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Original Article

Ubiquitination regulation of inflammatory responses through NF-κB pathway

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Abstract: The development of inflammation is mutually affected with damaged DNA and the abnormal expression of protein modification. Ubiquitination, a way of protein modification, plays a key role in regulating various biological functions including inflammation responses. The ubiquitin enzymes and deubiquitinating enzymes (DUBs) jointly control the ubiquitination. The fact that various ubiquitin linkage chains control the fate of the substrate suggests that the regulatory mechanisms of ubiquitin enzymes are central for ubiquitination. In inflammation diseases, the pro-inflammatory transcription factor NF-κB regulates transcription of pro-labour mediators in response to inflammatory stimuli and expression of numerous genes that control inflammation which is associated with ubiquitination. The ubiquitination regulates NF-κB signaling pathway with many receptor families, including NOD-like receptors (NLR), Toll-like receptors (TLR) and RIG-I-like receptors (RLR), mainly by K63-linked polyubiquitin chains. In this review, we highlight the study of ubiquitination in the inflammatory signaling pathway including NF-κB signaling regulated by ubiquitin enzymes and DUBs. Furthermore, it is emphasized that the interaction of ubiquitin-mediated inflammatory signaling system accurately regulates the inflammatory responses.

Keywords: Ubiquitination, NF-κB pathway, inflammation, regulation

Introduction

Inflammation is the defensive process of living tissue when vascular system responds to the injury factor. Inflammatory response is a complex process involving interaction between many factors and cellular points. The immune system, representing the first defense line against pathogen invasion or danger signals, is mediated by pathogen-recognition receptors [1]. When the body suffers damage or pathogen invasion, the immune system will be activated and gather a lot of inflammatory cells, secrete varieties of cytokines and inflammatory mediators leading to inflammation. The inflammatory response is one of the important performances of lots of diseases including cardiovascular disease and metabolic diseases [2]. The inflammatory response also induces the occurrence of tumor and promotes tumor development and metastasis. The development of inflammation is mutually affected with damaged DNA and the abnormal expression of protein modification [3]. Ubiquitination is the process that ubiquitin molecules modify the target protein by an enzymatic reaction cascade. Ubiquitination regulates protein stability, activity and regulation of protein function, thereby controls cellular function [4].

NF-κB, as a transcription regulator in cells, induces varieties of genes expression via stimulating agents, such as viruses, tumors, B cell activating factor and lymphatic toxin, and thereby produces a variety of cytokines participated in inflammatory response. Studies have indicated that involvement of TLR, NLR and RIG-I-mediated inflammatory pathways leads to activation of transcription factors including NF-κB and results in the release of pro-inflammatory cytokines and chemokines which is associated with ubiquitination [5, 6]. Some

studies have shown that TLR, NLR and RLR rely on the linear and K63-polyubiquitination to mediate the NF-κB activation [7-9]. In addition, ubiquitin enzymes and DUBs also play a significant role in regulation of NF-κB activation which promotes the expression of inflammatory cytokines [10, 11]. In this paper, recent discoveries regarding molecular regulation of the inflammation by ubiquitination are summarized and possible mechanism which is associated with ubiquitination targeting the inflammation to impact inflammatory signaling is discussed.

The inflammation system

Inflammation is a defensive response to stimuli. In inflammatory process, tissue and cells are damaged by injury factors, and on the other hand the inflammatory congestion and exudative reactions kill the injury factors. The inflammatory response is one of the important performances of lots of diseases including cardiovascular disease, metabolic diseases, the initiation and development of tumor [2]. In addition, damaged tissue is repaired and healed by substrate and interstitial regenerated cells. NF-κB signal transduction pathway is closely related to inflammatory lesions. In recent years, the signal pathway, as the target of anti-inflammatory therapy, is a hot spot and many studies have shown that the development of inflammation through NF-κB pathway is associated with ubiquitination [12, 13].

The ubiquitin system

Ubiquitin (UB) is a highly conserved small protein with a molecular mass of 8.5 kDa, containing 76 amino-acid residues, which are widely present in eukaryotic cells [14]. The ubiquitin molecule consists of seven lysine sites (K6, K11, K27, K29, K33, K48 and K63) at the C-terminal glycine (Gly) point and at the N-terminal methionine (Met1) site. The C-terminal glycine of ubiquitin molecule can be combined with the e-amino group of any one lysine molecule to form multi-monoubiquitination [15]. Therefore, the substrate protein plays an important control function conducted by the variety of ubiquitin chain connection. Ubiquitination is a process, which uses three different types of enzymes, an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase to attach the substrate proteins [16] (**Figure 1**). Ubiquitination, the ubiquitin depending on enzymes to modify the target protein, plays an important role in the regulation of various biological functions such as injury repair and inflammatory immunity.

Central role of ubiquitin enzymes in ubiquitination

Attachment of UB to proteins is catalyzed by Ubiquitin-activating enzyme E1, Ubiquitin-conjugating enzyme E2 and Ubiquitin ligase E3 [17]. The specific process of ubiquitination: E1 enzyme activates ubiquitin through adhesion to the tail of ubiquitin molecule. Then the activated ubiquitin molecule will be transferred to the E2 enzyme. At last, the E2 enzyme and some different types of E3 enzyme can decorate the target protein of ubiquitination by co-recognizing it. The downstream of all ubiquitination reactions are regulated by E1 ubiquitin-activating enzyme in human body. Various E2 and E3 enzymes are combined, by binding specific protein substrates, to catalyze different types of ubiquitin chains. The E2 enzyme plays a central role in regulating ubiquitin chain assembly, which controls the length of ubiquitin chain and the type of connection. E3 ligases, capable of decorating the target protein, confer the substrate upon some ubiquitin modifications through regulating ubiquitin transfer from E2 enzyme to the target protein [18-20]. According to the protein domain type of the substrate identified by E3, E3 enzymes can be categorized into two groups, RING type and HECT type [21]. RING and U-box domains, highly similar in structure and function, serve as a scaffolding role to link a catalytically active E2 enzyme to a protein substrate. E3 ligases containing HECT domains catalytically activate themselves and transfer ubiquitin from E2 to their cysteine for directly connecting substrate protein. E2 and E3 play a crucial role in the type of ubiquitin chain linkage, especially for HECT domain E3 [22-24]. Depending on the relative ratio of E3 enzymes to target protein, the monoubiquitination and multi-monoubiquitination of target proteins can be identified [25-27]. Therefore, in series of enzyme cas-

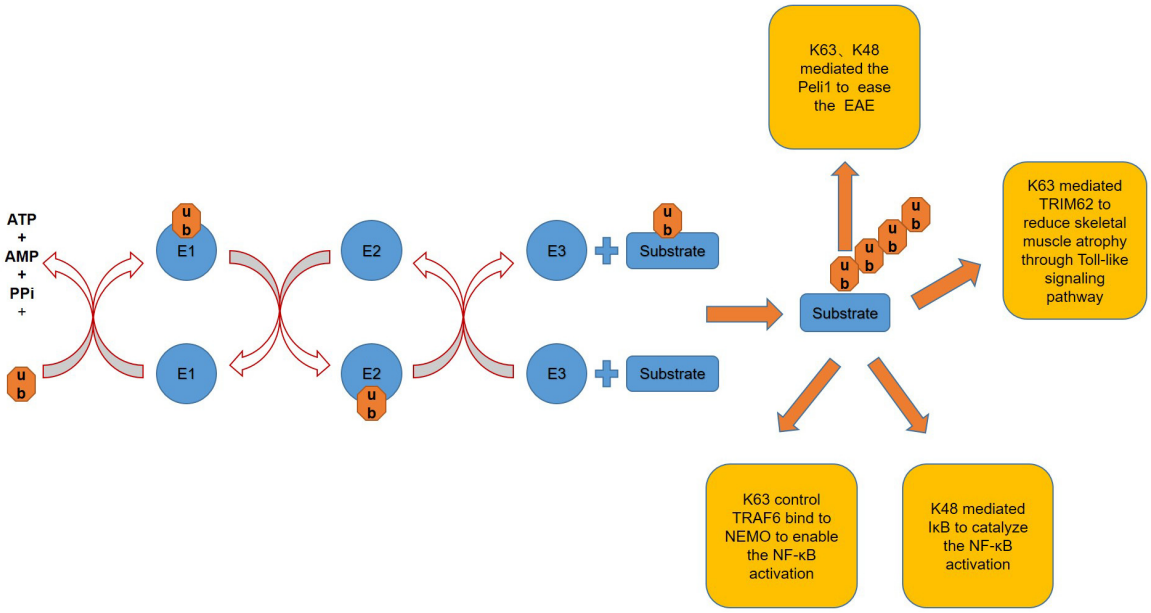

Figure 1. Ubiquitin cascade produces diverse ubiquitin chains that can affect different varieties of cellular processes. Ubiquitin-activating enzyme (E1) transfers ubiquitin to ubiquitin-conjugated enzyme (E2) with an ATP participation. Ubiquitin ligase (E3) then transfers the ubiquitin molecule to the substrate. Repeating this sequence of ubiquitin transfer results in a variety of length of the ubiquitin chain, K48 and K63-linked polyubiquitin chain bring into their biological function. K63- and K48-mediated polyubiquitination play an important role in a large number of biological and pathological functions by NF-κB signaling pathway.

cade reactions, E3 plays a crucial role in target protein of specific recognition and the regulation of ubiquitination system.

DUBs are proteases that reverse the reaction of ubiquitination, which is the main way in the degradation of protein under the substrate [28, 29]. The active site thiol is charged by DUBs that can separate ubiquitin from target protein and hydrolyze the connection between cellular thiol and amines for conjugating the protein, thereby releasing ubiquitin molecules [27]. DUBs play a key role in regulating ubiquitin-mediated signaling pathway. In inflammation, NF-κB, a ubiquitous transcription factor, plays a key role in regulating genes. It has been suggested that activation of NF-κB and regulation of inflammation are related to ubiquitination [30, 31]. Next, the release of NF-κB factor regulated by various type of ubiquitination is described.

Types of ubiquitin linear modification in signaling pathway

Different assembly modes of ubiquitin chain have various biological functions [32]. K48-linked polyubiquitin chain (Lys48) and K63-linked polyubiquitin chain (Lys63) play an important role in regulating activity process of NF-κB pathway [33]. The following discussion is focused on the function of Lys48 and Lys63.

K48-linked polyubiquitination

To regulate the stability of protein through negative feedback adjustment is the major function of K48-ubiquitin chains. Lack of K48-linked polyubiquitin chains account for the death of cells [34]. In the process of cell signal transduction, K48 ubiquitination can promote or block the transmission of signals by regulating the degradation of signal proteins [33]. For example, K48 ubiquitination controls the degradation of signal inhibitor IκB which promotes transduction of the classical signal pathway of NF-κB [35, 36] (**Figure 1**). IκBs, the inhibitor proteins of NF-κB, bind NF-κB dimers and mask its nuclear localization signal, thereby enabling NF-κB remaining in the cytoplasm [37]. Exposure of cells to various extracellular stimuli leads to the rapid phosphorylation. After phosphorylation, the IKK phosphate acceptor

sites on IκB serve as an important part of a specific recognition site for E3RS (IκB/β-TrCP), a SCF-type E3 ubiquitin ligase, thereby explaining how to form the ubiquitin chains and promote the ubiquitination and degradation of IκB through catalysis [38]. Then, exposure of nuclear localization signal translocates NF-κB to the nucleus where it regulates the gene transcription [39].

K63-linked polyubiquitination

K63-linked polyubiquitination modulates non-proteolytic functions, including protein trafficking, kinase activation and phosphatase activation, DNA repair, NF-κB activation and chromatin dynamics [40]. The signaling molecules of K63-linked polyubiquitin play an important role in signal transduction including NF-κB, T-cell receptor, Toll-like receptor, RIG- I-like receptor, NOD-like receptor, DNA damage response pathways and Akt activation [41]. For instance, TRAF6 (TNF receptor-associated factor 6), an RING domain protein, serving as an Ub E3, plays a significant role in the activation of IKK by interleukin-1 (IL-1) and TLR pathways [42]. The E2 dimeric complex, composed of Ubc13 and a Ubc-like protein called Uev1A, is responsible for the activation of IKK. TRAF6, binding with the E2 dimeric complex (Ubc13/Uev1A), catalyzes the target proteins of K63-linked poly-ubiquitination including TRAF6 and NEMO themselves. Then, the protein kinase complex consisting of TAK1, TAB1 and TAB2 will be activated [43]. The K63 polyubiquitin chains bind to the domains of TAB2 and TAB3, which leads to the activation of TAK1. The activated TAK1 phosphorylates IKKb and then enables the downstream activation of NF-κB signal pathway [44].

Inflammation and its signaling pathways regulated by ubiquitination

Inflammation is a non-specific immune response to harmful stimuli where the ubiquitin system plays an important role. The first line of defense to resist invading pathogens is mediated by pathogen-recognition receptors. It is generally believed that inflammation is caused by series of interaction between genetic factors, the environment, the abnormal intestinal microbial immune response and other factors [45]. Some recent studies have strongly proved that the abnormal modification of protein, such as ubiquitination, plays a crucial role in the pathogenesis of inflammation [46]. Recently, ubiquitination has emerged as an essential role in signal transduction of inflammation, including TLRs, NLRs and RLRs. All of these can inhibit activation of NF-κB and MAPKs signaling pathway [47]. To illuminate the relationship between inflammation and ubiquitination, including three ubiquitin ligases (A20, CYLD and the E3 ligase Ubc13/uev1A) [48-51], the regulation of these receptors for inflammation is discussed below.

Toll-like receptors

TLRs, major components of innate immunity, activate specific signaling pathways and inflammatory responses through recognizing conserved pathogen components [52]. TLR4 is the only TLR that uses all four adaptors. In contrast to other TLRs, TLR4 signaling can induce inflammatory cytokines through activating MyD88 and TRIF-dependent pathways. Upon the binding of bacterial lipopolysaccharide, TLR4 gathered upon TIRAP on the plasma membrane, where TLR4 subsequently promotes the gathering of MyD88, resulting in activation of NF-κB, MAPK and production of pro-inflammation cytokines [53, 54]. MyD88 is essential for the downstream signal of various TLRs. Children with deficiency of this signal molecules result in the recurrence of [55]. IL-1R-associated kinase (IRAK)-4, a serine kinase with a N-terminal death domain, interacts with MyD88 to activate other IRAK family members such as IRAK-1 and IRAK-2 [56]. TNFR-associated factor 6 (TRAF-6) acts as an E3 ubiquitin protein ligase. Combination of TRAF-6 and IRAK-1 on the Lys63 chain with E2 ubiquitin-conjugating enzymes Ubc13 and Uev-1A influences activation of IRAK, which interacts with TRAF-6 and catalyzes formation of the polyubiquitin [57, 58] (**Figure 1**) (**Table 1**). The complex of TGF-β-activated kinase 1 (TAK1) is activated by K63-linked poly-ubiquitin chains and linked with the novel zinc finger type ubiquitin-binding domain of TAB2 and TAB3, thus, regulating the kinase TAK1 complex [59]. The IKK complex, composed of IKK-a, IKK-b, and NF-κB essential modulator (NEMO) also bind the K63-linked poly-ubiquitin chains

Table 1. Modification of NF-κB signaling via ubiquitin enzymes and DUBs

Pathway	Substrate	Classification	Ubiquitin modification	Consequence	Reference
TNF-α	A20	DUBs	Remove K63-ub from RIP1 and TRAF6	Inhibit TNF-α induced NF-κB signaling	[68, 69]
	OTULIN	DUBs	Inhibit the production of M1-ub	Inhibit TNF-α induced NF-κB signaling	[72]
TLR	Ubc13/uev1A	RING E3 ligase	Transfer of K63 on TRAF6	Inhibit NF-κB signaling	[45]
	XIAP	RING E3 ligase	Transfer of K63 on RIP2	Inhibit NF-κB signaling	[54]
NOD1/2	RIP2	-	Remove K63-ub from RIP2	Inhibit NF-κB signaling And promote IKK activation	[55]
	CYLD	DUBs	Remove K63-ub from RIP2	Inhibit NF-κB signaling	[64, 65]
RIG-I	TRAF3/TAK1	-	Prevent interaction between TRAF3 and TBK1	Inhibit NF-κB signaling	[60]
	DUBA	DUBs	Remove K63-ub from TRAF3	Inhibit NF-κB signaling	[71]

Abbreviations: Ubc13, ubiquitin conjugating enzyme E2N, XIAP, X-linked inhibitor apoptosis protein.

[60, 61]. Thus, Phosphorylated IκB proteins suffer from the degradation by the ubiquitin system, suggesting that the freed NF-κB is translocated into the nucleus and activates the expression of pro-inflammation cytokine genes [62]. In contrast to other TLRs, TLR4 signaling can induce the expression of inflammatory cytokines through activating MyD88 and TRIF-dependent pathways [63] (**Figure 2**).

NOD-like receptors

NLRs family consists of evolutionarily conserved cytosolic proteins that respond to pathogen- or damage-associated molecular patterns (PAMPs or DAMPs) [64]. NLRs typically include several leucine-rich repeats (LRRs) at the C termini which can recognize PAMP or DAMP and, then, activate the inflammasome [65, 66]. Mutations of NLRs are frequently linked to inflammation diseases. For example, the emerged evidences suggested that NALP3/cryopyrin was associated with various kinds of inflammatory syndromes, while the mutations of NOD2 were related to the Crohn's disease [67]. Cumulative evidences have demonstrated that NLRP3 inflammasome priming plays an important role in regulating ubiquitin [68-70]. NOD1 and NOD2, the cascade downstream of NLR signal, induce the transcriptional upregulation of pro-inflammatory cytokines and activate NF-κB via an adaptor, RIP2/RICK [71] (**Table 1**). Many studies have suggested that NLRs relying on the K63 polyubiquitination played a central role in NOD-mediated activation of IKK and NF-κB [72, 73] (**Figure 2**). NOD1 and NOD2 could be stimulated by bacterial ligands which produced muramyl dipeptide to gather RIP2 via the gathering CARD domain containing protein kinase to activate NF-κB and MAPK signaling pathways [74]. In addition, the activation of signal requires the ubiquitin ligases. RIP2, TRAF6 and NEMO, the target proteins of K63 polyubiquitination, catalyzed by Ubc13/Uev1A and TRAF proteins [75, 76]. The sites of RIP2 and NEMO ubiquitination are important for activating IKK by NODs. TAK1 kinase complex was gathered and activated by RIP2 polyubiquitination which promoted the activation of IKK. This post-translational modification leads to the linear polyubiquitination of RIP2 and promotes the expression of pro-inflammatory cytokines and chemokines [75] (**Figure 2**). Therefore, the assembly and disassembly of K63-linked polyubiquitin chains and the expression of pro-inflammation cytokines are essential for regulating NLR signal.

RIG-I-like receptors

Formation of RIG-I is known to be modulated by ubiquitination. E3 ubiquitin ligases, TRIM25 and RNF135, mediate K63-linked polyubiquitination of RIG-I which results in RIG-I oligomerization [77]. This modification is associated with the activation of RLR signal. TRAF3 and TBK1 gathered by RIG-I complex stimulate the production of interferon TRAF6 and activate the NF-κB signal (**Table 1**) (**Figure 2**). Deficiency of TRIM25 had an effect on cell activation which induced the response of type 1 interferons to RNA virus infection, suggesting that protein plays a key role in the viral signaling pathway [78, 79]. The signal activation of RIG-I downstream required another RING domain protein, TRAF3. Many experiment results indicated that lack of TRAF3 RING domain caused

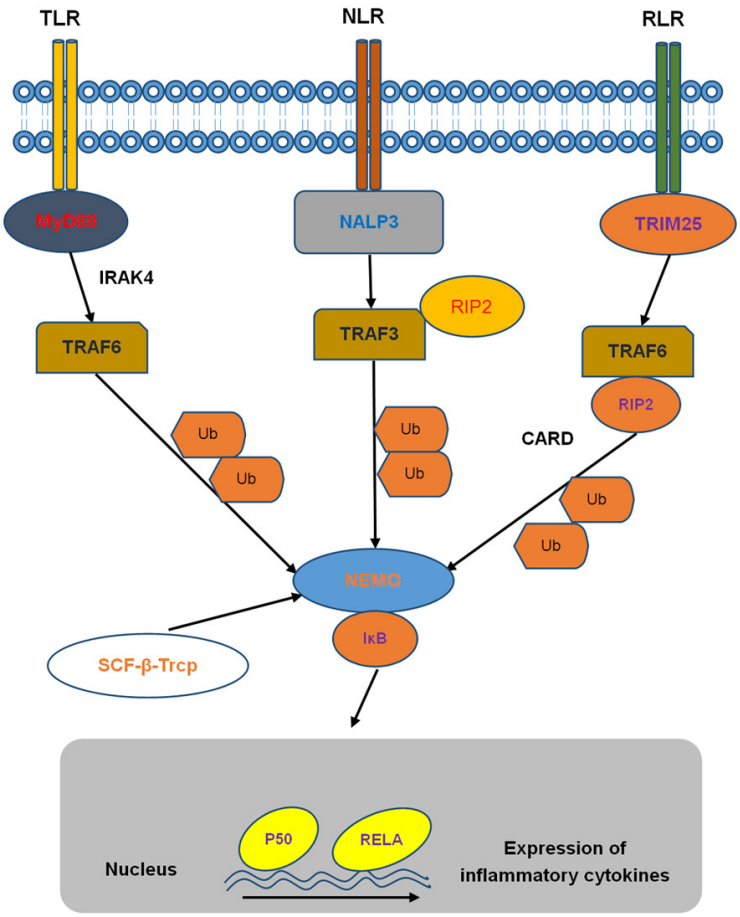

Figure 2. Ubiquitination plays a central role in the activation of NEMO and IκB by multiple signaling pathways, including those emanating from Toll-like receptors (TLRs), NOD-like receptors (NLRs) and RIG-I-like receptors (RLRs). In TLRs-mediated signaling, TLR4 promotes the gathering of MyD88, leading to activation of NF-κB and the production of pro-inflammation cytokines. In NOD2-mediated signaling, NALP3 protein promotes K63-linked polyubiquitination of RIP2, thus allowing the degradation of the IκB and NEMO to enable translocation of NF-κB proteins p50 and RelA to the nucleus, whose kinase activation stimulated the expression of inflammatory cytokines. RIG-I signaling pathways are regulated by TRAF6, which promote K63-linked polyubiquitination on TRAF6 and RIG-I to stimulate NEMO gathering.

abnormal induction of type 1 interferons, suggesting that its E3 activity was important for activating TBK1 [80]. Multiple TRAF proteins, including TRAF2, TRAF3, and TRAF6, bind to MAVS (mitochondrial antiviral signaling) to activate IKK and TAK1. However, K63-linked chains were removed by DUB cylindromatosis (CYLD), which counteracted the K63-linked poly-ubiquitination in RLR signaling (**Table 1**). Therefore, the bind between RIG-I and MAVS was abrogated [81]. In addition, the TRIM25-related ubiquitination of RIG-I caused the negative regulation of RIG-I signal by linear ubiquitination [82]. Therefore, activation of RLR pro-inflammatory signal relies on K63-linked poly-ubiquitination for emerging antiviral responses and producing inflammatory cytokines.

DUBs associated with inflammation and pathways

Deubiquitination also plays an important role in regulating homeostatic NF-κB activation, which could lead to excessive inflammation and cancer. However, the aberrant activation of NF-κB linking to various inflammation is associated with the defects in DUBs. Recently, several researches indicated that DUBs negatively regulated activation of NF-κB [83, 84]. The NF-κB pathway, which is activated by a range of trigger proteins, including TNFR, IL-1R and TLR receptor, stimulated the IKK complex activation. The IKK complex contains catalytic subunits IKKα and IKKβ and regulatory subunit NEMO [85].

CYLD, a tumor suppressor protein and a DUB, is known to inhibit IKK [12]. CYLD contains a UBP domain, whose activation is associated with K63-linked polyubiquitin chains, and is usually mutated in cylindroma patients [86]. CYLD removed the K63 polyubiquitin chains from a range of NF-κB signaling proteins [87]. Experiment results indicated that K48-K63 branching was shown to protect the K63 linkages from CYLD-mediated deubiquitination, which suggests that CYLD has a key role in deubiquitinating several NF-κB signaling proteins [7]. Deficiency of CYDL resulted in the expression of pro-inflammatory gene. CYLD-deficient mice displayed a myriad of phenotypes, suggesting that CYLD inhibited expression of IKK and NF-κB [88, 89]. Similar to CYLD, A20, another DUB inhibiting NF-κB, plays an important role in regulating inflammatory

signal. A20 stimulated by TNF receptors was negatively regulating the activation of NF-κB signal. Some studies demonstrated that A20, as an NF-κB-responsive gene inhibited activation of NF-κB, which is not only in response to TNF receptors, but also including many pro-inflammatory stimuli such as IL-1, LPS, T- and B-cell receptor antigens, NOD2 ligands and PRRs activation [90]. With the stimulation of TNF-α, mice deficient of A20 are likely to have inflammation in multiple organs and defective cells, which leads to activation of NF-κB (**Table 1**). DUB activity of A20 from multiple NF-κB signaling intermediates decomposed the K63 polyubiquitin. In addition, DUB activity of A20 removes K63 polyubiquitin chains from RIP1 and TRAF-6 which accordingly inhibits NF-κB function [91, 92]. Upon stimulation of TNF-α, A20 also mediates ITCH, an HECT domain that E3 required for RIP1 degradation, to negatively regulate activation of IKK [93]. Indeed, many examples illustrated that A20 gathered from ubiquitinated proteins at TNF-R1 signaling complex to modulate ubiquitin-dependent signal to prevent activation of IKK and the downstream signal of NF-κB, suggesting that A20 derived from K63 polyubiquitin had a key role in restricting the inflammatory responses [94]. There are many other DUB operator inhibiting the function of NF-κB pathway. For example, a new DUB called DUBA has shown that TRAF3, detached from K63 polyubiquitination, inhibits the activation of TBK1 [95] (**Table 1**). Lots of researches have reported that OTULIN, another DUB, regulates activation of LUBAC and LUBAC-mediated NF-κB to inhibit the production of M1 polyubiquitin. Lacking OTULIN leads to inhibit the TNF-induced activation of NF-κB and expression of pro-inflammatory gene [96] (**Table 1**). Furthermore, the expression of a catalytically inactive OTULIN C129A mutant which bound the ubiquitin and exerted a dominant-negative effect, led to negatively regulating activation of NF-κB signal [97, 98]. In summary, DUBs play an important role in regulating ubiquitination-associated inflammation.

Conclusions

Ubiquitination is a post-translational modification that exists in eukaryotic cells which convey a wealth of information through a variety of unique linkage types. More and more studies demonstrate that ubiquitination plays an important role in many diseases and physiological processes, including cancer and inflammatory responses; however, there still lacks full understanding of the mechanisms of these processes. Inflammatory signal is regulated by several of these linkages, including K48, K6 and K27, especially K63-linked and linear chains. Tightly controlled activation of signaling pathways via regulating different types of ubiquitination and modulation of many other molecular events by ubiquitination has profound impact on inflammation. Ubiquitin enzymes and DUBs have a crucial role in ubiquitination, suggesting that how to regulate these enzymes and illustrate the regulatory mechanisms are important for inflammation. NF-κB as a signaling transduction factor, released by inflammatory stimulation, is related to ubiquitination. In summary, in recent years, understanding of ubiquitin as a key role in inflammation has greatly increased, but there are many existing problems to be solved. For example, the specific mechanism of ubiquitin-modifying enzymes regulates the ubiquitination-directed molecular signals and their biological functions, and how to establish a tandem interaction network between ubiquitination and other post-translational modifications which identified key ubiquitination-associated proteins through NF-κB signaling pathway. The study is important for human beings to explain the mechanism of inflammation.

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Disclosure of conflict of interest

None.

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