of viral particles. Dying cells do not only release viral particles but also damage-associated molecular patterns (DAMPs), e. g. double stranded (ds) RNA. Viral replication is inhibited via induction of an inflammatory host response ensuring disease tolerance.

*Toll-like receptor (Tlr) 3*$^{-/-}$: In the absence of TLR3 the infected cells die but no activation of an inflammatory host response is achieved. This results in uncontrolled viral replication leading to infection of additional cells finally resulting in diminished disease tolerance by the host.

*Chronic proliferative dermatitis mice (cpdm)*: In the absence of SHANK-associated RH-domain-interacting protein (SHARPIN), no inflammatory host response is initiated. Infected cells die resulting in release of both, viral particles and DAMPs, including dsRNA. TLR3 is activated in uninfected bystander cells which, in the absence of SHARPIN, are prone to die. This increase in dying cells allows control of viral replication. However, if the initial viral dose is too high, the aberrantly increased levels of cell death will cause lung damage beyond repair leading to diminished disease tolerance.

*Tlr3*$^{-/-}$*cpdm*: In the absence of both, TLR3 and SHARPIN, infected cells still die but no TLR3-induced death of bystander cells occurs. No inflammatory host response is

achieved in the absence of TLR3. Thus, the result is uncontrolled viral replication with diminished disease tolerance.