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The Role of E3 Ligases in Macrophage-mediated Inflammation*

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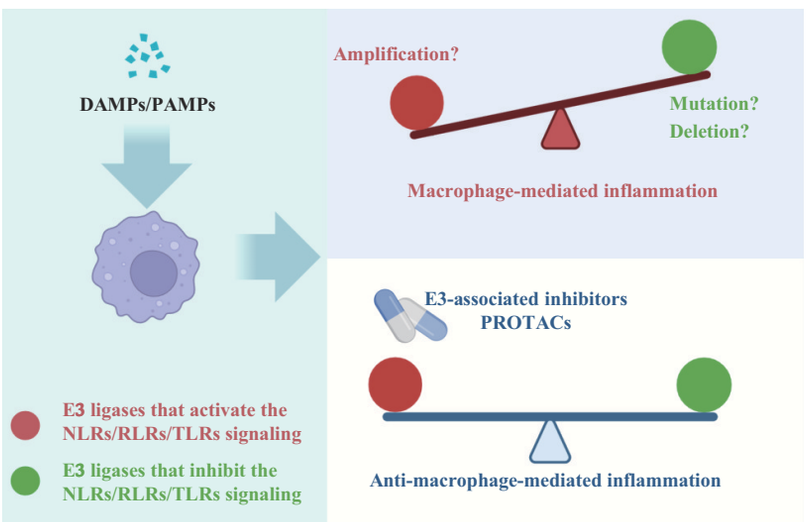
Graphical abstract



Abstract Macrophages, existed in almost all organs of the body, are responsible for detecting tissue injury, pathogens, playing a key role in host defense against a variety of invading pathogens triggering inflammatory responses. Emerging evidence suggests that macrophage-mediated immune responses are efficiently regulated by the ubiquitination modification, which is responsible for normal immune responses. However, numerous studies indicates that the aberrant activation or inhibition of macrophage-mediated immune responses occurs in inflammation, mainly caused by dysregulated ubiquitination modification due to E3 ubiquitin ligases mutations or abnormal expression. Notably, E3 ubiquitin ligases, responsible for recognizing the substrates, are key enzymes in the ubiquitin-proteasome system (UPS) composed of ubiquitin (Ub), ubiquitin-activating E1 enzymes, ubiquitin-conjugating E2 enzymes, E3 ubiquitin ligases, 26S proteasome, and deubiquitinating enzymes. Intriguingly, several E3 ubiquitin ligases are involved in the regulation of some common signal pathways in macrophage-mediated inflammation, including Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors

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(CLRs) and the receptor for advanced glycation end products (RAGE). Herein, we summarized the physiological and pathological roles of E3 ligases in macrophage-mediated inflammation, as well as the inhibitors and agonists targeting E3 ligases in macrophage-mediated inflammation, providing the new ideas for targeted therapies in macrophage-mediated inflammation caused aberrant function of E3 ligases.

Key words E3 ligases, macrophage, NLRs/RLRs/TLRs, targeted therapies
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Macrophages, an essential component of the innate immune system, originate from monocytes, which are derived and differentiated from hematopoietic progenitor cells in the bone marrow. Once released into the bloodstream, monocytes undergo further maturation and differentiation to become tissue-resident macrophages. These specialized macrophages are found in various tissues, including the liver (Kupffer cells), lungs (pulmonary macrophages), bone tissue (osteoblasts), and brain tissue (microglial cells) [1]. These tissue-resident macrophages play crucial roles in maintaining tissue homeostasis and tumor progression, phagocytosing pathogens, and regulating immune responses. As part of innate immunity, macrophages preserve phagocytic activity and initiate a protective inflammatory response to recognize and eliminate microorganisms such as bacteria, fungi and viruses, as well as damaged and senescent cells, and are an important element of the body's non-specific immunity[2]. The innate immune system is the first line of defense against pathogens such as viruses and bacteria, and activates macrophages through intracellular signaling cascades and inflammatory responses to noxious stimuli (*e. g.*, pathogens, dead cells, irritants). Janeway[3] first proposed the concept that the innate immune system has pattern recognition receptors (PRRs), which are cell surface or cytoplasmic receptors that play important roles in recognizing invading pathogens and mediating host immunity, including nucleotide-binding oligomerization domain-like receptors (NLRs), retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), and Toll-like receptors (TLRs) [4]. These PRRs form a signaling network in organisms and work together to regulate natural immunity, inflammatory responses, and acquired immune responses[5]. Crucially, PRRs recognize exogenous pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), viral or bacterial DNA or RNA, as well as endogenous damage-associated molecular patterns (DAMPs) such as heat shock proteins and other substances released during tissue cell damage, rapidly activating the intrinsic immune response and promoting the transcription of inflammatory cytokines and type I IFN genes, finally releasing inflammatory cytokines and type I IFN for rapid clearance of pathogens or necrotic materials[6].

Inflammatory activation of macrophages is a powerful weapon of host cells against microbial infections, in which inflammasomes are important molecular structures for the body to fight against pathogens or recognize its own danger molecules[7]. When macrophages are stimulated by PAMPs or DAMPs, inflammasomes are activated, prompting the maturation and release of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-18, which induce cellular proptosis and participate in intrinsic immune defense[8-9]. The nucleotide-binding oligomerization domain leucine-rich repeat and pyrin domain-containing protein 3 (NLRP3) inflammasome is the most extensively studied inflammasome to date, and the molecular mechanisms of its activation and its relationship to disease pathogenesis are emerging research hotspots.

During viral infection, activation of macrophages is mediated through PAMPs-PRRs interactions to elicit an antiviral response through the production of cytokines such as type I IFN, IL-1β, and inducible nitric oxide synthase (INOS), leading to apoptosis in virus-infected cells. In this process, TLRs and RLRs play a pivotal role in warning the host cells against any invading pathogens as well as initiating the immune response of the innate and acquired immune systems[10-11]. Macrophage activation is also regulated by ubiquitination and deubiquitination, and dysregulation of ubiquitination can lead to a variety of inflammatory diseases. Therefore, ubiquitination, as an important protein post-translational modification, plays an important role in innate immune signal

transduction.

In this review, we first introduce the ubiquitination systems and their roles in the NLRs/RLRs/TLRs signaling pathway, and further explores how the body uses the ubiquitination systems to modulate the immune response mediated by the NLRs/RLRs/TLRs signaling pathway, and finally highlights some drugs that target E3 ligases in inflammation-related therapies, and suggests perspectives for a more precise targeting of therapies.

1　The ubiquitin system

Restoring homeostasis in response to noxious stimuli requires changes in the abundance and function of cellular proteins. The primary mechanism of this “protein quiescence” is mainly dependent on ubiquitination modification. The ubiquitin-proteasome system (UPS) is a common post-translational modification process that degrades approximately 80% of the intracellular proteins[12], and this system is consisted of Ubiquitin (Ub), E1 activating enzyme, E2 binding enzyme, E3 ligase, E4 ubiquitin chain assembly factor, 26S proteasome and deubiquitinase (DUB)[13]. Ub, a small 8.5 ku protein containing 76 amino acids, can be covalently tagged to target proteins *via* a cascade of enzymatic reactions, including E1, E2 and E3 ligases[13-14].

Ubiquitination modification is an ordered process. Firstly, Ub is activated by E1 in the presence of ATP, and the G76 at Ub terminus forms a thiol-lipid bond with E1 cysteine. Subsequently, Ub is translocated to E2 *via* the E1 intermediate. Finally, E3 ligases transfer Ub to substrates either directly or indirectly by recruiting E2. In the proteasome degradation pathway, the ubiquitination modified substrate is recognized by the 26S proteasome complex for ubiquitin-mediated degradation (Figure 1)[15].

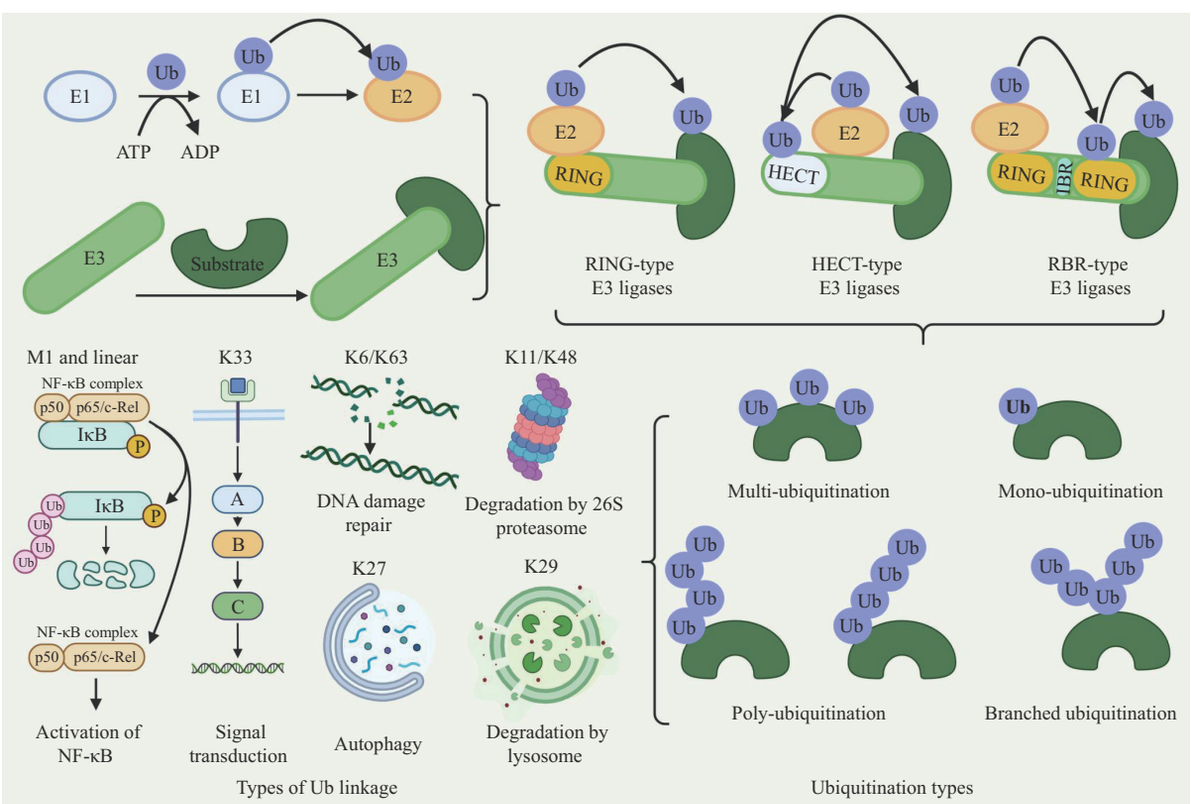


Fig. 1　The types and functions of E3 ligase family

The UPS in its most basic form is represented by 4 components—the ubiquitination machinery (Ub, E1, E2 and E3 enzymes). Ubiquitination: firstly, the Ub molecule is activated by E1; secondly, Ub is shifted to E2 by the E1 intermediate; finally, E3 ligase (the E3 ligase family is divided into 3 types (HECT-type, RING-type, and RBR-type E3s)) transfers Ub directly or indirectly to substrates by recruiting E2, and E3 ligases catalyze substrates to undergo mono-ubiquitylation, multi-ubiquitylation, branched ubiquitylation or polyubiquitination. The substrate binds to lysine residues of the Ub polypeptide to form 8 poly-Ub chains with different fates: K11, K48, K29, K6, K63, K27, K33, and M1 and linear (created with BioRender.com).

Ub contains seven lysine sites (K6, K11, K27, K29, K33, K48, and K63), as well as a Met1 site for Ub chain elongation. Specifically, K11 and K48 ubiquitin linkage form is responsible for the degradation of substrates, while K6, K63, K27, K29 and K33 ubiquitin linkage is mainly for nondegradative ubiquitination involved in the DNA damage repair, protein autophagy, lysosomal degradation, and signal transduction[16-18]. During the ubiquitination process, the E3 ligase is responsible for the specific recognition and labeling of the substrate[19]. Moreover, E3 ligases are classified into 3 main types based on their structural and biochemical features, including homologous to E6-associated protein C-terminus (HECT), really interesting new gene (RING), and RING-in-between-RING (RBR)[20]. The inflammatory and antiviral responses of macrophages to invading pathogens are mainly based on NLRs, RLRs and TLRs signals, and E3 ligase regulates them in many ways. For NLRs, RLRs and TLRs, most K48-linked ubiquitination is an inhibitory signal, while most K63-linked ubiquitination is an activating modification. Therefore, we will further discuss the mechanism by which E3 ligases regulate the inflammatory response and antiviral response of macrophages to invading pathogens according to different ubiquitin ligation types (Table 1).

Table 1 E3 ligase regulating NLRs， RLRs， and TLRs signaling

Pathway	E3	Types	Sub-types	Substrates	Type of Ub linkage	Interaction	Ubiquitination site	Reference
NLRs	CHIP	RINGs	Homodimers	NLRP3	K48	CHIP-TPR NLRP3-LRR	CHIP-H261	[21]
	SPOP	RINGs	CRLs	NLRP3	K48	Not identified	Not identified	[22]
	TRIM16	RINGs	Monomers	NLRP3	K48	Not identified	TRIM16-box domain	[23]
	TRIM31	RINGs	Monomers	NLRP3	K48	TRIM31-the second C-terminal coiled-coil domain NLRP3-PYD	TRIM31-C16, C36 NLRP3-K496	[24]
	MARCH7	RINGs	Monomers	NLRP3	K48	NLRP3-NACHT, LRR	Not identified	[25]
	AMFR	RINGs	Monomers	NLRP3	K6, K11, K48, K63	NLRP3-NACHT	NLRP3-NACHT, Linker1, Linker2	[26]
	ARIH2	RBRs	−	NLRP3	K48 K63	NLRP3-NACHT	ARIH2-C300	[27]
	TRIM65	RINGs	Monomers	NLRP3	K48 K63	TRIM65-RING domain NLRP3-NACHT	Not identified	[28]
	RNF125	RINGs	Monomers	NLRP3	K63	NLRP3-LRR	NLRP3-LRR	[29]
	Cbl-b	RINGs	Monomers	NLRP3	K48	Cbl-b-UBA region NLRP3-LRR	NLRP3-K496	[29]
	FBXL2	RINGs	CRLs	NLRP3	Not identified	NLRP3-PYRIN W73	NLRP3-K689	[30]
	Parkin	RBRs	−	NLRP3	Not identified	Not identified	Not identified	[31]
	TRIM24	RINGs	Monomers	NLRP3	Not identified	Not identified	Not identified	[32]
	Cullin1	RINGs	CRLs	NLRP3	K63	NLRP3-PYD, NACHT, LRR	NLRP3-K689	[33]
	MARCH5	RINGs	Monomers	NLRP3	K27	NLRP3-NACHT	NLRP3-K324, K430	[34]
	Pellino2	RINGs	Monomers	NLRP3	K63	Not identified	Not identified	[35]
	LUBAC	Linear ubiquitin	−	ASC	M1	Not identified	Not identified	[36]
	HUWE1	HECTs	−	NLRP3	K27	HUWE1-BH3 domain NLRP3-NACHT	NLRP3-K21, K22, K24	[37]
	TRIM33	RINGs	Monomers	DHX33	K63	Not identified	DHX33-K218	[38]
RLRs	RNF122	RINGs	Monomers	RIG-I	K48	RNF122-TM domain RIG-I-CARD	RIG-I-K115, K146	[39]
	RNF125	RINGs	Monomers	RIG-I	K48	RNF125-N-terminal region RIG-I-CARD	RNF125-C72, C75	[40]

Continued to Table 1

Pathway	E3	Types	Sub-types	Substrates	Type of Ub linkage	Interaction	Ubiquitination site	Reference
	TRIM40	RINGs	Monomers	RIG-I	K48	TRIM40-CC domain RIG-I-CARD	Not identified	[41]
	c-Cbl	RINGs	Monomers	RIG-I	K48	RIG-I-CARD	Not identified	[42]
	CHIP	RINGs	Homodimers	RIG-I	K48	Not identified	Not identified	[43]
	Parkin	RBRs	–	RIG-I	K48	Not identified	Not identified	[44]
	TRIM25	RINGs	Monomers	RIG-I	K63	RIG-I-CARD T55	RIG-I-K172	[45]
	RNF135	RINGs	Monomers	RIG-I	K63	RIG-I-CARD	RIG-I-K909, K907, K888, K851, K849, K788	[46]
	TRIM4	RINGs	Monomers	RIG-I	K63	Not identified	RIG-I-K154, K164, K172	[47]
	MEX3C	RINGs	Monomers	RIG-I	K63	Not identified	RIG-I-K99, K169	[48]
	TRIM25	RINGs	Monomers	MDA5	K63	Not identified	MDA5-K174	[49]
	TRIM40	RINGs	Monomers	MDA5	K27, K48	Not identified	MDA5-K23, K43, K68	[41]
	TRIM65	RINGs	Monomers	MDA5	K63	Not identified	MDA5-K743	[50]
	RNF122	RINGs	Monomers	MDA5	K27, K48	Not identified	Not identified	[51]
TLRs	RNF170	RINGs	Monomers	TLR3	K48	TLR3-TIR	TLR3-K766	[52]
	Triad3A	RINGs	Monomers	TLR4, TLR9	K48	Not identified	Not identified	[53]
	Smurf1	HECTs	–	MyD88	K48	Not identified	MyD88-K231, K262	[54]
	WWP2	HECTs	–	TRIF	K48	WWP2-the second WW domain TRIF-TIR	WWP2-C838	[55]
	TRIM38	RINGs	Monomers	TRIF	K48	TRIM38-PRY/SPRY domain TRIF-TIR	TRIM38-C16	[56]
	c-Cbl	RINGs	Monomers	MyD88 TRIF	K48	Not identified	Not identified	[57]
	Pellino1	RINGs	Monomers	MyD88	K48	Pellino-CTE domain	Not identified	[58]
	Nrdp1	RINGs	Monomers	MyD88	K48	Nrdp1-ring-finger domain, B-box domain MyD88-intermediate domain	Not identified	[59]
	Nrdp1	RINGs	Monomers	TBK1	K48	TBK1-coil-coil domain	Not identified	[59]
	TRAF6	RINGs	Monomers	NEMO	K63	RING finger	NEMO-K124	[60]
	TRAF6	RINGs	Monomers	IRF7	K63	Not identified	Not identified	[61]
	Pellino	RINGs	Monomers	RIP1	K63	Not identified	Not identified	[62]
	RNF128	RINGs	Monomers	TBK1	K63	RNF128-PA domain	TBK1-K30, K401	[63]
	TRIM24	RINGs	Monomers	TRAF3	K63	Not identified	TRAF3-K429, K436	[64]

2 E3 ligases in the NLRs

2.1 NLRs family

The NLRs family includes 23 cytoplasmic PRRs, which can recognize PAMPs and DAMPs and involve in the formation and activation of inflammasomes, some non-inflammasome functions, as well as the secretion of inflammatory cytokines[1]. Of the 23 human NLR families, the NLRP3 receptor protein is one of the most studied and mature members. NLRP3 inflammasome is an inflammatory complex composed of NLRP3, apoptosis-associated speck-like protein containing a CARD (ASC) and Caspase-1, which is mainly expressed in peripheral giant cells, monocytes, conventional dendritic cells and central microglia[65]. The NLRP3 receptor consists of 3 domains, the N-terminal Pyrin domain (PYD), which plays an important role in the formation of supramolecular complexes, the nucleotide binding and oligomerization domain (NACHT), which regulates protein oligomerization, and the C-terminal leucine-rich repeat domain (LRR), acting as a signal transducer[66-67]. Besides, ASC consists of two structural domains: the N-terminal PYD domain and

the C-terminal Caspase recruitment domain (CARD), while Caspase-1 consists of an N-terminal CARD domain and two catalytic domains, the latter consisting of a central large catalytic (p20) domain and a C-terminal catalytic small subunit (p10) domain[68]. The activation of NLRP3 inflammasome is generally believed to require two signals, the first signal and the activation signal or the second signal[69]. The initial step is triggered by the first signal, when cells are stimulated by risk factors like TNF-α, PAMPs and DAMPs, NF-κB pathway could induce the transcription and expression of NLRP3 and pro-IL-1β[70-71]. The second signals include mitochondrial reactive oxygen species, K^+ efflux, Ca^{2+} circulation, misfolded proteins, lysosomal destruction, and viral RNA[72-73]. After the second signal appears, the LRR domain of NLRP3 binds to its ligands, exposing its NACHT domain, forming a highly ordered NLRP3 protein oligomer through ATP polymerization, recruiting ASC and Caspase-1 to form a complex-inflammasomes, and producing activated Caspase-1[74]. The PYD domain of ASC binds to the PYD domain of NARP3, and its CARD domain recruits the CARD domain of Caspase-1 through the homotypic structure[75]. Activated Caspase-1 (also known as IL-1β convertase) can cleave the inactive IL-1β precursor at D116 residue in cells to form activated mature IL-1β secreted into cells and induce inflammatory cascade reactions[76-77]. Importantly, the expression of NLRP3 protein is the rate-limiting step of NLRP3 inflammasome activation, therefore the protein level and the activity of NLRP3 is quite important for immune response[78]. The protein level and activity of NLRP3 is largely regulated by the UPS, and a large number of studies have demonstrated the important role of E3 ligases in regulating the level of NLRP3. Herein, we would summarize the NLRP3-associated E3 ligases in immune response pathways (Figure 2, 3).

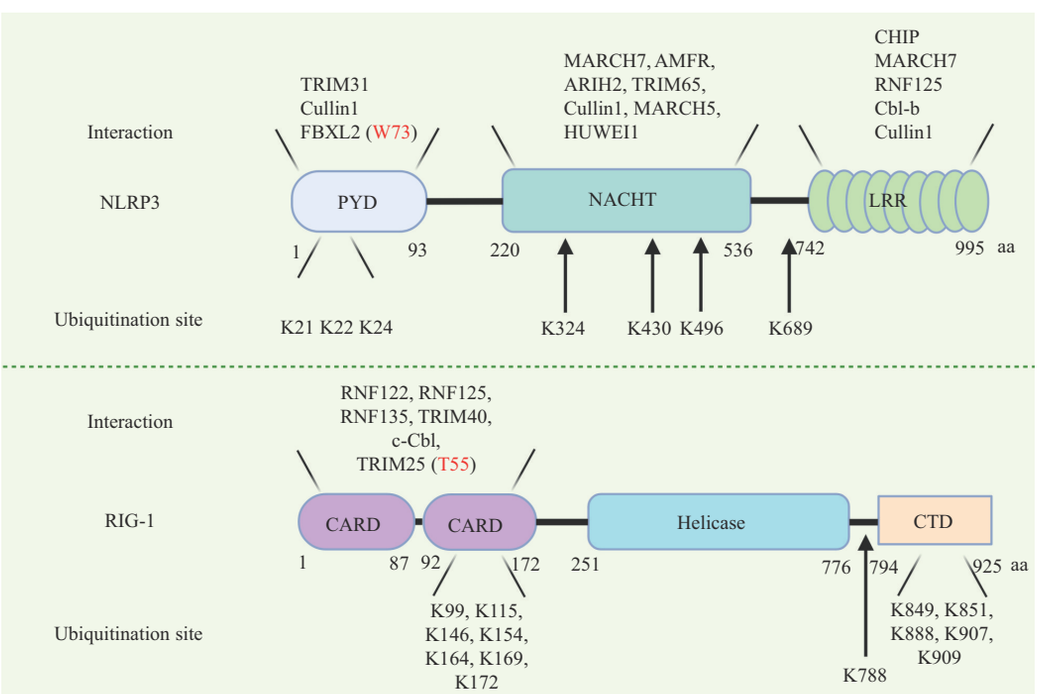


Fig. 2　Structure of NLRP3 and RIG−I and the E3 ligases involved in ubiquitination NLRP3 and RIG−I

NLRP3 contains three structural domains: PYD, NACHT and LRR. Those that can interact with the PYD structure are TRIM31, Cullin1, and FBXL2. Proteins interacting with NACHT structural domains are MARCH7, ARIH2, Cullin1, HUWEI1, AMFR, TRIM65, and MARCH5. Proteins interacting with LRR structural domains are CHIP, MARCH7, RNF125, Cbl-b, and Cullin1. The ubiquitination sites of NLPR3 are K21, K22, K24 in the PYD domain, K324, K430, K496 in the NACHT domain, and K689 between the NACHT and LRR domains. RIG-1 contains two CARD domains, the Helicase domain and the CTD domain. Among them, RNF122, RNF125, RNF135, TRIM40, c-Cbl, and TRIM25 interact with the CARD structure. The ubiquitination sites of RIG-1 are K99, K115, K146, K154, K164, K169, K172 in the second CARD domain, K788 locating between the Helicase structural domain as well as the CTD structural domain, and K909, K907, K888, K851, K849 in the CTD domain. The red text indicates the sites where the E3 ligase binds to its substrate (Created with BioRender.com).

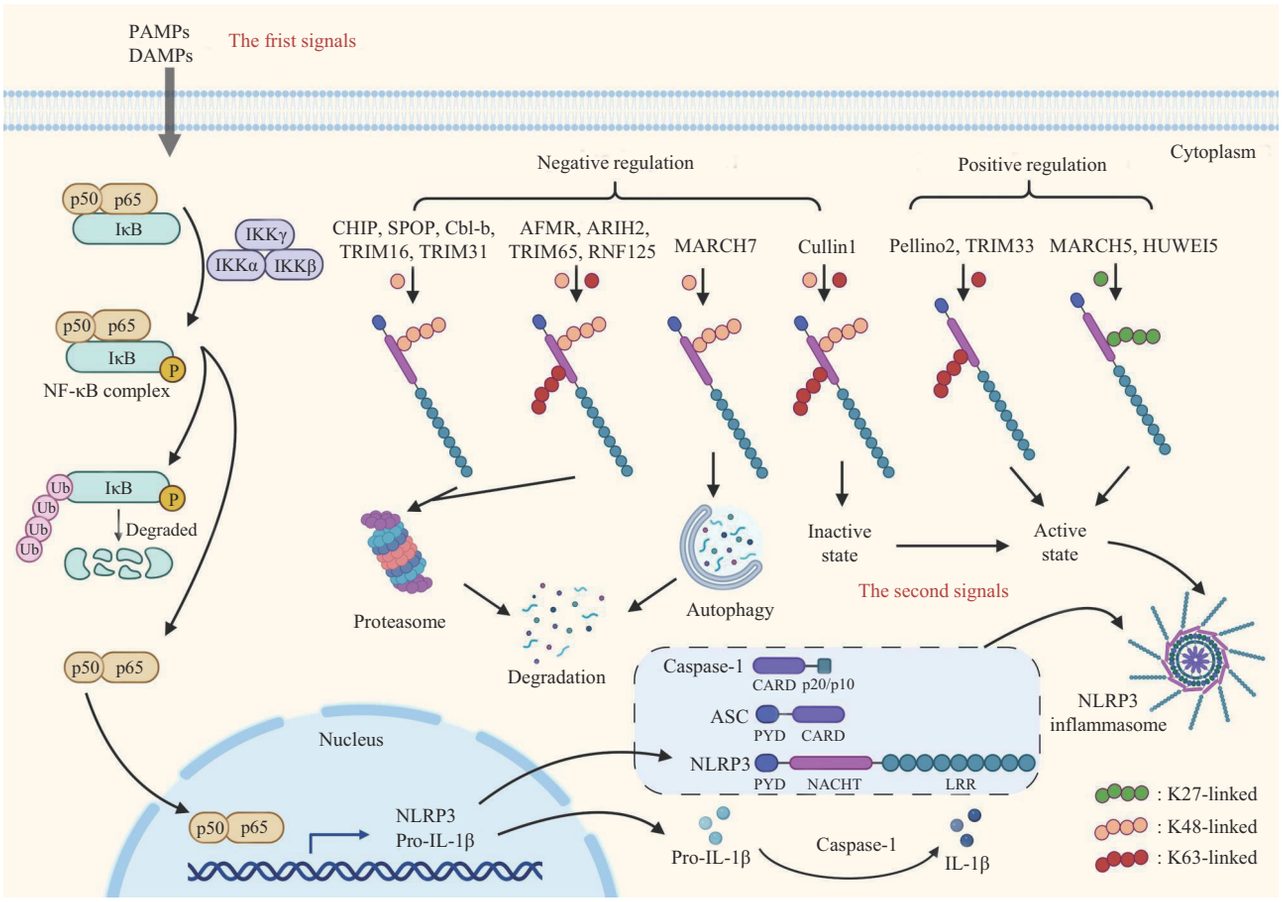


Fig. 3　Overview of ubiquitin−mediated regulation of NLRP3 inflammasome activation

The activation inflammasome requires a first signal (PAMPs, DAMPs), that activates the NF-κB subunits (p50 and p65), leading to upregulation of *NLRP3* and *pro-IL-1β* genes in macrophages. After various regulation by E3 ligases, the NLRP3 at active state assembles the active NLRP3 inflammasome complex with ASC and Caspase-1 under the mediation of the second signal (Created with BioRender.com).

2.2　Negative regulation of NLRP3 by E3 ligases

E3 ubiquitin ligases have been discovered to be capable of negatively regulating the inflammasome by targeting NLRP3 or other inflammasome components such as ASC and Caspase-1. One of the main regulations is to control the level of NLRP3 through the ubiquitin proteasomal degradation pathway.

2.2.1　K48-linked ubiquitination

(1) CHIP

Carboxy-terminus of Hsc70-interacting protein (CHIP) is a U-box E3 ligase composed of a three tetratricopeptide repeat (TPR) domain that interacts with Hsc70/Hsp70 and a U-box domain with E3 ligase activity[79]. Zhang *et al.*[21] found that in mouse proximal tubule (BUMPT) cells, there is an HSP70-dependent interaction between the TPR domain of CHIP and the LRR domain of NLRP3, inducing K48-linked ubiquitination of NLRP3. CHIP-mediated ubiquitination and degradation of NLRP3 attenuates pyroptosis in septic kidney injury, suggesting that CHIP/NLRP3 may be a potential therapeutic target for S-AKI[21].

(2) SPOP

Speckle-type POZ protein (SPOP) is a substrate-binding adaptor of the Cullin3/RING-box1 E3 ubiquitin ligase complex. SPOP mediates both degradative and nondegradative polyubiquitination of a variety of substrates with different biological functions. It is considered as a tumor suppressor in prostate cancer and endometrial cancer, but it plays an oncogenic role in kidney cancer[19]. And in inflammation-related studies, SPOP was found that it can directly interact with NLRP3, and induce its degradation by promoting its K48-linked

polyubiquitination. In high glucose-stimulated podocytes, specific knockdown of SPOP can dramatically facilitate inflammatory response in glomeruli of diabetic nephropathy mice *via* enhancing NLRP3 inflammasome and NF-κB signaling pathways[22].

(3) TRIM16

Tripartite motif containing 16 (TRIM16) does not have the RING domain that most E3 ligase members contain, but TRIM16 still exerts its E3 ligase activity through the B box domain[80]. Shi *et al*.[23] found that TRIM16 can interact with NLRP3, promoting K48-linked ubiquitination of NLRP3 and facilitating the degradation of NLRP3. In mice in which an adenoviral vector encoding TRIM16 was injected directly into the left ventricle, a significant reduction in infarct size after myocardial ischemia-reperfusion surgery can be found improving the inflammatory response caused by NLRP3[23].

(4) TRIM31

In mouse peritoneal macrophages, THP-1 cells, and HEK293T cells, Song *et al*. [24] verified that TRIM31 can directly interact with NLRP3, promoting K48-linked polyubiquitination of NLRP3 and facilitating NLRP3 degradation through the proteasome. Besides, they also found that the second C-terminal coiled-coil domain of TRIM31 and the PYD domain of NLRP3 are required for the NLRP3-TRIM31 interaction, and the C16, C36 sites of the RING structural domain of TRIM31 are ubiquitination active sites. When *TRIM31* is deficient *in vivo*, it will enhance the activation of the NLRP3 inflammasome and exacerbate aluminous aluminum-induced peritonitis[24]. However, another study reported that a protective role for NLRP3 in a model of inflammatory bowel disease, where *TRIM31* deficiency could in turn reduce the severity of dextran sodium sulfate-induced colitis[81]. Further studies by Zhao *et al*. [82] showed that Akt was activated and could promote phosphorylation of the S5 site of NLRP3 under LPS induction, which would inhibit TRIM31-mediated NLRP3 ubiquitination at K496 and limit NLRP3 oligomerization, preventing overactivation of inflammasome in the absence of the second signal. Interestingly, when NLRP3 is activated by the second signal, protein phosphatase 2 (PP2A) can mediate dephosphorylation of the S5 site of NLRP3, which in turn activates NLRP3 oligomerization and activates NLRP3 inflammasome assembly, but also promotes TRIM31-mediated NLRP3 ubiquitination and degradation[83]. As a result, the Akt/TRIM31/PP2A can jointly regulate the protein level and oligomerization trend of NLRP3, thus setting a tight regulatory threshold for NLRP3 activation, which may play a role in preventing excessive inflammatory responses following activation of NLRP3 inflammasome. Therefore, targeting TRIM31 under different pathophysiological conditions may be another key therapeutic target for intervening in NLRP3 inflammatory disorders.

(5) MARCH7

Membrane associated ring-CH-type finger 7 (MARCH7) is classified as one of the non-canonical members of the MARCH family, a subfamily of the RING (really interesting new gene)-type E3 ubiquitin ligases, due to the absence of the transmembrane domain and its RING-CH domain at the C-terminal[84]. In a separate study, the neurotransmitter dopamine functions as an anti-inflammatory stimulus and activates the E3 ligase, MARCH7 can directly interact with NLRP3, promoting K48-linked polyubiquitination of NLRP3[25]. Yan *et al*.[25] also found that in HEK293T cells, overexpressed MARCH7 not only interacted with full-length NLRP3, but also could interact with the NACHT and LRR domains alone but not with the PYD structural domain. Although the link-type of ubiquitination between MARCH7 and NLRP3 is K48, the final pathway of degradation of NLRP3 is induced by autophagy in bone marrow-derived macrophages[25]. Consistently, Cai *et al*.[85] found that ubiquitin specific peptidase 5 (USP5) is a key scaffolding protein that attracts the E3 ligase MARCH7 to NLRP3 and promotes autophagic degradation of NLRP3. However, this does not mean that NLRP3 is not immune to proteasomal degradation by MARCH7. In a study of nonalcoholic fatty liver disease (NAFLD), lncRNA GAS5 was reduced in the livers of NAFLD patients, mice fed a high-fat diet, and obese mice with leptin deficiency. And lncRNA GAS5 served as a sponge of miR-28a-5p, targeting the 3' untranslated region of MARCH7 to inhibit the expression of MARCH7, reducing the proteasomal degradation of NLRP3 protein, and enhancing pyroptosis, and alleviate NAFLD[86].

2.2.2 Both K48- and K63-linked ubiquitination

(1) AMFR

Autocrine motility factor receptor (AMFR, also

known as gp78) is a membrane-bound E3 ligase that usually binds to insulin-induced gene 1 (Insig-1) and plays a key role in a variety of biological events such as cholesterol synthesis and antiviral immunity[87-88]. AMFR mediated nondegradative polyubiquitination of NLRP3, which inhibited NLRP3 inflammasome activation by suppressing the oligomerization and subcellular translocation of NLRP3[26]. Xu *et al*. [26] found that the AMFR-mediated K48- and K63-linked ubiquitination sites of NLRP3 were located in the NACHT domain, whereas the K6- and K11-linked ubiquitination sites were in the Linker1 and Linker2 domains. Notably, Insig-1 is required for interaction with AMFR/NLRP3 and ubiquitination of NLRP3. In *AMFR* or *Insig-1* deficient myeloid cells, NLRP3 inflammasome-dependent inflammation was found to be aggravated, resulting in LPS-induced systemic inflammation and alum-induced peritonitis[26].

(2) ARIH2

Ariadne homolog 2 (ARIH2) is an E3 ubiquitin ligase of the RBR family characterized by highly conserved domains: a RING1 domain, an in-between RING domain, and a RING2 domain[89]. Kawashima *et al*.[27] found that ARIH2 interacts with the NACHT domain of NLRP3 and promotes its K48- and K63-linked polyubiquitination, and the C300 site of the RING2 domain of ARIH2 was the active ubiquitin ligase site. However, the need to determine the *in vivo* role of ARIH2 in *ARIH2*-deficient mice remains a challenge because *ARIH2*-deficient mice have embryo-lethal abnormal inflammation, and the conditional knockout mice may be appropriated for this study[90].

(3) TRIM65

Tang *et al*.[28] found that TRIM65 levels in PMA-differentiated and LPS-primed THP-1 cells were reduced in a time-dependent manner upon treatment with the NLRP3 agonist MSU. Further studies showed that the RING domain of TRIM65 could bind to the NACHT domain of NLRP3 to promote the ubiquitination of NLRP3 K48- and K63-linked polyubiquitination of NLRP3, thereby inhibiting NLRP3 inflammasome assembly, Caspase-1 activation and IL-1β secretion[28]. TRIM65 is highly expressed in a variety of tumors, including hepatocellular carcinoma[91], colorectal cancer[92], bladder cancer[93], and so on. It has also been shown that over-activated NLRP3 inflammasome is closely related to tumor development[94]. However, the relationship between TRIM65 overexpression and over-activated NLRP3 inflammasome in some tumors is worthy of further investigation.

(4) RNF125 and Cbl-b

Here, the two E3 ligases, ring finger protein 125 (RNF125) and casitas-B-lineage lymphoma protein-b (Cbl-b) are presented together because they are both involved in one of the mechanisms of NLRP3 activation. When mice receive a sublethal dose of LPS, LPS induces oligomerization of Caspase-11, which cleaves the GSDMD to release its N-terminal structural domain (NTD), which further triggers the activation of NLRP3 inflammasome[29]. Firstly, RNF125 targets the NLRP3 LRR domain for K63-linked polyubiquitination, and then the K63 ubiquitin chain attached to the NLRP3 LRR domain recruits Cbl-b by binding to its UBA region; finally, Cbl-b promotes K48-linked polyubiquitination of NLRP3 at K496 of NACHT domain and targets NLRP3 for degradation by the proteasome, thereby controlling the NLRP3 inflammasome[29].

2.2.3 Other E3 ligases

The E3 ubiquitin ligase F-box L2 (FBXL2) is a member of the conserved F-box family proteins that determine the specificity of SCF ubiquitin ligase complex and FBXL2 can target TRAF proteins for degradation to regulate inflammation[95-96]. Han *et al*.[30] found that FBXL2 can bind to the W73 site of NLRP3 in the PYRIN domain, and further identified K689 is likely a major ubiquitin modification site in NALP3 protein. FBXL2 ubiquitinates NLRP3 in the resting state and hence contributes to its degradation by the proteasome. However, upon LPS sensing, the levels of the E3 ligase FBXO3 increased, inducing ubiquitination and degradation of FBXL2[30], and reduced levels of FBXL2 lead to elevate NLRP3 expression, thus increasing inflammasome activation. It has been reported that the E3 ligase Parkin could also be involved in NLRP3 modulation, and the enhanced NLRP3 signaling in *Parkin* knockdown microglia is accompanied by a loss of antiapoptotic signaling protein 20 (A20) induction, which inhibits NLRP3 inflammasome activation by inhibiting NF-κB activation, while *Parkin*-deficient cells have increased NLRP3 activation[31]. Hang *et al.* [32] found the TRIM24 expression decreased in ectopic endometrium of endometriosis compared with that in normal endometrium and TRIM24 could interact with NLRP3 in human endometrial stromal cells (hESCs). TRIM24 overexpression promotes the ubiquitination

of NLRP3 in hESC, and then NLRP3 is degraded by the proteasome system and TRIM24 can negatively regulate the cell death and migration of hESCs mediated by the NLRP3 inflammasome signaling pathway[32]. However, more research on the specific ubiquitination types and modification sites of TRIM24-mediated NLRP3 ubiquitination remain to be completed. The majority of the E3 ligases mentioned above could induce the proteasomal degradation of NLRP3 protein, preventing the activation of NLRP3 small inflammasomes, but here, we also noticed a special mechanism. Cullin1 is an essential component of the Skp1-Cullin1-F-box E3 ligase. Studies have shown that Cullin1 interacts with NLRP3 and promotes NLRP3 ubiquitination, leaving it in an inactive state instead of protein degradation, hence limiting NLRP3 inflammasome activation[33]. Sensing the activation signal (the second signals), Cullin1 dissociates from NLRP3 to form an active inflammasome.

2.3 Positive regulation of NLRP3 by E3 ligases

2.3.1 MARCH5

Membrane associated ring-CH-type finger 5 (MARCH5) is an E3 ubiquitin ligase located on the mitochondrial outer membrane, which can maintain mitochondrial homeostasis by eliminating protein aggregation accumulated on mitochondria[97-98]. To generate oxidized mtDNA fragments that bind to NLRP3, new mitochondrial DNA synthesis is necessary, and there is growing evidence that damaged mitochondrial DNA and RNA can be released into the cytoplasm and trigger NLRP3 and other inflammatory signaling[99-100]. In HEK293T cells, MARCH5 can interact with the NACHT domain of NLRP3 to produce K27-linked polyubiquitination on the K324 and K430 residues of NLRP3[34]. Further, ubiquitinated NLRP3 can bind to NEK7 and enhance NLRP3 oligomers, resulting in abnormal inflammatory vesicle activation and the creation of ASC spots and the generation of IL-1β[101].

2.3.2 Pellino2

Structurally, Pellino-2 consists of an N-terminal forkhead-associated (FHA) domain that recognizes phosphothreonine residues and mediates association with interleukin-1 receptor-associated kinase-1 (IRAK1), and a C-terminal RING-like domain that catalyzes the poly-ubiquitination of substrates[102]. Interestingly, Pellino2 could play a dual role in the regulation of NLRP3. On the one hand, LPS promotes the binding of Pellino2 to NLRP3, thereby promoting K63-linked polyubiquitination of NLRP3 in wild-type macrophages[35], finally resulting in NLRP3 inflammasome assembly, ASC oligomerization, downstream Caspase-1-mediated pro-IL-1β and pro-IL-18 processing, and pyroptosis. On the other hand, Pellino2 could also promote ubiquitination of IRAK1, thereby restricting the interaction between IRAK1 and NLRP3 and preventing the inhibitory effect of IRAK1 on NLRP3 activation[35].

2.3.3 LUBAC

The linear ubiquitination assembly complex (LUBAC) is the only known ubiquitin ligase complex that can catalyze the formation of linear ubiquitin chains and consists of HOIP, HOIL-1, and Sharpin[103]. Rodgers *et al*.[36] found that ASC is a novel LUBAC substrate and that HOIL-1L is an important regulator of NLRP3/ASC inflammasome activation in BMDMs and is required for the assembly of NLRP3/ASC inflammasome and linear ubiquitination of ASC.

2.3.4 HUWE1

HECT, UBA, and WWE domain containing 1 (HUWE1, also known as MULE or ARF-BP1), modulates a wide array of cellular functions through the targeting of a number of substrates for ubiquitination, such as p53, and histones involved in the cell cycle, stem cell lineage commitment, and tumorigenesis[104-105]. Recent studies have shown that the BH3 domain of HUWE1 can bind to the NACHT domain of NLRP3, and then promote K27-linked polyubiquitination of NLRP3 at K21, K22 and K24 sites in the PYD domain of NLRP3[37], which in turn promotes NLRP3 inflammasome assembly, ASC oligomerization and Caspase-1 activation, suggesting that HUWE1 can also be regarded as a positive regulator of NLRP3 inflammasome[37].

2.3.5 TRIM33

TRIM33, also known as transcription intermediary factor 1g (TIF1g), has been studied mostly in oncology, acting as a tumor suppressor or promoter in different tumors. For example, in non-small cell lung cancer, breast cancer, glioma and clear cell renal cell carcinoma, TRIM33 acts as a tumor suppressor and its expression is reduced, whereas in B-lymphoblastic leukemia, pancreatic cancer and cervical cancer, TRIM33 acts as a tumor promoter and prevents apoptosis of tumor cells[106]. And in studies

related to inflammation, TRIM33 can directly bind to the RNA helicase DHX33 in the cytosol, and then promote K63-linked polyubiquitination of DHX33 at K218 site[38]. Activated DHX33 is essential for the formation of the DHX33-NLRP3 signalosome complex. Weng *et al*. [38] found that *TRIM33* knockdown in THP-1-derived macrophages and human monocyte-derived macrophages inhibited NLRP3 inflammasome activation because DHX33 could not be ubiquitinated and activated.

3 E3 ligases in the RLRs

3.1 RLRs family

RLRs, one of the classical PRRs, are widely distributed in the cytoplasm of innate immune cells and normal tissue cells[107]. RLRs family members include laboratory of genetics and physiology 2 (LGP2), retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated antigen 5 (MDA5) [108], and only RIG-I and MDA5 have an N-terminal Caspase activation and recruitment domain (CARD) that can mediate downstream signaling. Although LGP2 does not have CARD, it can regulate the activation of RIG-I and MDA5[109-110]. The C-terminal domains (CTD) of RIG-I and MDA5 are mainly involved in recognition of viral RNA. Because RIG-I and MDA5 differ in CTD, different types of viral RNA can be recognized. For example, RIG-I recognizes relatively short dsRNA (<1 kb), whereas MDA5 recognizes longer dsRNA (>1 kb) [111-112]. The RLRs family recognizes cytoplasmic RNA viruses and sends a signal to activate the downstream mitochondrial antiviral signaling proteins (MAVS) to recruit tumor necrosis factor receptor-associated factors (TRAFs), which activates inhibitor κBs kinase (IκBs kinase) and finally phosphorylates interferon regulatory factors (IRFs) and NF-κB proteins into the nucleus to produce type I IFN and cellular inflammatory factors and exert antiviral immunity[113-114]. Ubiquitination plays a dual role in the regulation of RLRs signaling pathway molecules, which can either activate or inhibit their activation, and a vast variety of E3 ubiquitin ligases are involved in this process. Among them, RIG-I and MDA5 play a significant role in the activation of RLRs signaling pathway, so we will focus on the regulation of E3 ubiquitin ligases on them in the following (Figure 2, 4).

3.2 Regulation of RIG−I by E3 ligases

RIG-I mainly undergoes modification of ubiquitin chains linked by K48 and K63. These two types of ubiquitin modifications play a pivotal role in the activation and regulation of RIG-I signaling.

3.2.1 K48-linked ubiquitination

The K48-linked ubiquitination of RIG-I by multiple E3 ligases leads to proteasomal degradation. RNF122, significantly expressed in macrophages, contains a transmembrane (TM) domain in the N-terminal and RING finger domain in the N-terminal, suggesting its potential E3 ubiquitin ligase activity[115]. The TM domain of RNF122 can directly interact with the CARD of RIG-I and promote the K48-linked polyubiquitination of RIG-I at K115 and K146 in the CARD domain[39]. *RNF122* deficiency selectively increases RIG-I-triggered macrophage type I IFN and proinflammatory cytokine production and more resistance to lethal RNA virus infection and increased type I IFN production in *RNF12*-deficient mice[39]. RNF125, also named T cell RING protein identified in activation screen (TRAC-1), is an E3 ubiquitin ligase serving a positive regulatory role in T cell activation[116]. Arimoto *et al.* [40] discovered that the N-terminal region of RNF125 could interact with the CARD domain of RIG-I and the C-terminal region of RIG-I. IFN-α and the dsRNA analogue poly(I:C) stimulate the production of RNF125, which promotes the K48-linked polyubiquitination and degradation of RIG-I, hence limiting type I IFN production[40]. TRIM40 contains an N-terminal RING-finger domain, B-box domain, and an internal coiled-coil (CC) domain[117]. Zhao *et al.*[41] found that the CC domain of TRIM40 could interact with the CARD of RIG-I and promote the K27- and K48-linked polyubiquitination of RIG-I and knockout of *TRIM40* has been shown to significantly enhance RLRs signaling and anti-viral immune response in mice.

Riok3, an atypical kinase can recruit and interact with TRIM40 to induce RIG-I ubiquitination[118]. Siglec-G, a lectin family member, is upregulated by RNA virus infection *via* a RIG-I-dependent mechanism. Siglec-G could inhibit type I IFN production by boosting c-Cbl binding to the CTD domain of RIG-I and facilitating K48-linked ubiquitination and proteasomal degradation at K813 of RIG-I[42]. In addition, CHIP was also found to bind

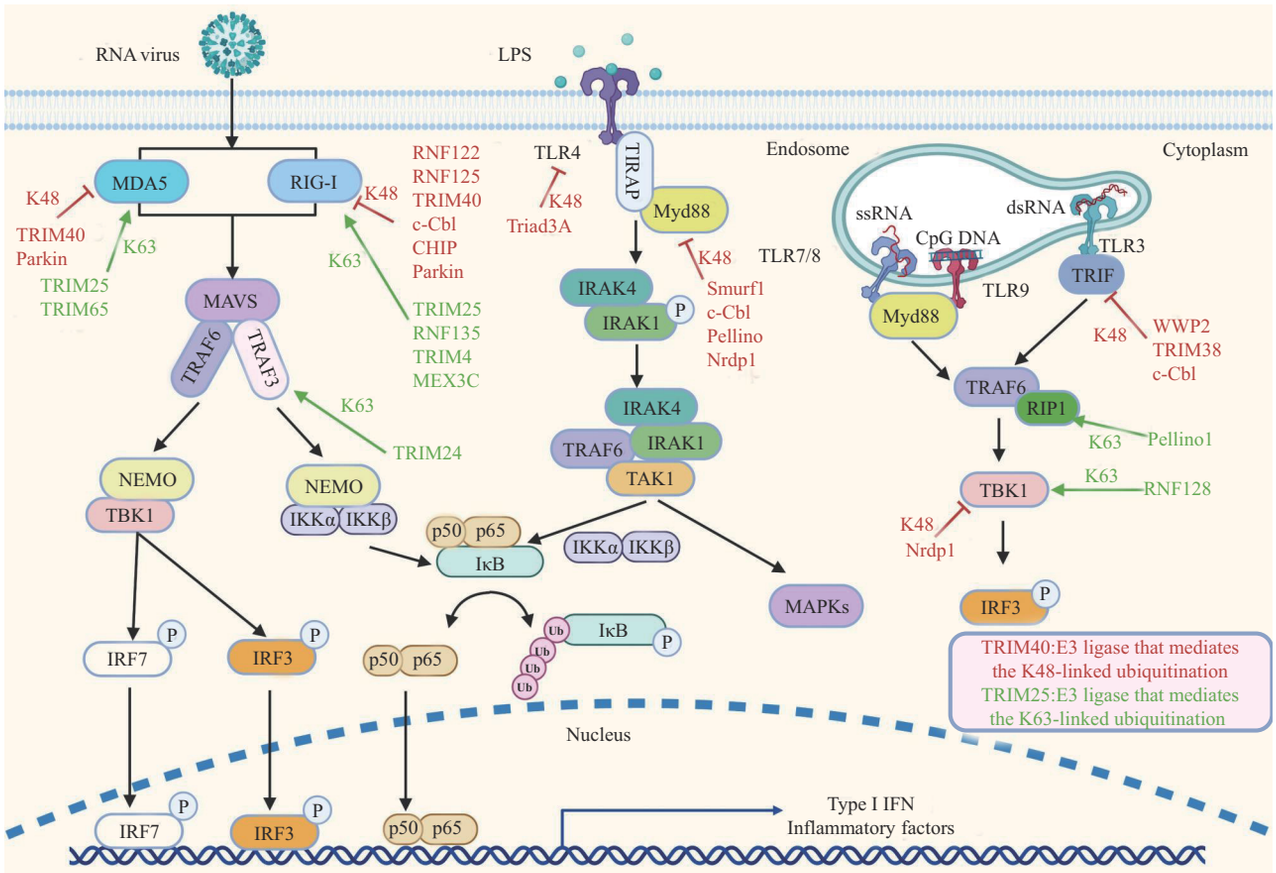


Fig. 4　Overview of ubiquitin−mediated regulation of RLRs/TLRs signaling activation

The RIG-1 and MAD5 recognize cytoplasmic RNA viruses and send a signal to activate the downstream MAVS to recruit TRAFs, which activates IκB kinase and finally phosphorylates IRFs and NF-κB proteins into the nucleus to produce type I IFN and cellular inflammatory factors and exert antiviral immunity. TLR4 is mainly involved in the recognition of bacterial LPS. TLR3 recognizes double-stranded RNA derived from RNA viruses. TLR7 and TLR8 recognize viral single-stranded RNA. TLR9 recognizes both viral and bacterial DNA. Upon recognition of PAMPs, TLRs activate downstream signals by recruiting TIRAP and MyD88. And then MyD88 recruits IRAK4, which phosphorylates IRAK1 and interacts with TRAF6 and TAK1, thereby activating MAPKs and NF-κB signaling pathways. In the MyD88-independent/TRIF-dependent pathway, TLR3 indirectly recruits TRIF through TRAM and interacts with RIP1 and TRAF6 to activate TBK1, which in turn phosphorylates IRF3 and induces the production of type I IFN and inflammatory factors (Created with BioRender.com).

to the CARD domain of RIG-I and promote the K63-linked ubiquitination of RIG-I[43]. Interestingly, further studies revealed that mixed lineage leukemia 5 (MLL5) plays an important role in CHIP-induced ubiquitination of RIG-I. MLL5 translocates from the nucleus to the cytoplasm during virus infection, enhancing MLL5/CHIP/RIG-I interaction and triggering RIG1 degradation[43]. The E3 ubiquitin ligase Parkin can directly interact with RIG-I and MDA5 and promote K48-linked polyubiquitination and degradation of RIG-I and MDA5, thereby limiting the activation of innate immune cascade signaling triggered by RLRs[44]. However, the specific binding sites as well as ubiquitination sites remain to be confirmed.

3.2.2　K63-linked ubiquitination

K63-linked polyubiquitin modification can stabilize the CARD tetramer of RIG-I, drive mitochondrial accumulation of RIG-I, assist RIG-I binding to MAV, and activate MAV targeting mitochondrial signaling to stimulate downstream signaling pathways[10]. TRIM25, which can bind to the CARD domain of RIG-I, is the major E3 ubiquitin ligase that mediates K63-linked polyubiquitination at K172 of the N-terminal CARD of RIG-I[46]. Further research revealed that TRIM25 could not bind to RIG-I after the mutation of T55 residue in the first CARD of RIG-I[45]. Zinc finger CCHC-type

containing 3 (ZCCHC3) can inhibit the replication of some viruses because it contains multiple zinc finger domains, and Oshiumi *et al*.[119] found that ZCCHC3 can recruit TRIM25 to the complex of RIG-I and MDA5 to promote the K63-linked polyubiquitination of RIG-I and MDA5, thus activating the transduction of antiviral signals, as well as the production of type I IFN and inflammatory factors. On the basis of this, another study found that Reticulon3 was substantially increased and accumulated in the endoplasmic reticulum (ER) during RNA virus infection, potentially impairing TRIM25-mediated K63-linked polyubiquitination of RIG-I and thereby limiting IRF3 and NF-κB activation[120]. Besides, it has been proposed that the role of ring finger protein 135 (RNF135, also known as Riplet/REUL) is synergistic with TRIM25 in promoting RLRs signaling. Mechanistically, RNF135 could bind to the CTD domain of RIG-I and boost K63-linked polyubiquitination of RIG-I, hence improving the interaction between TRIM25 and RIG-I[46]. They also found that RNF135 can bind to K909, K907, K888, K851, K849, and K788 of RIG-I CTD and mediate its K63-linked ubiquitination, of which the K788 was the most significant site[46]. Yan *et al*.[47] discovered that TRIM4 may directly interact with RIG-I in HEK293T cells and increase K63-linked polyubiquitination of RIG-I at K154, K164 and K172 in the CARD domain. Mutation of the K154, K164, and K172 residues drastically reduced TRIM4's increased effect on RIG-I-mediated IFN-β promoter activation. In addition, Kuniyoshi *et al*.[48] found that the 382−599 region between the KH and RING-finger domains of mex-3 RNA binding family member C (MEX3C) is responsible for binding to RIG-I and further promoted K63-linked polyubiquitination of RIG-I at K48, K99 or K169 sites of RIG-I. Mechanistically, MEX3C colocalizes with RIG-I in virus-infected cells' stress granules, and overexpression of MEX3C promotes ubiquitination of RIG-I, activating the IFN-β promoter. After infection with RIG-I-recognized RNA viruses, embryonic fibroblasts, macrophages, and conventional dendritic cells generated from Mex3C-deficient animals exhibit type I IFN production deficiencies, limiting RIG-I-induced antiviral responses. However, among the three RIG-I ubiquitination sites mentioned above, the MEX3C-mediated enhancement of IFN-β promoter activity was reduced only when K99 or K169 was mutated, but not when K48 was mutated[48].

It is worth noting that the SARS-CoV-2 papain-like protease (SCoV2-PLpro) effectively inhibited RLRs signaling, significantly reducing the K63-linked polyubiquitination of RIG-I, MDA5, MAVS, TBK1, TRAF3, TRAF6, and IRF3, as well as the K48-linked polyubiquitination of IκBα, which are known to be essential for innate immune signal transduction[121]. In addition, overexpression of sorting nexin 5 or FK506 binding protein 8 could enhance the K48-linked ubiquitination of RIG-I while decreasing K63-linked ubiquitination[122]. Overexpression of HSPBP1, on the other hand, favorably controlled the anti-viral signaling pathway by reducing RIG-I K48-linked ubiquitination and increasing RIG-I K63-linked ubiquitination[123].

3.3 Regulation of MDA5 by E3 ligases

Based on the review of MDA5 studies, we found that although the MDA5 protein is mainly regulated by phosphorylation. Polyubiquitination modification of the MDA5 protein has also been reported in recent years. The MDA5 domain shares about 23%−35% amino acid identity with RIG-I, so some E3 ligases ubiquitinating RIG-I will also ubiquitinate MDA5[124]. For example, TRIM25-mediated ubiquitination of RIG-I as discussed above also occurs in MDA5, and the K63-linked ubiquitination site is located at K174 residue in the CARD domain of MDA5 whereas K23, K43 and K68 in the CARD domain of MDA5 are required residues for TRIM40-mediated K27- and K48-linked ubiquitination and degradation, and K743 residue in the MDA5 helicase domain is required residues for TRIM65-mediated K63-linked ubiquitination[41, 49-50]. Another study discovered that PRRS viral infection increased RNF122-mediated K27- and K48-linked ubiquitination and degradation of MDA5, reduced IFN production, and ultimately enhanced virus proliferation[51]. However, as with the regulation of RIG-I, how these E3 ligases synergistically govern MDA5-mediated innate immune responses needs to be investigated further.

4 E3 ligases in the TLRs

4.1 TLRs family

TLRs are type I transmembrane proteins with varying numbers of LRRs in the extracellular region, and the cytoplasmic region is highly homologous to the cytoplasmic region of the interleukin-1 receptor

(IL-1R) family members, known as the Toll-IL-1 receptor (TIR) domain, which are responsible for the activation of downstream signaling[125]. Currently, 13 TLRs family members have been identified in mammals that recognize different PAMPs, and among them, TLR3, TLR7, TLR8 and TLR9 are localized in intracellular vesicles and are primarily engaged in the identification of virus-associated ligands[126-127]. TLR3 recognizes double-stranded RNA derived from RNA viruses, and TLR7 and TLR8 recognize viral single-stranded RNA, while TLR9 could recognize both viral and bacterial DNA[128-130]. TLRs distributed on the cytoplasmic membrane are mainly involved in bacterial recognition, like TLR4, which is mainly involved in the recognition of bacterial LPS. Upon recognition of PAMPs, TLRs activate downstream signals by recruiting adaptor proteins containing TIR domains, including myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like (Mal, also known as TIRAP), TRIF-related adaptor molecule (TRAM), and so on[131-132]. Activation of TLRs can signal through two distinct pathways, MyD88-dependent and MyD88-independent/TRIF-dependent. Among them, all TLRs except TLR3 mediate immune signal transduction through MyD88-dependent pathways. In the MyD88-dependent pathway, TLRs recruited the downstream signaling molecule MyD88 through their TIR domain, and then MyD88 recruits IL-1 receptor-related kinase-4 (IRAK4), which phosphorylates IRAK1 and interacts with TRAF6, thereby activating MAPKs and IKK-NF-κB signaling pathways and releasing a variety of pro-inflammatory cytokines and chemokines[133]. In the MyD88-independent/TRIF-dependent pathway, TLR3 indirectly recruits TRIF through TRAM and interacts with RIP1 and TRAF6 to activate TBK1, which in turn phosphorylates IRF3 and induces the production of type I IFN and inflammatory factors[134]. In the TLRs signaling, polyubiquitination of intermediate signaling molecules MyD88, TRIF, TRAF6, TAB2/3, NF-κB basic regulator (NEMO), and TRAF3 is required for activation of downstream signaling molecules NF-κB and IRF3. Ubiquitination, like the two signals mentioned above, has a dual role in TLR signaling; some can enhance signal activation while others repress it. In TLR signaling, ubiquitination is classified as K48-linked ubiquitination or K63-linked ubiquitination. Overall, K48-linked ubiquitination inhibits excessive innate immune responses, whereas K63-linked ubiquitination increases TLR signaling during pathogen infection (Figure 4).

4.2 K48−linked ubiquitination

In the TLRs signaling, several E3 ligases have been discovered to directly regulate the expression of TLRs themselves *via* the K48-linked ubiquitination. RNF170 could facilitate K48-linked polyubiquitination of K766 in TLR3's TIR domain and promote TLR3 degradation *via* the proteasome pathway[52]. Similarly, Triad3A, an E3 ubiquitin ligase, promotes the K48-linked ubiquitination of TLR4 and TLR9, which leads to their destruction *via* the proteasome pathway and, ultimately, impairs signals transmission[53]. MiR-191 expression is downregulated in endotoxin-tolerant macrophages and facilitates of Triad3A and TRAF3 expression, which can further inhibit the expression of TLR4 and TLR9[135].

TGF-β also induces the K48-linked ubiquitination and proteasomal degradation of MyD88 and Smad6[54]. Smurf1 selectively mediates MyD88 ubiquitination at K231 and K262 residues *via* the MH1-like domain (amino acids 1−180) interacting to MyD88[54]. Yang *et al*.[55] discovered that the second WW domain of WWP2 interacts with the TIR domain of TRIF and promotes K48-linked ubiquitination of TRIF, which was suppressed by the WWP2 C838A mutation, demonstrating that C838 residues are responsible for WWP2 ubiquitination. Furthermore, *WWP2*-deficient mice exhibited increased susceptibility to poly (I∶C)-induced death than the control littermates.

Xue *et al*. [56] found that TRIM38 negatively regulates TLR3-mediated type I IFN signaling by targeting TRIF, and the PRY/SPRY domain of TRIM38 interacts with the N-terminus of TRIF, promotes the K48-linked ubiquitination. Furthermore, the PRY/SPRY domain of TRIM38 was found to interact with TRAF6 and mediate its K48-linked ubiquitination[136]. Han *et al*.[57] found that c-Cbl can negatively regulate TLRs signaling by mediating proteasomal degradation of MyD88 and TRIF, and that the degradation of MyD88 and TRIF is dependent on Sky-induced phosphorylation on Y227 of MyD88 and Y375 of TRIF, respectively. In addition, Pellino could bind to MyD88 *via* its CTE domain and accumulates on the plasma membrane in a MyD88-dependent way, increasing MyD88 ubiquitination and degradation and therefore negatively influencing TLR

signaling[58]. Neuregulin receptor degradation protein-1 (Nrdp1) contains four domains: the ring-finger domain, the B-box domain, the coiled-coil domain and the carboxy-terminal ErbB3-interacting region[59]. Nrdp1 could directly bind and polyubiquitinate MyD88 and TBK1, resulting in MyD88 breakdown and TBK1 activation. The ring-finger domain and the B-box domain of Nrdp1 bind the intermediate domain of MyD88, and the coiled-coil domain of Nrdp1 recognizes the coiled-coil domain of TBK1[59].

4.3 K63−linked ubiquitination

Many downstream signaling molecules of TLRs, such as TRAFs, NEMO (NF-κB-essential modulator) and TBK1, can be modified by K63-linked ubiquitination.

TRAF6 is a signal adaptor molecule essential for TLRs signal transduction[137]. The E3 ubiquitin ligase activity of TRAF6 is reflected in two aspects. On the one hand, TRAF6 can mediate K63-linked autoubiquitination. On the other hand, TRAF6 can synthesize the unanchored K63 ubiquitin chain, recruit and activate the downstream of TAK1 and IKK, and eventually increase the production of a range of inflammatory factors. However, the role of TRAF6 autoubiquitination in TLRs signaling is debatable. Lamothe *et al*. [60] demonstrated that mutations in TRAF6's major ubiquitination site K124 significantly reduced TRAF6-mediated NEMO ubiquitination as well as TAK1 and IKK activation. TAK1 activation was further investigated, and it was discovered that the RING finger domain of TRAF6 is needed for TAK1 activation but not for TRAF6 autoubiquitination[138]. In the MyD88-dependent activation pathway, MyD88 recruits signaling intermediates including IRAK1, IRAK4, and TRAF6 through its death domain, and TRAF6 can further promote IRF7 ubiquitination and induce type I IFN production[61].

Interestingly, when E3 inactivated mutants of TRAF6 are expressed in *TRAF6*$^{-/-}$ cells, TLR/IL-1 signaling is partially preserved, whereas when Pellino1 and Pellino2 inactivated mutants are expressed in *TRAF6*$^{-/-}$ cells, TLR/IL-1 signaling is completely abrogated[139], indicating that TRAF6 and other E3 ligases such as Pellino1 and Pellino2 promote the formation of MyD88-dependent A previous study discovered that Pellino1 mediates K63-linked polyubiquitination of receptor-interacting protein 1 (RIP1) and that Pellino1 deletion alters RIP1 ubiquitination and impairs NF-κB signaling in response to TLR3/4 ligand poly (I:C) stimulation[62].

Besides, *Pellino1*$^{-/-}$ mice were immune to LPS-induced septic shock[62]. The PA domain of RNF128 captures target proteins for cytosolic ubiquitination, and the RING finger domain facilitates the ubiquitination of captured substrates[140]. Song *et al*.[63] discovered that RNF128 may physically bind with TBK1 *via* its PA domain and then mediate K63-linked ubiquitination of TBK1 at K30 and K401 residues. Infection with the vesicular stomatitis virus promotes the translocation of TRIM24 to mitochondria, where it binds to TRAF3 and directly mediates the K63-linked ubiquitination of TRAF3 at K429/K436 residues[64], thus promoting its binding to MAVS and TBK1, thereby activating downstream antiviral signals.

5 E3−associated inflammation inhibitors

Disorders of ubiquitination are associated with a variety of diseases, including cancer and inflammation. Therefore, E3 ligases have great potential as pharmacological targets for drug development[141]. The Food and Drug Administration (FDA) currently has approved many proteasome inhibitors, including Bortezomib, Carfilzomib, and Ixazomib[142-144]. Importantly, E3 ligase inhibitors have been proven to be more efficient, selective, and less harmful in the genesis of cancer[145]. Moreover, because numerous E3 ligases have been shown to modulate macrophage-mediated signaling, the discovery of E3 ligase inhibitors for the treatment of inflammation is particularly intriguing. Furthermore, because E3 ligases are the most critical components of the Ub conjugation machinery and mediate degradation with great substrate specificity[146], targeting the active site of E3 enzymes or their interactions with substrates offers promising options for developing drugs with fewer side effects. At present, NLRP3 inflammasome inhibitors have been studied widely, we sought to summarize them in this part (Table 2).

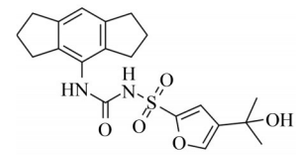
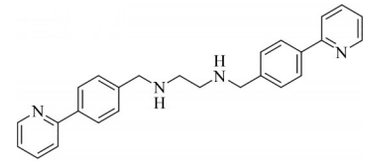
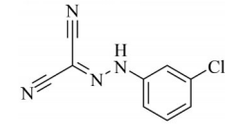
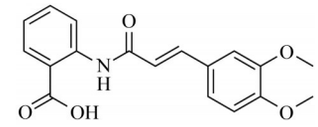
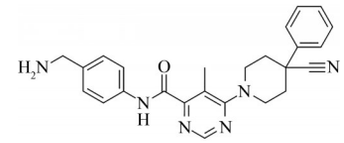
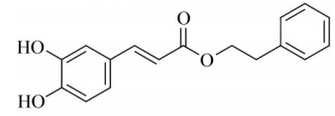
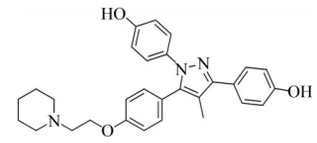
The diary sulfonylurea compound MCC950 is the most potent and specific NLRP3 inhibitor[147].

Parkin deficiency increases Caspase-1 activation and IL-1β production relative to *Parkin* wildtype cells due to the development of the NLRP3 inflammasome complex, which can be reversed by the NLRP3 inhibitor MCC950[31]. Unfortunately, despite showing promising therapeutic effects on NLRP3 targeting in preclinical immunopathological models such as immune encephalomyelitis, Alzheimer's disease, traumatic brain injury, and atherosclerosis, MCC950's phase II clinical trial for the treatment of rheumatoid arthritis was halted due to hepatotoxicity[148]. BC1215 is primarily thought to be an FBXO3 inhibitor. FBXO3 is an E3 ligase that promotes the activation of the NLRP3 inflammasome. BC1215 can prevent FBXL2 ubiquitination and degradation by inhibiting the interaction of FBXO3 with FBXL2[149]. Stabilization of FBXL2 leads to increased ubiquitination of NLRP3, thereby inhibiting its activation. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a mitophagy inducer. Parkin is diffusely expressed in the cytoplasm of HEK293T cells without CCCP treatment, but following CCCP treatment, it is recruited to punctate bodies colocalizing with mitochondria to negatively inhibit type I IFN responses and antiviral immunity[44].

Tranilast is an anti-allergic medication that can stabilize mast cell and basophil cell membranes, prevent degranulation, and so decrease the release of histamine, 5-hydroxytryptamine, and other allergic reaction mediators. A study by Chen *et al*. [150] identified a regulatory mechanism for the inhibition of NLRP3 inflammasomes by tranilast, which increases K63-linked ubiquitination of NLRP3, leading to NLRP3 oligomerization and restricted NLRP3 inflammasomes assembly. Furthermore, anti-atherogenic effects of tranilast were detected in mice lacking low-density lipid receptors and apolipoproteins[150]. Additionally, BI8622 can target and inhibit the E3 ligase HUWE1. HUWE1 can cause inflammasome aggregation, ASC spot formation, and persistent Caspase-1 activation by mediating K27-linked polyubiquitination of AIM2, NLRP3, and NLRC4[37]. Caspase-1 activation in NLRP3, AIM2, and NLRC4 inflammasomes was significantly reduced in BMDM treated with BI8622[37]. One of the main active components of propolis, caffeic acid phenethyl ester (CAPE), has an effect on herpes virus, adenovirus, and influenza virus[151]. CAPE, on the other hand, has a specific lethal impact on tumor cells. CAPE did not change NLRP3 or IL-1 transcription, but rather improved NLRP3 binding to Cullin1, promoted NLRP3 ubiquitination, and exerted anti-tumor effects in AOM/DSS mice models[152]. In addition to these drugs related to NLRP3 inflammasome signaling, we also found methyl-piperidino-pyrazole (MPP), a drug that can target the TIR domain of MyD88[153]. MPP can inhibit TLRs signaling by preventing dimerization of the MyD88 TIR domain, and administration of MPP analogs to mice protects them from TLR4-dependent inflammation[153].

However, the lack of specificity of E3 ligases can result in off-target effects and treatment failure, and many E3 ligase inhibitors are still at the cellular stage and far from the clinical stage. In order to improve medication development, targeted proteolytic chimeras (PROTACs) controlled at the substrate level are receiving increasing attention[154]. The protein of interest (POI) is not directly inhibited by the PROTACs mechanism. PROTACs, on the other hand, bind the E3 ligase to the intracellular POI, tag it with ubiquitin, and then activate the UPS to remove the POI[155]. HDAC6 is involved in the assembly and activation of NLRP3 inflammasome in mouse cells. In order to degrade it, Bockstiege *et al*. [156] designed a PROTAC named PROTAC A6, which is composed of the pan-HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and the E3 ligase ligand thalidomide. PROTAC A6 can specifically target HDAC6 in THP-1 macrophages, significantly reducing IL-1β release. Compared with the traditional small molecule inhibitors, PROTACs have a distinct mode of action for reducing POI. Furthermore, PROTACs have the advantage of having a longer and more powerful biological effect on the target.

Table 2　E3 ligase-associated inflammation inhibitors

Target point	Compounds	Disease models	Regulatory mechanism	Outcome	Molecular structure	Reference
NLRP3	MCC950	Microglia and bone marrow-derived macrophages from *Park2*$^{-/-}$ and *Pink1*$^{-/-}$ mice	MCC950 blocking Parkin deficiency leads to high levels of Caspase-1 activation and IL-1β release, inhibits NLRP3 activation	Inhibits NLRP3 inflammasome activation		[31]
FBXO3	BC-1215	U937, THP-1, primary human alveolar macrophages	C-1215 inhibits FBXO3, increases the E3 ligase FBXL2, and decreases ubiquitin mediated NLRP3 degradation	Inhibits NLRP3 inflammasome activation		[149]
Parkin	Carbonyl cyanide 3-chlorophenylhydrazone (CCCP)	HEK293 and Raw264.7 cells	Following CCCP treatment, Parkin was recruited to the mitochondria and mediated mitophagy negatively regulated type I IFN response and antiviral immunity	Inhibition of type I IFN response and antiviral immunity		[44]
NLRP3	Tranilast	J774A.1, BMDM, atherosclerosis in *Ldr*$^{-/-}$ and *ApoE*$^{-/-}$ mice model	Increased K63-linked polyubiquitination	Inhibition of NLRP3 inflammasome, protectionagainst atherosclerosis		[151]
HUWE1	BI8622	BMDM, *Huwe1*-deficient mice	BI8622 can target and inhibit the E3 ligase HUWE1, significantly reducing Caspase-1 activation in the NLRP3, AIM2, and NLRC4 inflammasome in BMDM	Inhibits NLRP3 inflammasome activation		[37]
Cullin1	Caffeic acid phenethyl ester (CAPE)	Bone marrow-derived macrophages (BMDMs), THP-1 cells, and azoxymethane/dextran sulfate sodium (AOM/DSS)-induced colon cancer mouse model	Increased interaction between NLRP3 and Cullin1 and promoted NLRP3 ubiquitination	Inhibition of NLRP3 inflammasome activation, anticancer		[152]
MyD88	Methyl-piperidino-pyrazole (MPP)	GyrB-TIRAP RAW264.7 Lps-induced sepsis model in mice	MPP inhibits TLRs signaling by preventing dimerization of the MyD88 TIR domain	Inhibits TLRs activation		[153]

6 Conclusion

In this review, we summarize the role of E3 ligases in NLRs/RLRs/TLRs signaling. When mediating K48-type ubiquitination, they usually inhibit NLRs/RLRs/TLRs signaling due to substrate degradation, while when mediating K63-type ubiquitination, the opposite effect occurs. The studies of E3 ligases have been a fascinating frontier in biological processes and cell communication pathways, playing a powerful role in innate immune defense. However, the present results do not explain how E3 ligases recognize specific substrates and the mechanism of selectivity for specific lysine of substrates, which of course awaits a deeper dissection of the structure of E3 ligases. In addition, a particular protein is often regulated by several different E3 ligases, such as NLRP3 K496 residue can act as a ubiquitination site for TRIM31 as well as Cbl-b[23, 29]. But when a protein can be regulated by different E3 ligases, it is not clear which E3 plays a more critical role and which E3 has more potential to be targeted. The functional and molecular mechanisms of how these E3s work together to regulate inflammation are unclear, leaving a question mark as to whether they contribute to this process or whether there is mutual inhibition. Then we should take care to clarify whether these different enzymes have redundant functions or whether they perform related functions in a cell type and context dependent manner.

In contrast to proteasome inhibitors, which non-selectively limit protein degradation in the entire system, drugs that target individual E3 ligases and DUBs may be able to selectively modulate protein levels with less toxicity and greater specificity in the future. Striking a balance between the function of E3 ligase, to add ubiquitin, and that of DUBs, to remove ubiquitin, plays a crucial role in maintaining cellular equilibrium. Studies have shown that in response to the control of inflammatory processes by ubiquitination, pathogens have evolved mechanisms to exploit the ubiquitination system to promote infection by mimicking host E3 ligases and DUBs[157]. Taking advantage of this feature, therapeutic inhibitors are being developed to alter the outcome of intracellular infections. Small molecule inhibitors of E3 ligases have great preventive potential and may become first-line treatments for innate immune diseases. These inhibitors interfere with the interaction between E3 ligases and substrates and inhibit their enzymatic activity. This is the real “targeted therapy”, not only targeting E3 ligases, but also targeting the evolution of pathogens. In any case, further studies on E3 ligases and inflammatory responses as well as pathogen immune evasion will help us elucidate the complex biology of the initiation and development of inflammation, leading to more precise treatments in the future.

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E3连接酶在巨噬细胞介导的炎症中的作用*

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摘要 巨噬细胞几乎存在于人体的所有器官中，负责检测组织损伤、病原体，在宿主抵御各种入侵病原体引发炎症反应的过程中发挥关键作用。研究表明，巨噬细胞介导的免疫反应受到泛素-蛋白酶体系统（ubiquitin-proteasome system，UPS）的严格调控，其失调或异常激活可能是许多炎症发病机制的关键因素。负责识别底物的E3连接酶是UPS中的关键酶，它包含多种亚家族蛋白，参与调控巨噬细胞介导的炎症中的一些常见信号通路，如核苷酸结合寡聚化结构域样受体（nucleotide-binding oligomerization domain-like receptors，NLRs）、维甲酸诱导基因I样受体家族（retinoic acid-inducible gene 1-like receptors，RLRs）和Toll样受体（Toll-like receptors，TLRs）。本文总结了巨噬细胞介导的炎症相关E3连接酶的最新研究进展，并讨论了E3与其底物结合导致NLRs/RLRs/TLRs信号转导异常激活或失活的一些潜在机制。此外，还探讨了针对E3连接酶在巨噬细胞介导的炎症中作用的相关抑制剂和激动剂，展望了针对巨噬细胞介导的炎症中异常E3连接酶的靶向疗法的未来前景。

关键词 E3连接酶，巨噬细胞，NLRs/RLRs/TLRs，靶向治疗

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